Peroxisome proliferator–activated receptor γ (PPARγ)-2 is a member of the nuclear hormone receptor superfamily that is expressed predominantly in adipocytes and is thought to have a role in energy homeostasis, adipogenesis, and insulin sensitivity. A functional single nucleotide polymorphism (SNP) that predicts a proline to alanine substitution (Pro12Ala) within the coding region of this gene has previously been associated with obesity and type 2 diabetes in several populations. In this study, we identified several novel SNPs in the promoter region of PPARγ2 and genotyped them, along with the previously identified Pro12Ala SNP. In 241 nondiabetic Pima subjects, the Pro12Ala was associated with whole-body insulin action (P = 0.05), hepatic insulin action (P = 0.03), and fasting plasma insulin concentrations (P = 0.01). One of the promoter SNPs positioned within a putative E2 box was in high linkage disequilibrium ([D′] = 0.98) with the Pro12Ala. This promoter SNP was similarly associated with whole-body insulin action (P = 0.04) and hepatic insulin action (P = 0.05), but not fasting plasma insulin concentrations. Functional studies in transfected 3T3-L1 cells demonstrated that this single base substitution in the putative E2 box significantly altered transcriptional activity from a luciferase reporter construct. These data indicate that this promoter SNP, via its effect on PPARγ2 expression, may also have functional consequences on PPARγ2-activated pathways, and perhaps both the promoter SNP and the Pro12Ala contribute to PPARγ2-related phenotypes. Diabetes 52:1864–1871, 2003

Obesity is a metabolic risk factor for type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease (1). The development of obesity requires either an increasing number of differentiated adipocytes or an increasing size of preexisting adipocytes due to additional storage of energy as triglycerides (2). This process of adipocyte differentiation and development is controlled by a number of transcription factors that include the nuclear receptor peroxisome proliferator–activated receptor γ (PPARγ) (3,4), the family of CCAAT enhancer binding proteins (C/EBPs) (5) and the basic helix-loop-helix leucine zipper transcriptional factor ADD1/SREBP1 (6). C/EBP-β and -δ and ADD1/SREBP1 induce the expression of PPARγ, which triggers the adipogenic process by transactivation of adipose-specific genes involved in lipid storage and metabolism, such as aP2 (fatty acid binding protein), PEPC (phosphoenolpyruvate carboxykinase), AOX (acyl-CoA oxidase) and LPL (lipoprotein lipase) (7,8).

The critical role of PPARγ in adipogenesis, energy homeostasis, and insulin sensitivity has led to studies on the expression of this gene. Two alternatively spliced isoforms of PPARγ, namely PPARγ1 and PPARγ2, are expressed in adipose tissues, heart, muscle, and liver. In these tissues, PPARγ1 is much more abundant than PPARγ2 (9). Both isoforms possess ligand-dependent and -independent domains. However, PPARγ2 has an additional 28 amino acids in the NH2-terminus that renders its ligand-independent activity 5–10 times higher than that of PPARγ1. This ligand-independent activation requires insulin stimulation (10). It has been shown that natural PPARγ ligands, such as prostaglandins (11), fatty acids (12), or synthetic ligands such as thiazolidinediones (TZDs), bind to PPARγ and lead to ligand-dependent activation. In particular, activation of the PPARγ by TZD, an antidiabetic drug, can lead to increased insulin sensitivity and improved glucose tolerance in patients with type 2 diabetes (13) and is currently an effective treatment for some individuals with this disease.

The Pima Indians of Arizona have an extremely high prevalence of type 2 diabetes. Their diabetes is characterized by obesity, dysfunction of insulin secretion, insulin resistance (decreased glucose disposal), and increased hepatic glucose output (hepatic insulin resistance) (14).
Previous studies have suggested that genetic variation in PPARγ may constitute a predisposing factor for obesity, type 2 diabetes, or insulin resistance, and a Pro12Ala substitution in exon B of PPARγ has been associated with BMI and type 2 diabetes in several populations (15–19). To determine the genetic impact of PPARγ on obesity and type 2 diabetes in Pima Indians, we screened the PPARγ gene for functional genetic variants that could contribute to the development of these diseases.

