Complex Haplotypes of the PGC-1α Gene Are Associated With Carbohydrate Metabolism and Type 2 Diabetes

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Peroxisome proliferator–activated receptor coactivator-1α (PGC-1α) is a transcriptional coactivator implicated in transcriptional programs of hepatic gluconeogenesis, oxidative phosphorylation, and insulin release by β-cells. To study associations of the PGC-1α gene locus with carbohydrate metabolism and type 2 diabetes in humans, we identified several polymorphisms in the promoter region that were located in a haplotype block distinct from a second haplotype block containing part of intron 2 and extending beyond exon 13. Each block contained five common haplotypes. Oral glucose tolerance testing revealed associations of promoter haplotype combinations with 30- and 60-min postload plasma glucose levels, whereas haplotypes in both blocks were associated with indexes of β-cell function. The associations of promoter haplotypes are supported by functional studies showing that some polymorphisms are located in transcription factor binding sites and affect transactivation in an allele-specific manner. By comparing patients with type 2 diabetes and control subjects, we observed borderline significant differences of four-loci haplotype distributions in the downstream haplotype block. Moreover, the haplotype that was associated with the strongest insulin response to glucose conferred the lowest risk of type 2 diabetes (P < 0.01). Thus, the PGC-1α gene locus influences carbohydrate metabolism and contributes to type 2 diabetes in the population studied. Diabetes 53:1385–1393, 2004

Insulin resistance, impaired compensatory upregulation of insulin secretion, and enhanced hepatic gluconeogenesis are the hallmarks of type 2 diabetes (1). Peroxisome proliferator–activated receptor coactivator-1α (PGC-1α) may play a pathogenic role in each component of this triad. PGC-1α interacts with hepatocyte nuclear factor (HNF)-4α, the glucocorticoid receptor, and the forkhead transcription factor FOXO 1 to execute transcriptional programs of insulin-regulated gluconeogenesis (2–4). In addition, PGC-1α enhances glucose uptake in muscle cells by the induction of GLUT4 expression via coactivation of the MADS box transcription enhancer factor (MEF) 2C (5). MEF2 family members, which are released from class II histone deacetylases in response to calcium signaling, transactivate the PGC-1α promoter (6,7). This autoregulatory mechanism may result in the stable induction of PGC-1α, which has been implicated in exercise-induced mitochondrial biogenesis and muscle fiber–type switching (8). Finally, elevated levels of PGC-1α in pancreatic β-cells have been shown to blunt the glucose-induced increase in cellular ATP levels and insulin exocytosis (9).

Studies in humans also support a role of PGC-1α in the pathogenesis of type 2 diabetes. Expression of PGC-1α and its target genes involved in oxidative phosphorylation were downregulated in muscle tissue of type 2 diabetic patients in comparison to control subjects (10). Moreover, skeletal muscle expression of PGC-1α and PGC-1β, another PGC-1 family member (11), was reduced in both diabetic subjects and family history–positive nondiabetic subjects compared with control subjects (12). Hence, genetic influences that affect PGC-1α and -1β expression in muscle may contribute to the disorder. Indeed, single nucleotide polymorphisms (SNPs) in the transcribed sequence of PGC-1α have been associated with type 2 diabetes in Danish, British, and Japanese but not in French populations (13–16). To gain further insight into the role of PGC-1α in humans, we identified and characterized sequence substitutions across its gene locus, determined their haplotype arrangement, and studied their association with carbohydrate metabolism and type 2 diabetes.

