The concordance rate of non-insulin-dependent diabetes mellitus (NIDDM) in identical twins has been reported to be from 60 to 95% (1, 2), but segregation analyses have not been consistent with Mendelian inheritance of a single major disease gene (3). Therefore, NIDDM is a probable multifactorial disorder (4). Although multiple loci might influence diabetes susceptibility, the defects in single genes may possibly be found by studying phenotypes that predispose individuals to NIDDM, such as degree of insulin resistance and β-cell function. Insulin resistance and obesity are major risk factors for the disease in Pima Indians (5), and insulin action in vivo aggregates in families (6), as does obesity (7). At maximally stimulating insulin concentrations, insulin action has a trimodal frequency distribution, which is consistent with the hypothesis that maximal insulin action is determined by a single gene with a codominant mode of inheritance (8). Recent evidence has suggested that a putative gene exists on chromosome 4q determining maximal insulin action in Pima Indians (9). A relatively low acute insulin resistance (AIR), as measured by an intravenous glucose tolerance test (IVGTT), also predicts the development of NIDDM in insulin-resistant Pima Indians (10), and we have recently begun a search for genetic markers of AIR in this population.

We studied two candidate genes with polymorphic dinucleotide repeats in a large group of Pima Indians for linkage to AIR (11). Both the liver/islet glucose transporter gene (GLUT2) and the glucokinase gene are thought to be components of the glucose-sensing apparatus (12) and may regulate insulin release from β-cells in response changes in external glucose concentrations. GLUT2 is the major glucose transporter of the pancreatic β-cells and hepatocytes (13) and has a higher $K_m$ for glucose than $V_{max}$ than any other known glucokinase (14–16). The cloning and characterization of the human GLUT2 gene has been reported recently (17). A functional defect in high-$K_m$ glucose transport could result in impaired β-cell response to plasma glucose concentrations and lead to uncorrected hyperglycemia (18). Only one positive association between GLUT2 and NIDDM (19) has been observed among several population studies (20–22), and linkage analyses between NIDDM and GLUT2 also have produced negative results (23,24). However, these studies do not exclude the possibility of a defect in GLUT2 that leads to a relatively low AIR in the Pima Indians.

The rate of glucose metabolism is controlled largely by glucokinase in pancreatic islet β-cells; thus, this enzyme controls the first rate-limiting reaction in glycolysis (12). Significant expression of glucokinase appears restricted to β-cells and hepatocytes (25). Mutations in the glucokinase gene have been previously shown to contribute to the dominantly inherited, early-onset form of NIDDM (26–29) and gestational diabetes (30). The identified mutations have been suggested to lead to early-onset NIDDM by a gene-dosage mechanism (26–29), and the demonstration of decreased enzymatic activity of some of these mutations is
consistent with this hypothesis (31). Many missense mutations in the glucokinase gene significantly change the $K_m$ and $V_{max}$ of the mutant protein (31), whereas four nonsense mutations identified previously result in the synthesis of a truncated form of glucokinase (29-31). We have studied the contributions of possible mutations in the GLUT2 and glucokinase genes to the variation in AIRs and NIDDM in the Pima Indians.

RESEARCH DESIGN AND METHODS

The Pima Indian volunteers in this study are participants in a longitudinal study of risk factors for the development of NIDDM (32), performed by the National Institutes of Health (NIH) in Phoenix, Arizona. The study was conducted with the approval of the Arizona State University Human Subjects Institutional Review Board, by the ethics committees of the National Institute of Diabetes and Digestive and Kidney Diseases and the Indian Health Service, and by the tribal council of the Gila River Indian Community. Informed consent was obtained from all subjects. Diabetic subjects were identified by a 2-h postload plasma glucose concentration >7.8 mmol/l and by the National Health and Nutrition Examination Survey (NHANES III) criteria (33). Of the 796 subjects analyzed for the analyses reported in this study.

A subset of volunteers was admitted to the clinical research ward for 8-16 days and fed a weight-maintaining diet. After 3 days, each subject underwent several tests after an overnight fast including a 75-g oral glucose tolerance test (33), underwater weighing to determine body composition (34), and an IVGTT to measure AIR. A 25-g intravenous injection of glucose (50% solution) was given over 3-4 min, and time 0 was when 12.5 g had been infused. Samples were collected at 3, 4, 5, 6, 8, and 10 min for determination of glucose and insulin concentrations.