RESEARCH DESIGN AND METHODS

Subjects and clinical characteristics. A total of 985 full-blooded Pima Indian subjects are part of our ongoing longitudinal study of the etiology of type 2 diabetes among the Gila River Indian Community in central Arizona (20). All individuals who are ≥5 years of age are invited to participate in a standardized health examination every 2 years. To determine diabetes status, a 75-g orally administered glucose tolerance test is given and the results are interpreted according to the criteria of the World Health Organization (21). All studies were approved by the Tribal Council of the Gila River Indian Council and the Institutional Review Board of the National Institutes of Diabetes and Digestive and Kidney Diseases.

For detailed metabolic testing, individuals are admitted to our clinical research ward for 7–10 days, and only individuals found to be healthy by medical history, physical examination, and routine laboratory tests and who are not taking medications are studied. Oral glucose tolerance is measured after 2–3 days on a weight-maintaining diet of mixed composition. Subjects ingest 75 g of glucose, and blood for plasma glucose and insulin measurements is drawn before ingesting the glucose and at 30, 60, 120, and 180 min thereafter. Subjects also receive a 25-g intravenous injection of glucose over 3 min to measure the acute insulin response. Blood samples are collected before infusion and at 3, 4, 5, 6, 8, and 10 min after infusion for determination of plasma glucose and insulin concentrations. The acute insulin response is calculated as the mean increment in plasma insulin concentrations from 3 to 5 min.

The hypereosinophilic-euglycemic clamp technique is used to determine basal glucose appearance and insulin-stimulated glucose disappearance (uptake) rates (22). Briefly, insulin is infused to achieve physiologic and maximally stimulating plasma insulin concentrations (137 ± 3 and 2,394 ± 68 μU/ml, respectively) for 100 min for each step. Plasma glucose concentrations are held constant at ~100 mg/dl by a variable 20% glucose infusion. Trifluorinated glucose is infused for 2 h before the insulin infusion to calculate rates of postabsorptive glucose appearance rates and to calculate glucose disappearance rates during the lower dose of insulin infusion. During the last 40 min of the low- and high-dose insulin infusion, the rate of insulin-stimulated glucose disposal is calculated, adjusted for steady-state plasma glucose and insulin concentration, and normalized to estimated metabolic body size (EMBS = fat-free mass + 17.7 kg) as described (22,23). The rate of endogenous glucose output (measured by a primed [30 μCi/min], continuous [0.3 μCi/min] [3-3H]glucose infusion) was determined during the physiological dose of insulin infusion. Endogenous glucose output is assumed to be zero during maximally stimulating insulin infusion (22,23). Body composition was estimated by underwater weighing until January of 1996 and is currently measured by dual energy X-ray absorptiometry (DPX-I; Lunar Radiation, Madison, WI). A conversion equation derived from comparative analyses is used to make estimates of body composition comparable between methods (24).

The measurement of energy expenditure (EE) and substrate oxidation in the respiratory chamber has been described previously (24). Briefly, after an overnight fast, subjects enter the chamber for 23 h and are fed calories to maintain the energy according a previously determined equation. The rate of energy expenditure is measured continuously and calculated for each 15-min interval and then extrapolated for 24 h (24-h EE). Carbon dioxide (VCO2) and oxygen (VO2) production are measured for every 15-min interval. The 24-h respiratory quotient (24-h RQ) is calculated as a ratio of 24-h VCO2 and 24-h VO2. The 24-h carbohydrate, lipid, and protein oxidation are determined based on the 24-h RQ, 24-h EE, and 24-h urinary nitrogen excretion (24).