RESEARCH DESIGN AND METHODS

For haplotype characterization, we studied 40- to 65-year-old male and 45- to 70-year-old female participants of the Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk (SAPHIR). The overall study aim...
and recruitment procedures have been described (17). Associations of geno-
types, haplotypes, and haplotype combinations with estimates of glucose
metabolism were determined in a subset of 405 SAPHIR participants, who had
fasting plasma glucose levels 110 mg/dl and underwent an oral glucose
tolerance test (OGTT). After a 12-h overnight fast, subjects ingested a solution
containing 75 g glucose, and plasma samples were obtained at 0, 30, 60, and
120 min for determination of glucose and insulin. Insulin sensitivity/resistance
was estimated using the homeostasis model assessment (HOMA) insulin
resistance index ([HOMA_R]) (18), the composite insulin sensitivity
index ([HOMA_S]), and the metabolic clearance rate and S estimated by Stumvoll
et al. (20). For metabolic clearance rate and S calculations, average values of
60- and 120-min rather than 90-min glucose values were used. β-Cell function
was assessed by the HOMA-based insulin release index (HOMA IR),
first-phase and second-phase insulin release (20), the CIR (corrected insulin
response at 30 min), the 30- to 0-min insulin ratio (I_30/I_0), the 30-min
insulin-to-glucose ratio (I_30/G_30), and the insulogenic index estimated as
I_30/I_0/G_30 = G_30/(I_0)/(G_30) (21). A total of 506 unrelated patients with type
2 diabetes were recruited from diabetes outpatient clinics of the Landesklini-
klinik Salzburg and the Hospital Hallein. Patients who were <73 years of age
and <63 years of age at the time of diabetes diagnosis were included. SAPHIR
participants who were not using hypoglycemic medications and had fasting
blood glucose levels <110 mg/dl served as control subjects. Study populations
comprised only white Europeans, mainly of Bavarian or Austrian-German
descent living in the same geographic region. Participants provided informed
consent, which was approved by the local ethics committee.

Laboratory analyses. Plasma glucose, insulin, cholesterol, triglyceride, HDL
cholesterol, and HbA1c levels were measured as described (22). Genomic DNA
was extracted from peripheral white blood cells.

Identification of SNPs, genotyping, and haplotyping procedures. SNPs
in the promoter region of the human PGC-1α gene were identified by
amplification of a 5,551-bp fragment from genomic DNA of six human subjects
using the CCGTAACCGCACATCTCTGAGGCGCTCCTTG-3 and 5'-CGGAT
CGCTTCCAGCTCCTGAATGACGCCAG-3' forward and reverse primers,
respectively (NCBI glycan gene annotation no. NT_006316). Restriction enzyme
tsites for MluI and KpnI, respectively, are in bold. The resulting PCR products
were ligated into the pG3 basic vector (Promega, Madison, WI). Individual
clones and PCR products were sequenced from both directions by dye-
terminator sequencing using an ABI Prism 310 genetic analyzer (Applied
Biosystems, Foster City, CA). To delineate the phase of polymorphisms in 17
additional subjects, regions harboring SNPs were sequenced in PCR products
and in one clone representing the sequence from one chromosome. Sequence
differences between PCR products and clones thus provided the sequence of
the second chromosome.

Four SNPs that unambiguously predicted phased haplotypes were typed in
our population by restriction fragment length polymorphism (RFLP) detec-
tion with or without artificially introduced restric-
tion sites, using mismatch primers as well as real-time PCR. For the latter
method, allele-specific hybridization probes conjugated with a
fluorophore at the 5'-end were used with minor groove binder ligand and quencher moiety at
the 3'-end (Applied Biosystems). For quality control, 30% of real-time PCR-
based typings for SNP –1422 T/C were confirmed using an RFLP assay. Assay
details are shown in Table 1 in the online appendix (available at http://diabetes.
diabetesjournals.org).

To search for additional sequence variants in the transcribed region, we
sequenced 12 alleles from the transcriptional start site to 300 bp beyond the
more-downstream-located polyadenylation signal, but excluding the large
intron 2. The +1302G/A, +1654G/A, and 1704A/G polymorphisms in exon 8
and the +2962A/G polymorphism in the 3'-untranslated region of the PGC-1α
gene (Genbank accession no. NM_013251) were typed in our populations as
described (22,23). Haplotypes were estimated from unphased genotypes using
the expectation-maximization algorithm of Terwilliger and Ott (24). Four-loci
genotype determinations in both the promoter and the transcribed region were
available in 1,698 SAPHIR participants. In the 405 glucose-tolerant
SAPHIR participants who underwent an OGTT, four-loci genotypes in
the promoter or transcribed region were available in 361 or 398 subjects,
respectively. In the 506 patients with type 2 diabetes and in the nondiabetic
1,489 SAPHIR participants with four-loci promoter genotype determinations,
four-loci genotypes of the transcribed region were available in 494 and 1,478
subjects, respectively.