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Fasting plasma insulin levels used in these analyses are the mean of two measurements taken before the intravenous injection of glucose. Measurements of plasma insulin and glucose concentrations were determined as described recently (9).

**Table 1**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers (5' to 3')</th>
<th>Product size (bp)</th>
<th>MgCl$_2$ concentration (mM)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1a-ATAACTAGGCTAGGCGAGAG</td>
<td>197</td>
<td>2.0</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>1b-CTCCGCTAGGCTAGGCTAGTGC</td>
<td>301</td>
<td>1.5</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>2a-CACCCTGAGGACCTCCTAG</td>
<td>475</td>
<td>2.0</td>
<td>54</td>
</tr>
<tr>
<td>4A</td>
<td>2b-CTTCTGTCCTCCACCTGGAG</td>
<td>235</td>
<td>1.5</td>
<td>58</td>
</tr>
<tr>
<td>4B</td>
<td>3a-ACATTAATGAGATCTGTTGAC</td>
<td>212</td>
<td>1.0</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>4Aa-GCTGGAATTTATATCCCTTGCG</td>
<td>295</td>
<td>1.5</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>4Bb-TGAGATAGTCCTGGGATTGAC</td>
<td>269</td>
<td>1.5</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>5a-GGTTAGTTCAAGATTATTTG</td>
<td>190</td>
<td>4.0</td>
<td>56</td>
</tr>
<tr>
<td>8</td>
<td>6a-ACTGAGCTCAGGAGCCATG</td>
<td>195</td>
<td>2.0</td>
<td>57</td>
</tr>
<tr>
<td>9 + 10</td>
<td>7b-GGACTAAGGAACAAGCAGAG</td>
<td>585</td>
<td>1.5</td>
<td>54</td>
</tr>
</tbody>
</table>

**Dinucleotide repeat analysis.** Multiplex polymerase chain reaction (PCR) was employed to screen two dinucleotide repeat regions in 796 Pima Indians: one in intron 4A of the GLUT2 gene (35) and the other, 10 kilobase downstream of the glucokinase gene (36). Oligonucleotide primers were synthesized on a DNA synthesizer at the NIH (model 391, Applied Biosystems, Foster City, CA). For GLUT2, the 5' primer (5'-ATTGCTGGAAGAAGCATATGACG-3') and the 3' primer (5'-TCTGTCATTGTCACCCCTAGTCAA-3') flanked 155-167 base pair (bp) of the region of dinucleotide repeats. For glucokinase, the 5' primer (5'-CCACACAAACTGCGGTATATG-3') and the 3' primer (5'-TTGGTGCACTGGAACGCACTGG-3') flanked 196-199 bp of the repeat region. The 5' primers were end labeled with 33P-ATP (3,000 Ci/mmol, Du Pont-NEN, Boston, MA) using T4 polynucleotide kinase. PCRs were conducted in 10 μl vol with 100 ng genomic DNA, 2 pmol end-labeled 5' primers, 5 pmol 3' primers, and a GeneAmp PCR core reagents kit (Perkin-Elmer/Cetus, Norwalk, CT). Samples were processed through 25 cycles of amplification consisting of denaturation for 30 s at 94°C, annealing for 30 s at 65°C, and extension for 45 s at 72°C with the use of a GeneAmp PCR system 9600 (Perkin-Elmer/Cetus). The labeled PCR products were heat denatured and separated on 6% sequencing gels. Autoradiography was performed for 16 h at room temperature.

**PCR-single-strand conformation polymorphism (SSCP) analysis.** Screening for mutations was performed on the coding region (11 exons) of the GLUT2 gene in 40 subjects with NGT but with a wide range of AIRs by using the PCR-SSCP technique. Sequence information for the primers is shown in Table 1. The 3' primers for each exon were end labeled with 33P-ATP, and PCR was conducted as described above, except for varying MgCl$_2$ concentrations (Table 1). Samples were processed through 25 cycles of 30 s denaturation at 94°C, 60 s annealing at optimum temperature (Table 1), and 45 s extension at 72°C. Exon 3 PCR products were cut with PvuII before electrophoresis to reduce the product size for increased sensitivity. Restriction digestions were performed using a 10 μl PCR product, 1.2 μl of 25 mM MgCl$_2$, and 1 μl PvuII (20 U/μl, BRL Life Technologies, Gaithersburg, MD). Samples were digested for 2 h at 37°C, cutting the PCR product into 211 and 284 bp fragments. PCR products of all exons were heat denatured and then separated on two different types of nondenaturing polyacrylamide gels.