Single nucleotide polymorphism identification and genotyping. Genomic DNA was obtained from peripheral lymphocytes. To identify sequence variants, all seven exons and ~12 kb of the PPARγ2-specific promoter region were PCR amplified and sequenced in DNA samples from 24 non-first-degree related Pima Indians. Among these 24 subjects, 13 were diabetic (BMI 31.86 ± 2.6 kg/m², age of onset of diabetes 40.4 ± 4.0 years), 10 were nonobese subjects (BMI 32.2 ± 2.9 kg/m²), and 1 had an unknown diabetes status. Sequences were aligned using the ClustalW alignment program (25,26) on an automated DNA capillary sequencer (model 3700; Applied Biosystems). Single nucleotide polymorphisms (SNPs) identified by sequencing were genotyped in DNA from 985 subjects using the TaqMan Allelic Discrimination (AD) Assay (Applied Biosystems). The TaqMan genotyping reaction was amplified on a GeneAmp PCR system 9600 (95°C for 10 min, following 45 cycles of 95°C for 15 s, and 60°C for 1 min), and fluorescence was detected on an ABI Prism 7700 sequence detector (Applied Biosystems). Sequence information for all oligonucleotide primers and probes is available upon request. The DNA region encompassing the Pro12Ala substitution was sequenced using primers forward 5′-ctagctagttgtaggtgctta-3′ and reverse 5′-gatgctagcaagccagagtcgtg-3′ and genotyped by AD using primers forward 5′-cttcctttagctagatgcttct-3′ and reverse 5′-ctacattcctgtctcaagata-3′ and Turbo Taq probes: Fast-ttcctaatgctgcag and Vic-ttctttagctagatgcttct (Applied Biosystems). The DNA region encompassing the promoter E2 box polymorphism at –2821 bp was sequenced with primers forward 5′-tcaaccaatggtgctta-3′ and reverse 5′-gatgctagcaacaagttcttggt-3′ and genotyped by AD using primers forward 5′-cttcctttagctagatgcttct-3′ and reverse 5′-ctacattcctgtctcaagata-3′ and Turbo Taq probes: Fast-ttcctaatgctgcag and Vic-ttctttagctagatgcttct (Applied Biosystems). The DNA region encompassing the promoter E2 box polymorphism at –2821 bp was sequenced with primers forward 5′-tcaaccaatggtgctta-3′ and reverse 5′-gatgctagcaacaagttcttggt-3′ and genotyped by AD using primers forward 5′-cttcctttagctagatgcttct-3′ and reverse 5′-ctacattcctgtctcaagata-3′ and Turbo Taq probes: Fast-ttcctaatgctgcag and Vic-ttctttagctagatgcttct (Applied Biosystems).

Statistical analyses. Statistical analyses were performed using the Statistical Analysis System of the SAS institute (Cary, NC). For continuous variables, the general estimating equation procedure was used to adjust for covariates. These analyses account for the correlation among family members (i.e., siblings). Fasting and 2-h plasma insulin concentrations and rates of glucose disposal during the low-dose insulin infusion were log-transformed before analysis to approximate a normal distribution. The association of SNPs with type 2 diabetes was performed by analysis of contingency tables, where the frequencies of the genotypic groups were compared between the diabetic and nondiabetic subjects and adjusted for appropriate covariates.

Functional analysis of the potential E2 box

Constructs

Minimal promoter. The basal promoter of PPARγ2 (nucleotide +120 to +910) was PCR amplified from genomic DNA using the primers forward 5′-tagctagttgtaggtgctta-3′ and reverse 5′-tagctagctagatgcttct-3′ and cloned into a pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen). The ~1-kb basal promoter fragment was then ligated into a pGL3-basic luciferase reporter vector (Promega) at the SacI and Xhol sites.

Polymorphic E box regulatory element. A 453-bp fragment of the PPARγ2 promoter (nucleotide −2,019 to −2,072) containing either a C or T nucleotide at position −2,021 in the potential putative E box was amplified with primers forward 5′-tagctagctagatgcttct-3′ and reverse 5′-ctagctagcaacaagtgctg-3′ and cloned into a pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen). The ~1-kb basal promoter fragment was then ligated into a pGL3-basic luciferase reporter vector (Promega) at the SacI and Xhol sites.

All constructs were confirmed by DNA sequence analysis.