To identify haplotype boundaries, regions in intron 2 as well as upstream and
downstream regions of PGC-1α that contained several SNPs (www.ncbi.
nlm.nih.gov/SNP) were sequenced in eight subjects to identify polymor-
phisms. Four SNPs at +22174, +16622, +55198, and +114481 relative to the
translational start site were typed in >84 randomly selected SAPHIR participants.

Plasmid constructs. PGC-1α promoter reporter plasmids encompassing
SNPs at positions −1894, −1774, −1679 and −1422 were constructed by
amplification of a 732-bp fragment from genomic DNA of patients with known
haplotypes. The reporter constructs −3959 TK-Luc, −3818 TK-Luc, −3614
TK-Luc, and −1422 TK-Luc were generated by amplifying −250 to 350-bp
fragments encompassing the respective sites using genomic DNA from
heterozygous subjects. Primers, PCR product fragment lengths, and restric-
tion enzymes used for cloning are listed in Table 2 in the online appendix.

Plasmids were cloned into a reporter vector harboring the firefly luciferase
gene under the TK promoter (25,26).

Primer sequences used to clone full-length HNF-1α into pcDNA6/V5-His
(Invitrogen, Carlsbad, CA) were based on GenBank NM_012120. A 728-bp
fragment was amplified from human liver cDNA using 5'-GAGAGGCCGGCCG
CAGCCGTAAACACGGAGCCG-3' and 5'-GAGAGCCCGCCGCAAGTGGCG
TGTTAGCTGGAGG-3' (stop codon underlined) as forward and reverse
primers, respectively. For construction of a full-length MEF2C expression
plasmid (Genbank accession no. NM_002397), we amplified a 1,438-bp frag-
ment from human muscle cDNA using 5'-GAGAGGCCGGCCGCAATATTTCG
GGGAGGAGGAGGAGG-3' and 5'-GAGAGCCGGCCGCAAGTGGCG
TGTTAGCTGGAGG-3' (stop codon underlined) as forward and reverse
primers, respectively. Nof restriction enzyme sites introduced are in bold. All
plasmid constructs were verified by sequencing.

Cell culture and transfection. INS-1E cells, kindly provided by Claes
Wollheim (Geneva, Switzerland); PAZ6 cells, and HepG2 cells were cultured
as described (17,22). Near-confluent cells were transfected with constant
amounts of reporter constructs and 500 ng/well of HNF-1α or MEF2C
expression plasmids or their empty pcDNA6/V5-His plasmid backbone using
Lipofectamine 2000 reagent, pRL-CMV (1 ng/well; Promega) was cotrans-
formed as transfection control. Transfection medium was replaced with fresh
culture medium 12 h later. Cells were harvested 24 h after transfection, and
firefly and Renilla luciferase activities were measured in cell extracts using a
Luc2 luminometer (Roushys Anhous, Salzburg, Austria) and the Dual-Luciferase
reporter assay system (Promega).

Computational analyses and statistics. For prediction of putative trans-
scription factor binding sites, we used MatInspector professional software
(www.genomatix.de), which locates individual matches by comparing the
sequences with weight matrix descriptions of binding sites (27). The matrix
library is based on Transfac release 3.3 (28). We considered matrix and core
similarities >80 and >95%, respectively.

Group differences of continuous variables were ascertained by ANOVA. We
made logarithmic transformations if the equal variance and normality assump-
tions of ANOVA were rejected. Measurements were adjusted for the effects of
age and BMI as indicated. Two-way ANOVA was used to compare reporter-
genotype activities from variant and wild-type alleles as well as transactivation
from PGC-1α promoter alleles by MEF2C and HNF-1α. For post hoc compar-
isons of means, Tukey's honest significant difference test was used.

Allele frequencies were estimated by gene counting. Agreement with Hardy-
Weinberg expectations was tested using a chi^2 goodness-of-fit test. The standard
of pairwise linkage disequilibrium statistics (D^2) were estimated (24).
For effects of individual SNPs on OGTT parameters, we used ANOVA with
analysis of variance for age, sex, and a combination of those variance. Differences in
the additive, dominant, or recessive models, respectively. Differences in genotype or allele
frequencies between patients with type 2 diabetes and control subjects were
determined using a chi^2 distribution with 2 or 1 degrees of freedom. Differences in
loci haplotype distributions between patients and control subjects were
ascertained by the log-likelihood ratio test with 15 degrees of freedom
(29). For testing of associations between haplotypes and quantitative or binary
traits, we used haplo.score software (http://www.mayo.edu/statgen/). This
method is based on efficient score statistics, which provides both global and
haplotype-specific tests and avoids the conclusion of rare haplotypes as well as
association for nongenetic covariates (30).