One type of gel was 5% hydrophilic long ranger gel (AT Biochem, Malvern, PA) with 1.2 X TBE, run at 25 W at room temperature. The other type was an 8% nondenaturing polyacrylamide gel with a 10% acrylamide-bisacrylamide concentration, 5% glycerol, and 1 X TBE that was run at 25 W at room temperature. Autoradiography was performed for 16 h at room temperature.

When a mobility shift was detected within an exon of GLUT2, these samples required direct sequencing using the dsDNA cycle sequencing system (BRL Life Technologies). PCRs were conducted in 10 μl vol using 100 ng genomic DNA, 10 pmol 5' and 3' primers, GeneAmp kit reagents, and optimum MgCl$_2$ concentration (Table 1). PCR conditions were the same as PCR-SSCP conditions described above. PCR products were concentrated on Centricon 100 microconcentrator columns (Amicon, Beverly, MA) to remove excess primers and dNTPs (dinucleotides, triphosphates), according to the instructions of the manufacturer. The instructions of the cycle sequencing system were followed for sequencing the PCR products except that 10 μCi of 33P-ATP was used per primer in the end-labeling reaction. After PCR amplification, samples were heat denatured and separated on 6% denaturing polyacrylamide gels and run...
GLUT2 GENE IN PIMA INDIANS

for 1.25 h at 70 W. Autoradiography was performed for 16 h at room
temperature.

Statistical analysis. Statistical analysis was performed using programs
of the SAS Institute (Cary, NC). Multiple linear regression analysis was
performed with the general linear model program. Sex, percentage of
body fat, and family membership were the independent variables. Each
individual within the same family was assigned the same value of an
indicator variable to denote family assignment. Family, therefore, is a set
of binary indicator variables signifying membership in each sibship. The
significance for the family effect was determined by the F statistic for
variability among families compared with within-family variability in
models that included the independent variables. The log_{10} of mean
plasma insulin concentrations from 3, 4, and 5 min after an IVGTT was
the dependent variable.

Sib-pair linkage analyses were performed using the SIBPAL proce-
dure from the Statistical Analysis for Genetic Epidemiology (S.A.G.E.)
package (see ACKNOWLEDGMENTS; 37). With this method, linkage was
assessed for each trait and each of the dinucleotide marker genes near
the GLUT2 and glucokinase genes. Support for linkage is indicated by a
significant regression of the squared sib-pair trait difference and the
estimated proportion of alleles that each sib pair shares identically by
descent. A maximum of 117 sib pairs from 38 nuclear families were
analyzed for the estimates of AIR and percentage of fat. Qualitative
sib-pair linkage analyses were performed on subjects with an onset of
NIDDM before age 45, and 470 sib pairs from 48 nuclear families were
analyzed. An age of onset of 45 was used because this may be the cutoff
age for individuals with familial NIDDM (as opposed to NIDDM as a
result of age). Phenotypic analyses were performed comparing individu-
als with the GLUT2 mutation at codon 110, and individuals without the
mutation and differences in measurements of AIR between these two
groups were analyzed with the use of 2 X 2 contingency tables and x^2
analysis. Thirteen subjects with the mutation and 167 subjects without
the mutation were analyzed.

RESULTS
The AIR aggregated in families both before and after adjust-
ing for sex and percentage of fat (P < 0.0002). Figure 1 shows the large differences among families (range between families) for AIR and the small mean variation within fami-
lies (mean range within families). If insulin action in vivo, as
determined by a hyperinsulinemic-euglycemic clamp (data not
shown), is included in the regression model along with the family class variable, family remains significant
(P < 0.0005). This indicates that AIR aggregates in families
independent of the familial aggregation of insulin action (6).