Transfection and luciferase assay

The pGL3 constructs were transiently transfected into a 3T3-L1 cell line (American Type Culture Collection) under both normal and differentiation-induced cellular growth conditions. 3T3-L1 cells were maintained in standard growth medium: Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL) modified to contain 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 10% FCS, and 1% penicillin-streptomycin in 5% CO2 and 95% air atmosphere at 37°C. Cells were transfected at 70–90% confluence in six-well plates. To control for transfection efficiency, 0.25 μg pRL-plasmid (renilla luciferase under control of TK promoter; Promega) was mixed with 0.75 μg of experimental DNA (1:3 ratio). The total DNA (1 μg) was mixed with 6 μl PLUS reagent (Gibco BRL) in 100 μl DMEM (serum-free) and incubated for 15 min at room temperature. LipofectAMINE (4 μl) (Gibco BRL) in 100 μl DMEM was added to the mixture and incubated for an additional 15 min. Parallel transfections were performed with a positive control vector, pGL3-promoter (beetle luciferase under control of cytomegalovirus promoter; Promega) and a negative control vector, pGL3-basic (a polynucleotide-beetle luciferase vector; Promega). Cells were rinsed with transfection medium (growth medium without antibiotics) and incubated with 1 ml of the DNA/liposome mixture for 3 h at 37°C. An additional 2 ml of transfection medium was added and incubated for 15 h. To maintain the transfected cells in the nondifferentiated state, the medium was replaced with standard growth medium. To induce differentiation of the transfected cells, the medium was replaced with differentiation medium (growth medium plus 1 μmol/l dexamethasone, 0.5 mmol/l 3-isobutyl-1-methylxanthine, and 0.01 mmol/l insulin [Sigma]). After 48 h, cells were harvested for the luciferase assay.

For each cell extract, dual luciferase assays were performed in triplicate using the standard protocol (Promega), and light was measured with a luminometer (BioScan). Beetle luciferase activity was normalized for renilla luciferase activity. Each transfection was repeated five to six times, and data were averaged. The difference in the average activity was analyzed by an unpaired t test.
RESULTS
Seven exons (exon B and 1–6) and ~12 kb of the PPARγ2-specific promoter were sequenced in 24 Pima Indians. Nine SNPs were identified (Fig. 1). Two of the SNPs were in the coding region, a C/G in exon B predicting a Pro12Ala substitution (SNP8) and a C/T in exon 6 predicting a silent His477His substitution (SNP9). The other seven SNPs were in the promoter. Based on the genotypes of the 24 Pima Indians, SNP 1 appeared to be in complete linkage disequilibrium with SNP 2. SNP 6 was a common variant (allele frequency 0.58) and was positioned within a putative 5′EF1 binding site relatively close to the transcriptional start site of the PPARγ2 gene. Therefore, SNP 6 (C-2821T) and SNP 8, the well-documented Pro12Ala variant, were selected for our initial detailed analysis.

The C-2821T and the Pro12Ala variants were further genotyped in DNA from 985 full-blooded Pima Indians for association analyses. Both SNPs were in Hardy-Weinberg equilibrium. In addition, both SNPs were modestly associated with BMI in 795 full-blooded, nondiabetic Pima Indians who were at least 18 years of age. For the Pro12Ala, only six individuals were homozygous for the alanine allele; therefore, for statistical purposes these subjects (Ala/Ala) were combined with subjects heterozygous for the proline/alanine alleles (Pro/Ala) and this combined group was then compared with subjects homozygous for the proline allele (Pro/Pro). Subjects with the Ala/Ala or Pro/Ala genotypes had a mean BMI of 34.2 ± 0.7 kg/m² (n = 117), whereas subjects with the Pro/Pro genotype had a mean BMI of 36.8 ± 0.3 kg/m² (n = 678) (P < 0.04 after adjusting for age, sex, birth year, and nuclear family membership). For the more common promoter C-2821T, subjects with the C/C, C/T, or T/T genotypes had mean BMI values of 37.6 ± 0.5 kg/m² (n = 262), 35.9 ± 0.4 kg/m² (n = 405), and 35.5 ± 0.7 kg/m² (n = 128), respectively (P < 0.03, after adjusting for age, sex, birth year, and nuclear family membership).