RESULTS

We identified eight polymorphic sites between −1422 and
−3959 (Table 3 in the online appendix and Fig. 1) by sequenc-
ing 12 promoter alleles up to −5551 relative to the
translational start site. Of the 2^16 (= 256) possible combina-
tions of these SNPs, only five haplotypes were observed in
the 46 alleles in which phased genotypes were determined
(Fig. 1B). Thus, SNPs at −3959, −3690, −1984,
−1774 as well as SNPs at −3614 and −1679 showed

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complete linkage disequilibrium. Four SNPs (at $-3959$, $-3818$, $-1679$, and $-1422$) that permitted unambiguous assignments of phased haplotypes were typed in 1,608 SAPHIR participants. All polymorphisms fulfilled Hardy-Weinberg expectations. Variant allele frequencies of promoter polymorphisms are shown in Table 3 in the online appendix. Standardized pairwise linkage disequilibrium statistics ($D_r$) were highly significant for the promoter polymorphisms (Table 1), and the five haplotypes delineated by phased genotyping accounted for 97% of all haplotypes defined by the respective sites (Fig. 1B).

We also ascertained haplotype structures across the transcribed region of $P_g-c_1$. Our sequencing studies confirmed previously identified polymorphic sites in introns 1, 2, 4, and 5 as well as in exons 8, 9, and 13, but they failed to detect additional sequence variants. Frequencies of the variant alleles are also shown in Table 3 in the online appendix. Typing of four informative polymorphisms in exons 8 (at $+1302$, $+1564$, $+1704$, and $+2962$) revealed strong linkage disequilibria (Table 1), and estimation of haplotype frequencies identified five common haplotypes (Fig. 1C) that accounted for 98% of haplotypes comprising the respective sites. However, at most, only weak linkage disequilibria were observed with the variant promoter sites (Table 1). Hence, two haplotype blocks, termed haplotype block 1 and 2, each characterized by five common haplotypes, were identified within $P_g-c_1$. Based on additional typing studies of SNPs in introns 2 and 5, the boundary between the blocks was located in intron 2 between SNPs at $+16022$ and $+55180$, relative to the translational start site. Haplotype block 1 extended to $22$ kb upstream of the translational start site, whereas haplotype block 2 extended $<20$ kb beyond the proximal polyA signal (Fig. 1A and Table 1).

Among the promoter polymorphisms typed, two sites displayed significant associations with oral glucose tolerance test parameters in glucose-tolerant subjects. Clinical characteristics of the study population are shown in Table 4 in the online appendix. The SNP at $-3959$ was associated with differences in 30-min postload insulin levels (Fig. 2) and parameters reflecting insulin release, such as first-phase, second-phase, and 30-min insulin-tolerance.
TABLE 1

Standardized pairwise linkage disequilibrium coefficients ($D'$) of polymorphic sites at the PGC-1α gene locus