In a sample of 181 subjects who were not first-degree relatives, 7 alleles and 16 genotypes were identified in the
GLUT2 repeat region (heterozygosity = 0.45); 3 alleles and 6
genotypes were detected in the glucokinase repeat region
(heterozygosity = 0.44). In this sample of the population, the
observed genotypic frequencies did not deviate significantly
from those predicted by Hardy-Weinberg equilibrium.

Sib-pair linkage analysis was used to test for linkage
between quantitative and qualitative traits and the marker
loci. The results of these tests are shown in Table 2. Borderline significance for linkage was observed between
GLUT2 and a measurement of AIR (log_{10} of mean plasma
insulin concentration from 3, 4, and 5 min after an IVGTT),
but no linkage was obtained between GLUT2 and NIDDM.
No significant linkage was observed between glucokinase
and AIR or NIDDM. Sib-pair linkage analyses also were
performed between each locus and ages of NIDDM onset
other than 45, and no significant linkage was observed (data
not shown).

To determine what might be responsible for this potential
linkage between AIR and GLUT2, genomic DNA from 40
subjects with NGT was screened for mutations in the entire
coding region of the GLUT2 gene using PCR-SSCP analysis.

Abnormally migrating bands were observed in exon 3;
whereas, in the other 10 exons, no abnormally migrating
bands were observed. An example of SSCP analysis of exon
3 from seven individuals is shown in Fig. 2A. Direct sequenc-
ing of genomic DNA was performed on all subjects with
different mobility shift patterns, using the published se-
dquence for comparison (13). A nucleotide substitution,
ACT H to ATT, was detected (Fig. 2B) that changes the
amino acid from Thr to Ile in the second membrane-spanning
domain of the GLUT2 protein (38). To determine the fre-
quency of this mutation, exon 3 was analyzed further in the
remaining subjects from the study population by both SSCP
and DNA sequencing. The observed allelic frequency of the
missense mutation at codon 110 was 3% of the study popu-
lation. In 796 Pima Indians, 755 subjects were homozygous
normal (Thr/Thr), Thr/Ile was observed in 38 subjects, and
Ile/Ile in 3 subjects. Although this mutation is rare in
Pima Indians, it was common in another population (38a).

Phenotypic analyses were performed to compare subjects
with (heterozygotes and homozygotes) and without the
mutation in GLUT2, and no significant associations were
observed with AIR or NIDDM (data not shown).

DISCUSSION
Insulin resistance and a relatively low AIR are risk factors
for the development of NIDDM in Pima Indians (5,10); therefore,
familial aggregation of insulin action or AIR possibly might
help explain the familial nature of NIDDM in this and other
populations. Insulin action in vivo has previously been shown
to aggregate in families (6), and recent evidence has
indicated that a putative gene on chromosome 4q contributes
to in vivo insulin action in Pima Indians (9). Our results
suggest that AIR also aggregates in families independent of
sex and percentage of body fat. This may be because of the
shared genetic background of siblings, their common envi-
ronment, or both. The potential influence of environmental
factors was reduced by including independent variables in
our linear models and measurements of acute insulin secre-
tion were made only after residing for 3–4 days in the clinical
research unit on a standard diet. Familial clustering of AIR
has also been observed in nondiabetic offspring of two
NIDDM parents in Caucasians (39). Thus, familial aggrega-
tion of AIR possibly is the result of a shared genetic
background that reflects an underlying genetic component.
To locate putative genetic components, we selected two
candidate genes and performed sib-pair linkage analyses
between the marker loci and measurements of AIR. GLUT2
and glucokinase are both candidates for a low AIR because
of their putative role as glucose sensors in pancreatic β-cells
(12).

No linkage between glucokinase and AIR or NIDDM was
observed with sib-pair linkage analysis. This suggests that an
allele does not exist at this locus that has an adverse effect
on AIR or development of diabetes in the Pima Indians.