Neither the Pro12Ala nor the C-2821T was associated with type 2 diabetes. The genotypic distribution and allele frequency for each of these SNPs in diabetic versus nondiabetic subjects is given in Table 1. Among a total of 985 subjects genotyped for Pro12Ala, 655 were diabetic (BMI 37.75 ± 0.40 kg/m², age of onset of diabetes 37.40 ± 0.63 years), and 328 were nondiabetic (BMI 35.76 ± 0.58 kg/m²). The minor allelic frequency of the Pro12Ala was 0.07 in the diabetic group as compared with 0.09 in the nondiabetic group (P = 0.7). Among 985 subjects genotyped for C-2821T, 655 were diabetic (BMI 37.74 ± 0.51 kg/m², age of onset of diabetes 37.31 ± 0.76 years) and 328 were nondiabetic (BMI 35.80 ± 0.74 kg/m²). The minor allele frequency for the C-2821T was 0.42 in the diabetic group as compared with 0.41 in nondiabetic group (P = 0.9).

We further assessed whether the Pro12Ala or the C-2821T variants were associated with metabolic predictors of obesity and type 2 diabetes in Pima Indians who had not yet developed diabetes. The means of various metabolic measurements in nondiabetic subjects, grouped by their Pro12Ala genotype (Table 2) or their promoter C-2821T genotype (Table 3) is given. Subjects with Ala/Ala or Pro/Pro genotypes had a lower mean fasting plasma insulin concentration, lower mean insulin-suppressed endogenous glucose output in response to physiological insulin concentrations (and therefore increased hepatic insulin sensitivity), and increased whole-body insulin sensitivity, as measured by glucose disposal in response to maximally stimulating insulin concentrations, as compared with Pro/Pro subjects (Table 2). Similarly, for the promoter SNP,
TABLE 2
Metabolic characteristics of nondiabetic subjects by Pro12Ala genotypes

<table>
<thead>
<tr>
<th></th>
<th>Pro/Pro</th>
<th>Pro/Ala + Ala/Ala</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (f/m)</td>
<td>203 (84/119)</td>
<td>38 (15/23)</td>
<td>0.42</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 ± 1</td>
<td>25 ± 1</td>
<td>0.25</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>33 ± 1</td>
<td>30 ± 2</td>
<td>0.18</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>92 ± 1</td>
<td>89 ± 1</td>
<td>0.12</td>
</tr>
<tr>
<td>2-h plasma glucose (mg/dl)</td>
<td>128 ± 2</td>
<td>118 ± 5</td>
<td>0.01</td>
</tr>
<tr>
<td>Fasting insulin (µU/ml)</td>
<td>42 ± 1</td>
<td>31 ± 3</td>
<td>0.08</td>
</tr>
<tr>
<td>2-h insulin (µU/ml)</td>
<td>226 ± 12</td>
<td>173 ± 26</td>
<td>0.005</td>
</tr>
<tr>
<td>Glucose disposal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-dose insulin clamp (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>2.49 ± 0.1</td>
<td>2.88 ± 0.1</td>
<td>0.26</td>
</tr>
<tr>
<td>High-dose insulin clamp (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>8.4 ± 0.1</td>
<td>9.5 ± 0.3</td>
<td>0.18</td>
</tr>
<tr>
<td>Basal glucose output (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>1.91 ± 0.01</td>
<td>1.94 ± 0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Hepatic insulin sensitivity (suppressed endogenous glucose output) (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>0.30 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Data are means ± SE. P values in bold are significant. EMBS, estimated metabolic body size.

the homozygous T/T group had a lower mean insulin-suppressed endogenous glucose output and increased whole-body insulin sensitivity, as compared with the C/C group, while the heterozygous (C/T) subjects had intermediate values (Table 3). In contrast to Pro12Ala polymorphism, the promoter C-2821T SNP was not associated with fasting plasma insulin concentrations.

The genotypic groups for the Pro12Ala and the C-2821T SNP were further analyzed for metabolic risk factors measured in our respiratory chamber (Tables 4 and 5). The Pro12Ala was not associated with any of these phenotypic measurements (Table 4); however, the promoter C-2821T SNP was modestly associated with lipid oxidation rate and lipid balance (Table 5).