<table>
<thead>
<tr>
<th>SNP</th>
<th>T/C</th>
<th>T/G</th>
<th>A/G</th>
<th>A/T</th>
<th>G/A</th>
<th>G/A</th>
<th>A/G</th>
<th>A/G</th>
<th>+114481</th>
</tr>
</thead>
<tbody>
<tr>
<td>−22174 T/C</td>
<td>+0.887</td>
<td>−0.912</td>
<td>−0.512</td>
<td>−0.312</td>
<td>+0.333</td>
<td>−0.333</td>
<td>−0.307</td>
<td>+0.069</td>
<td>+0.074</td>
</tr>
<tr>
<td>−3959 T/C</td>
<td>−0.968</td>
<td>−0.984</td>
<td>−0.305</td>
<td>+0.684</td>
<td>−0.018</td>
<td>−0.080</td>
<td>+0.055</td>
<td>−0.230</td>
<td>−0.192</td>
</tr>
<tr>
<td>−3818 T/G</td>
<td>−0.981</td>
<td>−0.955</td>
<td>+0.423</td>
<td>+0.348</td>
<td>+0.515</td>
<td>+0.094</td>
<td>+0.003</td>
<td>+0.051</td>
<td>+0.005</td>
</tr>
<tr>
<td>−1679 A/G</td>
<td>−0.951</td>
<td>−0.461</td>
<td>−0.305</td>
<td>+0.387</td>
<td>−0.385</td>
<td>−0.135</td>
<td>−0.170</td>
<td>−0.207</td>
<td>−0.479</td>
</tr>
<tr>
<td>−1422 T/C</td>
<td>−0.741</td>
<td>−0.244</td>
<td>−0.105</td>
<td>+0.138</td>
<td>+0.103</td>
<td>+0.127</td>
<td>+0.121</td>
<td>+0.162</td>
<td></td>
</tr>
<tr>
<td>+16022 T/C</td>
<td>+0.056</td>
<td>+0.043</td>
<td>+0.085</td>
<td>−0.262</td>
<td>−0.221</td>
<td>−0.126</td>
<td>+0.054</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+55180 A/G</td>
<td>+1.000</td>
<td>+1.000</td>
<td>+0.048</td>
<td>+0.938</td>
<td>−0.900</td>
<td>−0.725</td>
<td>−0.511</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+64456 A/T</td>
<td>+0.048</td>
<td>+0.938</td>
<td>−0.900</td>
<td>−0.725</td>
<td>−0.511</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+75656 G/A</td>
<td>0.924</td>
<td>0.921</td>
<td>0.926</td>
<td>−0.112</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>+75918 G/A</td>
<td>−0.970</td>
<td>−0.977</td>
<td>−0.549</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>+76058 A/G</td>
<td>0.980</td>
<td>0.431</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+94580 A/G</td>
<td>0.015</td>
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</tr>
</tbody>
</table>

SNPs at −22174, +16022, +55180, +64456, and +114481 were determined in >84 subjects. All other SNPs were determined in 1,608 subjects.

Polymorphisms are identified by their nucleotide positions in the genomic sequence relative to the translational start site. SNPs at +16022 and +55180 are located in intron 2, and SNP at +64456 is located in intron 5. SNPs at +75656, +75918, +76058, and +94580 in the coding region correspond to positions +1302, +1564, +1704, and +2962, respectively, in the mRNA sequence relative to the translational start site (23). $+/−$ linkage among sites. $D' > 0.90$ (●); $D' > 0.70$ (▲).

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**FIG. 2.** Associations of −3959 T/C genotypes with OGTT glucose and insulin levels and effects on PGC-1α gene expression in INS1-E cells. Time-dependent values for plasma glucose (A) and insulin (B) in response to 75 g glucose in glucose-tolerant patients differing by −3959 T/C genotypes. Error bars indicate SE. For T/T, T/C, and C/C subjects, $n$ was 238, 110, and 13, respectively. *$P < 0.02$, ANOVA, adjusted for age, sex, and BMI. C INS1-E cells were transiently transfected with −3959T TK-Luc and −3959C TK-Luc. Results are means ± SD and representative for one of two experiments, each performed in quadruplicate. **$P < 0.001$. D: Transcription factors and their binding matrices in relation to the PGC-1α promoter region encompassing the −959 T/C polymorphism. Variable nucleotides in the PGC-1α sequence and respective nucleotides in binding sequences of candidate transcription factors are in bold. Numbers refer to nucleotide positions relative to the transcriptional start site. The core sequences of binding sites are in upper case letters. Nucleotides underlined exhibit a high percentage of conservation. Binding matrices allowing for more than one nucleotide at a position are given in International Union of Pure and Applied Chemistry (IUPAC) standard codes for nucleic acids: $w = A,T; r = G,A; s = C,G$, and $m = A,C.$
glucose ratio ($P < 0.02$). The SNP at −1679 was associated with differences in 30- and 60-min postload glucose levels (Fig. 3) and the integrated area of glucose ($P < 0.015$, ANOVA). Linear regression models were most consistent with additive and variant-allele recessive effects at the −1679 sites, respectively. Neither polymorphism was associated with measures of insulin sensitivity such as HOMA$_{IR}$, $S_{(comp)}$, metabolic clearance rate, or $S_i$ (data not shown). Haplotype-specific analyses showed a consistent pattern of associations with parameters reflecting the insulin secretory response to glucose (Table 2), but not with 30- or 60-min glucose levels and integrated glucose areas. However, common four-loci promoter haplotype combinations showed associations with 30- and 60-min postload glucose levels and integrated postload glucose areas (Table 5 in the online appendix), a result