Results from sib-pair linkage analyses showed that GLUT2
was not linked to NIDDM, but linkage was suggested for
GLUT2 and estimates of AIR to glucose. Because linkage
of GLUT2 to AIR could not be excluded, the GLUT2 gene
was screened for mutations. Screening resulted in the identifica-
tion of a missense mutation in exon 3. The mutation, Thr^{110}
(Act) to Ile^{110} (Att), changes this codon from a nonionic
polar amino acid to a nonpolar aromatic amino acid. This
mutation is in the second membrane-spanning domain of the GLUT2 protein and, therefore, could increase the hydrophobicity of this region. It is unknown which membrane-spanning domains form the channel through which glucose is transported; thus, the second domain possibly could form part of the channel. An amino acid substitution within this region of a glucose transporter protein could thus affect the ability of the protein to transport glucose. Interestingly, this mutation in GLUT2 changes the amino acid to one that is conserved in the other members of the glucose transporter family. GLUT2 also differs from the rest of the glucose transporter family in that it has a high $K_m$ for glucose and a high $V_{max}$ (40). Therefore, we could speculate that this mutant GLUT2 has a decreased rate of glucose transport. Expression of this mutant GLUT2 requires additional analysis to test for the functional significance of the mutation.

Results from phenotypic analyses suggest that AIR is not associated with the GLUT2 mutation at codon 110. Although the sample size was very small, this suggests that the mutation in exon 3 does not affect the rate of insulin secretion from $\beta$-cells after a glucose challenge. In NIDDM-affected sib pairs from families with the GLUT2 mutation, 2 of 11 pairs showed concordance for the mutation. This is consistent with the lack of linkage between GLUT2 and NIDDM.

In conclusion, glucokinase does not appear to be linked to AIR or NIDDM in Pima Indians, and although GLUT2 was not linked to NIDDM, linkage between GLUT2 and AIR could not be excluded. PCR-SSCP analysis revealed a mutation in exon 3 in GLUT2, but it is unknown whether the mutation affects the functioning of this glucose transporter. Possibly, a mutation in the promoter region of GLUT2 is responsible for the potential linkage observed between GLUT2 and AIR; therefore, the identification of mutations in the promoter region in this population requires additional study. Although extensive analyses were performed on the entire coding region of

![FIG. 1. AIR in nondiabetic Pima Indians grouped by family. Subjects (x) are grouped by family (□), and the 55 families are ranked by family mean AIR (●). The AIR was calculated as the mean plasma insulin concentration from 3, 4, and 5 min after an IVGTT. The range of the mean family AIR and the mean range within families is indicated.](http://diabetesjournals.org/diabetes/article-pdf/43/4/558/337153/43-4-558.pdf)

**TABLE 2**

Sib-pair analysis for the GLUT2 and glucokinase locus and traits

<table>
<thead>
<tr>
<th>Traits</th>
<th>Sib pairs</th>
<th>GLUT2 $P$ values</th>
<th>Glucokinase $P$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of body fat adjusted for sex</td>
<td>117</td>
<td>0.7481</td>
<td>0.2645</td>
</tr>
<tr>
<td>$\log_{10}$ of fasting plasma insulin concentration taken before an IVGTT</td>
<td>117</td>
<td>0.5019</td>
<td>0.1808</td>
</tr>
<tr>
<td>$\log_{10}$ of mean plasma insulin concentration from 3, 4, and 5 min after an IVGTT</td>
<td>117</td>
<td>0.1384</td>
<td>0.4426</td>
</tr>
<tr>
<td>Above trait adjusted for percentage of fat and sex</td>
<td>117</td>
<td>0.0419</td>
<td>0.3243</td>
</tr>
<tr>
<td>$\log_{10}$ of concentration of peak plasma insulin from an IVGTT</td>
<td>116</td>
<td>0.2531</td>
<td>0.4303</td>
</tr>
<tr>
<td>NIDDM before age 45</td>
<td>470</td>
<td>0.2153</td>
<td>0.1300</td>
</tr>
</tbody>
</table>

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GLUT2, it is possible to miss >10% of mutations with PCR-SSCP analysis (41,42). Therefore, another possibility is that an undetected mutation in the coding region could be responsible for the inconclusive linkage of GLUT2 and AI. Although the functional significance of the mutation found in GLUT2 is unknown, it represents the first mutation reported in the human GLUT2 gene.

ACKNOWLEDGMENTS

The sib-pair analyses were performed by using the S.A.G.E. program package, which is supported by U.S. Public Health Service Resource Grant 1P41-RR-03655 from the Division of Research Resources, National Institutes of Health.

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