The combined genotype for the Pro12Ala and the C-2821T SNP was also determined in 981 Pima subjects (Table 6). Despite their difference in allele frequency, these two SNPs were in high linkage disequilibrium (\( D' = 0.98 \)) in Pima Indians. To assess whether either of these PPARγ2 SNPs could predict a phenotype independent of the other, we repeated all of the association analyses in Tables 1–4 but additionally adjusted each association for the genotypic effect of the other SNP. The association of Pro12Ala with fasting insulin levels remained significant (\( P < 0.04 \)) after adjusting for C-2821T as a covariant, and the association of the C-2821T variant with 2-h plasma glucose levels remained significant (\( P < 0.03 \)) after adjusting for Pro12Ala as a covariant. However, none of the other associations remained significant after adjusting for these covariants, suggesting that these two PPARγ2 SNPs are contributing the same genetic information in these associations and that there is no additive effect between these two SNPs.

To examine whether the C-2821T SNP alters a regulatory sequence that has functional consequences on the PPARγ promoter, we compared transcription levels from four reporter gene constructs. The first construct was a negative control, containing a promoter-less luciferase reporter gene, while the second construct was a positive control, containing the luciferase gene under the control of a 1-kb basal promoter, while the second construct was a positive control, containing the luciferase gene under the control of a 1-kb basal promoter of PPARγ2. The 1-kb basal PPARγ2 promoter contained a TATA-like element at –68 bp and a potential consensus C/EBP binding site (CCAAATT) at –56 bp. The third and fourth constructs were the experimental constructs for the C-2821T SNP. These plasmids contained a cloned 453-bp fragment (–3,046 to –2,593) of the PPARγ promoter ligated upstream of the minimal promoter, where one construct had a C nucleotide at position –2821 (C-2821) of this fragment and the other construct contained a T nucleotide at position –2821 (T-2821). In transiently transfected 3T3-L1 cells grown in normal growth medium (non-differentiating), the 1-kb promoter of PPARγ2 induced

TABLE 3
Metabolic characteristics of nondiabetic subjects by the promoter SNP C-2821T genotypes

<table>
<thead>
<tr>
<th></th>
<th>C/C</th>
<th>C/T</th>
<th>T/T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (f/m)</td>
<td>75  (22/53)</td>
<td>126 (61/65)</td>
<td>40 (16/24)</td>
<td>0.5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 ± 1</td>
<td>27 ± 1</td>
<td>26 ± 1</td>
<td>0.7</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>32 ± 1</td>
<td>33 ± 1</td>
<td>32 ± 2</td>
<td>0.4</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>92 ± 1</td>
<td>92 ± 1</td>
<td>93 ± 1</td>
<td>0.1</td>
</tr>
<tr>
<td>2-h plasma glucose (mg/dl)</td>
<td>129 ± 4</td>
<td>124 ± 3</td>
<td>132 ± 6</td>
<td>0.03</td>
</tr>
<tr>
<td>Fasting insulin (µU/ml)</td>
<td>42 ± 2</td>
<td>40 ± 1</td>
<td>39 ± 3</td>
<td>0.1</td>
</tr>
<tr>
<td>2-h insulin (µU/ml)</td>
<td>239 ± 23</td>
<td>203 ± 13</td>
<td>221 ± 31</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose disposal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-dose insulin clamp (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>2.4 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>High-dose insulin clamp (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>8.1 ± 0.2</td>
<td>8.8 ± 0.2</td>
<td>8.9 ± 0.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Basal glucose output (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>1.91 ± 0.02</td>
<td>1.91 ± 0.02</td>
<td>1.90 ± 0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Hepatic insulin sensitivity (suppressed endogenous glucose output) (mg · kg EMBS⁻¹ · min⁻¹)</td>
<td>0.32 ± 0.03</td>
<td>0.27 ± 0.08</td>
<td>0.21 ± 0.03</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Data are means ± SE. P values in bold are significant. EMBS, estimated metabolic body size.
**DISCUSSION**