TABLE 2
PGC-1α haplotype scores for indexes of insulin secretion

<table>
<thead>
<tr>
<th>Indexes</th>
<th>Block 1 haplotypes</th>
<th>Block 2 haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0000 0001 0101 0010 0000 0001 0011 0111 0000 0001 0111 1000</td>
<td>0000 0001 0101 0010 0000 0001 0011 0111 0000 0001 0111 1000</td>
</tr>
<tr>
<td>Frequencies</td>
<td>0.063 0.28 0.14 0.31 0.18 —</td>
<td>0.328 0.096 0.061 0.333 0.166 —</td>
</tr>
<tr>
<td>HOMA$_{secr}$</td>
<td>−0.89 −2.27†</td>
<td>−0.89 −2.27†</td>
</tr>
<tr>
<td>First phase</td>
<td>2.19† −1.48</td>
<td>2.19† 0.63 −0.85 −1.36 −1.43 0.091</td>
</tr>
<tr>
<td>Second phase</td>
<td>−1.96† −1.47 −0.07 0.41 2.09† 0.079 2.27† 0.05 −0.53 −1.63 −1.43 0.09</td>
<td></td>
</tr>
<tr>
<td>$I_{s0}/I_{c}$</td>
<td>−1.26 0.15 −0.61 0.45 0.78 0.634 2.76† −0.34 −1.63 −0.81 −1.25 0.022</td>
<td></td>
</tr>
<tr>
<td>$I_{s0}/G_{30}$</td>
<td>−1.96† −1.45 0.01 0.57 2.13† 0.126 3.14§ 0.45 −0.66 −2.07† 1.60 0.005</td>
<td></td>
</tr>
<tr>
<td>CIR$_{30}$</td>
<td>−1.44 −1.50 −0.09 0.83 1.64 0.290 2.89§ 0.73 −0.60 −1.98† −1.63 0.016</td>
<td></td>
</tr>
<tr>
<td>$\Delta I_{s0}/\Delta G_{30}$</td>
<td>−1.73 −1.80 −0.05 0.75 2.09† 0.103 2.19† 0.16 −1.56 −1.69 −0.68 0.069</td>
<td></td>
</tr>
</tbody>
</table>

*Global statistics for score comparisons of haplotypes with estimated frequencies $>0.01$; †$P < 0.05$, ‡$P < 0.01$, or §$P < 0.005$ for haplotype-specific statistics. Scores of haplotypes, which were assigned numbers in the order of the respective polymorphism along the PGC-1α gene, were estimated by score statistics (30). Block 1 haplotypes: 0, more common allele (−3959T, −3818T, −1679A, −1422T); 1, less common allele (−3959C, −3818G, −1679G, −1422C). Block 2 haplotypes: 0, more common allele (+1302G, +1564G, +1704A, +2962A); 1, less common allele (+1302A, +1564A, +1704G, +2962G).
compatible with the apparently recessive effect of the −1679 variant allele. None of the four SNPs in haplotype block 2 displayed an association with any of the OGTT parameters, but global and haplotype-specific statistics showed significant associations with calculated parameters of insulin response to glucose (Table 2). Haplotype block 2 combinations showed similar associations (data not shown).

Computational analyses of the 5′-flanking region of PGC-1α suggested that some polymorphic sites are located in cis regulatory elements that may affect interactions with various trans factors in an allele-specific manner, thereby modulating PGC-1α expression levels. Comparison of transfections with the −3959T TK-Luc and −3959C TK-Luc reporter constructs in INS1-E cells revealed lower reporter gene activity of plasmids containing the variant nucleotide (Fig. 2C), but no differences were noted in HepG2 and PAZ6 cells (data not shown). The inverse relationship of PGC-1α gene expression with indexes of insulin release in our study subjects is consistent with the proposed suppression of insulin release by PGC-1α (9). Transcription factors predicted to bind to the sequence harboring the polymorphic site at −3,959 are shown in Fig. 2D. We did not observe an effect of nucleotide substitutions at position −3818 on PGC-1α gene expression in HepG2, INS1-E, or PAZ6 cells (data not shown).