Previous studies have shown that the Pro12Ala substitution in the PPARγ receptor is associated with obesity and type 2 diabetes in several populations (15–19). In Pima Indians, the Pro12Ala was not associated with type 2 diabetes, but was modestly associated with BMI. The Pro12Ala substitution, however, was associated with several metabolic predictors of these diseases. Pima subjects with a Pro/Pro genotype had reduced hepatic and whole-body insulin sensitivity as compared with Ala/Ala or Pro/Ala subjects, although it should be noted that a difference in whole-body insulin sensitivity was only observed at maximally stimulating insulin concentrations, not at physiological insulin concentrations. This result might be expected in a population that has a high prevalence of extreme insulin resistance, such as the Pima Indians, because individual variation in insulin response would not be detected until high doses of insulin are infused. Furthermore, we found that mean fasting plasma insulin concentrations were higher in Pro/Pro subjects than in Ala/Ala or Pro/Ala subjects. Because high fasting insulin concentrations predict type 2 diabetes in Pima Indians (25) and because various diabetic animal models display hyperinsulinemia as the earliest detectable abnormality (26,27), these data indicate that the alanine allele may be protective for developing type 2 diabetes. This is consistent with a previous report by Deeb et al. (15) that showed that the alanine allele is associated with lower BMI, lower fasting plasma insulin concentrations, and improved insulin sensitivity in a Finnish population. Improved insulin sensitivity in alanine allele carriers has also been reported in a Swedish population (28).

Sequencing of the promoter region identified a novel C-2821T SNP that was similarly associated with whole-body insulin sensitivity (muscle) and hepatic insulin sensitivity. This promoter SNP has a different allele frequency as compared with the Pro12Ala variant (0.56 vs. 0.93 for the more common allele, respectively). However, in Pima Indians, the two SNPs are in high linkage disequilibrium.
and consequently it is difficult to assess whether the promoter SNP or the missense substitution, or both, contribute to the observed in vivo phenotypes. The mechanism(s) by which these variants function remains uncertain. However, it is possible that the Pro12Ala alters PPARγ2 activity and that the C-2821T alters PPARγ2 gene transcription. Previous studies have shown the alanine-containing protein to have a reduced ability to activate transcription via binding to PPAR response elements as compared with the proline-containing protein (15). Our in vitro studies comparing the –2821T and –2821C promoters demonstrated increased transcription from the –2821T promoter in both nondifferentiated and differentiated cells. Based on a consensus sequence search for potential transcription factor binding sites (MatInspector V2.2; TRANSFAC 4.0), we identified this polymorphism to be located in a binding site (ctcaACCtct) for the δ-crystallin enhancer binding protein δEF1 (29). δEF1 serves as a repressor of E2-box (CACCTG)–mediated gene activation, such as MyoD-induced myogenesis (30). Our in vitro studies also showed that PPARγ2 expression from a basal promoter alone is greater than expression with the additional δEF1 binding site–2821C allele, consistent with this site functioning as a transcription repressor.

Previous studies have shown that the PPARγ2 alanine allele encodes a protein with reduced activity (15), and this report shows that the PPARγ2–2821T allele has increased transcription. Yet, both of these alleles are associated with increased insulin sensitivity and decreased BMI. This apparent discrepancy has been previously addressed by Yamauchi et al. (31). They found that supraphysiological activation of PPARγ by agonist TZD increased triglyceride (TG) content of white adipose tissue (WAT), thereby decreasing TG levels in the liver and muscle, resulting in increased insulin sensitivity. However, moderate reduction of PPARγ activity in heterozygous PPARγ-deficient mice decreased TG content of WAT, skeletal muscle, and liver due to increased leptin expression, increased fatty acid combustion, and decreased lipogenesis, also resulting in increased insulin sensitivity (31). They conclude that, albeit by different mechanisms, both heterozygous PPARγ deficiency and PPARγ agonist ultimately result in improvement of insulin resistance.