The polymorphic site at −1679 was associated with postload glucose levels and had little effect in transient expression studies (Fig. 1 in the online appendix), but it was in perfect linkage disequilibrium with the SNP at −3614, which is located within a potential HNF-1α binding site (Fig. 3). Because HNF-1α has been implicated in the regulation of hepatic gluconeogenesis (31), we performed transient transfection studies using the −3614A TK-Luc and −3614C TK-Luc reporter constructs as well as co-transfections with the HNF-1α expression plasmid in HepG2 cells. PGC-1α expression from the variant −3614C allele was significantly decreased in both the absence and presence of HNF-1α overexpression (Fig. 3C). Thus, the reduced promoter activity of the −3614 variant allele paralleled the lower 30- and 60-min postload glucose levels in subjects homozygous for the −1679 variant allele (Fig. 3A). Reporter gene activities of the variant and wild-type alleles were similar in PAZ6 or INS1-E cells (data not shown). Because overexpression of HNF-1α in HepG2 cells resulted in an approximately threefold induction of luciferase activity from both the −3614A TK-Luc and −3614C TK-Luc constructs (Fig. 3C), HNF-1α may participate in the regulation of PGC-1α expression.

The SNP at −1422T is located within a putative MEF2C binding site previously identified in the murine PGC-1α promoter (6,7). The SNP at −774 is contained within another potential MEF2C binding site not conserved in the murine promoter. Contransfections studies with or without MEF2C expression plasmids revealed that constructs harboring the variant nucleotide at position −1422 increased reporter gene activity in HepG2 cells, in comparison to constructs harboring the wild-type allele (Fig. 1 in the online appendix).

To ascertain associations of the PGC-1α gene locus with type 2 diabetes, we determined genotype distributions in type 2 diabetic patients and control subjects. Clinical data of patients and control subjects are shown in Table 6 in the online appendix. None of the eight polymorphic sites displayed associations with type 2 diabetes in sex-specific or combined analyses in our population (data not shown). Four-loci haplotype distributions of haplotype block 2, but not haplotype block 1, showed borderline significant differences (Table 3). Score testing revealed that the common haplotype in block 2 that carries wild-type nucle-
otides at all four polymorphic sites was associated with the lowest risk of type 2 diabetes \((P < 0.01)\). The same haplotype was associated with the strongest insulin secretory response to glucose (Table 2). Conversely, the common block 2 haplotype that contained variant nucleotides at the three distal polymorphic sites, and displayed the lowest scores for insulin secretory indexes, exhibited the highest score for type 2 diabetes among common haplotypes. Thus, effects of PGC-1α on the insulin response to glucose are likely to contribute to its association with type 2 diabetes.

**DISCUSSION**

Previous studies in animal models and humans strongly support a role of PGC-1α in the pathogenesis of type 2 diabetes. In this work, we have delineated SNPs within contiguous regions of the PGC-1α gene promoter and the transcribed sequence. The SNPs in the promoter region comprise a haplotype block that is distinct from a second haplotype block harboring SNPs downstream of intron 2. Each block contains five common haplotypes. Other studies that applied high marker densities over contiguous genome regions also demonstrated blocks of variable length. Such blocks typically contain only few haplotypes separated by sites at which recombinations occurred in the ancestors of current populations (32,33). In glucose-tolerant subjects, haplotype block 1 showed discrete associations with postload glucose levels, whereas both haplotype blocks were associated with indexes of the insulin secretory response to glucose. Moreover, a common specific haplotype in block 2 revealed associations with β-cell function and type 2 diabetes risk.

Our transient transfection studies in human cell lines identified several promoter SNPs that modulate promoter function, but it is likely that other functional sites in haplotype block 1 might exist that affect carbohydrate metabolism and/or type 2 diabetes risk. Nucleotides at −1422 and −1774 are located within MEF2 binding sites. Even though both sites are required for full transactivation, only the −1422 site displayed allele-specific promoter effects, but by itself it showed no associations with OGTT parameters or type 2 diabetes. Because MEF2 has been implicated in skeletal muscle GLUT4 expression (5,34), and transcriptional responses of the PGC-1α promoter to MEF2 may play an important role in programs controlling muscle fiber-type conversion (7,8) and cardiac energy production (6), the −1422 site may contribute to the pathogenesis of other disease phenotypes or to type 2 diabetes in other populations.