Hepatic insulin sensitivity plays an important role in the pathogenesis of type 2 diabetes. Hyperglycemia results from excessive release of endogenous glucose due to increased gluconeogenesis, which is, in part, the consequence of decreased hepatic insulin sensitivity. The PPARγ2 variants could potentially influence suppression of gluconeogenesis by insulin via either a decreased release of free fatty acids or an increased release of adiponectin from adipocytes. Free fatty acids stimulate endogenous glucose production (32), whereas adiponectin inhibits endogenous glucose production in the liver (33).

Increased levels of alanine aminotransferase (ALT), a gluconeogenic enzyme, is also associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes in Pima Indians (34). Preliminary data indicate that the C-2821T SNP is associated with ALT levels in subjects with normal glucose tolerance (n = 209; P < 0.02 after adjusting for percent body fat and hepatic insulin sensitivity), whereas subjects with higher hepatic insulin sensitivity alleles (T/T) have lower ALT levels (B. Vozarova, unpublished observations). However, it remains unknown whether PPARγ2 influences hepatic insulin sensitivity through ALT. A recent study by Tschrirter et al. (35) showed that subjects with Ala/Ala or Pro/Ala genotypes have greater insulin clearance than subjects with a Pro/Pro, suggesting an alternate mechanism for increased hepatic insulin sensitivity in Ala allele carriers.

Performing multiple independent comparisons in a large dataset can result in false-positive associations. However, it remains debatable whether to adjust data for numbers of tests (36). The theoretical basis for advocating adjustment for multiple comparisons is the “universal null hypothesis,” where each hypothesis tested is independent to each other. Because the multiple hypotheses tested here are highly correlated, we did not perform a Bonferroni correction for independent comparisons. The reproducibility of our findings with those observed in other populations (15–19), combined with the known physiological role of PPARγ, lead us to believe that our associations are credible. However, we readily recognize that a conservative

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**TABLE 6**

Combined genotypes for the Pro12Ala and C-2821T SNP in 981 Pima subjects

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Pro/Pro</th>
<th>Pro/Ala</th>
<th>Ala/Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>326 (66.9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C/T</td>
<td>402 (66.7)</td>
<td>93 (60.2)</td>
<td>0</td>
</tr>
<tr>
<td>T/T</td>
<td>109 (72.5)</td>
<td>43 (65.1)</td>
<td>8 (50)</td>
</tr>
</tbody>
</table>

The percentage of diabetic subjects is given in parentheses.

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**FIG. 2.** Comparison of activity from promoters with either a C or T nucleotide in a putative E2 box. Activity was measured in transiently transfected 3T3-L1 cells in nondifferentiation medium (A) and differentiation medium (B). Luciferase activity is given as the ratio of firefly luciferase to renilla luciferase. Transfections were repeated five times under nondifferentiated conditions and six times under differentiated conditions. Negative control, 1-kb basal promoter, 1-kb basal promoter + C/C (–2821 bp), and 1-kb basal promoter + T/T (–2821 bp) are in indicated in white, black, and shaded boxes, respectively. P values (unpaired t test) are indicated between negative and 1-kb basal promoter or between C/C and T/T constructs.
adjustment for multiple comparison would render all of these associations nonsignificant.

In summary, a novel functional variation (C-282T) within the promoter of PPARγ2 has been identified. Similar to the well-characterized Pro12Ala, this promoter variant is associated with metabolic predictors of type 2 diabetes and obesity. In Pima Indians, this promoter polymorphism is in strong linkage disequilibrium with the Pro12Ala variant; therefore, the relative contribution of each SNP to the observed phenotypes remains unclear. However, in other populations there may be more genetic cross-over between the two variants, thus allowing better assessment as whether these SNPs interact with an additive effect or function independently. Further studies in other populations will be very useful in establishing the relative contribution of this promoter SNP, compared with the Pro12Ala, in determining obesity and type 2 diabetes. In addition, future studies are needed to fully evaluate all of the other common SNPs identified in the promoter of PPARγ and to elucidate the role of PPARγ in the development of type 2 diabetes and obesity.

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REFERENCES

5. Lin FT, Lane MD: CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. Proc Natl Acad Sci U S A 91:8757–5614, 1994