The −1679 polymorphic site as well as common haplotype combinations reflecting the integrated effects of promoter polymorphisms were associated with differences in 30- and 60-min postload glucose levels, and functionality of the perfectly linked polymorphic site at −3614 was established by transient transfection studies in HepG2 cells. Whether the association of the −3614/−1679 SNPs with decreased postload glucose levels reflects changes in glucose uptake or glucose production is not known, but both possibilities appear plausible. PGC-1α has been shown to participate in insulin-regulated hepatic gluconeogenesis by coactivating HNF-4α and FOXO1 (2–4). Moreover, hepatic gluconeogenesis contributes to glucose effective-ness ascertained in human studies (35,36). Conversely, PGC-1α enhances oxidative phosphorylation and glucose uptake in skeletal muscle (5,10,12). Hence, the variant allele or another functional site in linkage disequilibrium with it may have tissue-specific effects and enhance PGC-1α expression in skeletal muscle. Our cotransfection studies further suggest that HNF-1α contributes to the regulation of PGCG1α promoter activity. HNF-1α and HNF-4α, which is coactivated by PGC-1α, have been shown to activate the expression of each other (37). Mice lacking HNF-4α in liver exhibit higher PGC-1α mRNA levels than control mice (38). It is thus possible that HNF-1α is part of a regulatory loop that controls PGC-1α expression in liver.

The effects of the −3959 variant site were tissue-specific, since the less common allele reduced reporter gene activity in INS1-E cells but not in HepG2 or PAZ6 cells. An inverse association between PGC-1α expression and insulin secretion is supported by recent studies (9). The influence of this SNP was also apparent in a four-loci haplotype context because the haplotype harboring the −3959 variant nucleotide revealed the highest scores for insulin secretion indexes.

Haplotypes of block 2 revealed strong associations with derived estimates of the insulin secretory response to glucose. Associations of +1564G/A with lipid oxidation and early insulin secretion have been reported in Pima Indians (39). Even though we did not observe such an association, the results in Pima Indians are not inconsistent with our findings because haplotype block size, allele frequencies, and linkage disequilibrium relationships differ among populations (31). The absence of associations with individual polymorphisms in our population suggests that the block 2 SNPs studied are not major functional sites. Such site(s) may be located in introns and affect mRNA expression or splicing. Indeed, a PGC-1α isoform devoid of exon 8 and induced by exercise has been observed in rat skeletal muscle (40). Using RNase protection assays, we have recently observed distinct PGC-1α isoforms lacking specific interfaces in various human tissues (M. Xie, H.O., M. Roden, W.P., unpublished observations), but their pathophysiological significance awaits further analyses.

Given the role of PGC-1α in mitochondrial biogenesis and oxidative phosphorylation (10,12), the lack of associations with indexes of insulin resistance in either haplotype block was surprising. Imprecision in the ascertainment of insulin resistance may have contributed (41). Furthermore, combined effects of both haplotype blocks could not be determined because of sample size constraints. Interestingly, muscle-specific overexpression of PGC-1α in transgenic mice was shown to reduce GLUT4 expression (42).

The borderline-significant different haplotype distributions in block 2 between patients with type 2 diabetes and control subjects suggest, but do not prove, that the PGC-1α gene locus contributes to the susceptibility for type 2 diabetes. Importantly, the haplotype that displayed the lowest score for type 2 diabetes was associated with the highest scores for indexes of β-cell function, thus providing physiological plausibility and internal consistency of results. Associations of the +1564 site with type 2 diabetes have been reported in a Danish population (13), but these results are at variance with studies in French and
Japanese populations as well as with our findings. Nevertheless, two-loci haplotypes comprising the +1564 SNP displayed associations with diabetes in Japanese subjects. Among the three European populations, variant allele frequencies in subjects with type 2 diabetes were similar and ranged from 0.363 to 0.373, but they were slightly higher in French and Austrian than in Danish nondiabetic subjects (0.343 and 0.348 vs. 0.306). However, in all four populations, variant allele frequencies were higher in patients than in control subjects. It is therefore conceivable that the +1564 polymorphic site is not the main causal site, but is contained within haplotype block 2, that contributes to type 2 diabetes in several populations.

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