

# Variants in the Ca<sub>v</sub>2.3 (α1E) Subunit of Voltage-Activated Ca<sup>2+</sup> Channels Are Associated With Insulin Resistance and Type 2 Diabetes in Pima Indians

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**OBJECTIVE**—Linkage to type 2 diabetes has been reported on chromosome 1q21-25 in Pima Indians. Fine mapping identified single nucleotide polymorphisms (SNPs) near the *CACNA1E* gene associated with this disease. *CACNA1E* encodes the voltage-dependent calcium channel Ca<sub>v</sub>2.3 Ca<sup>2+</sup>, and mice lacking this channel exhibit impaired glucose tolerance and insulin secretion. Therefore, *CACNA1E* was investigated as a positional candidate gene.

**RESEARCH DESIGN AND METHODS**—*CACNA1E* was sequenced, and 28 SNPs were genotyped in the same group of Pima subjects who had been analyzed in the linkage study. Allele-specific expression was used to functionally evaluate a variant in the 3' untranslated region (UTR).

**RESULTS**—A novel G/A variant in the 3'-UTR was associated with young-onset type 2 diabetes (odds ratio 2.09 per copy of the G-allele [95% CI 1.31–3.33], adjusted *P* = 0.001) and had an effect on the evidence for linkage at chromosome 1q21-25 (*P* = 0.004). Among 372 nondiabetic Pima subjects who had undergone metabolic testing, the risk allele was associated with reduced insulin action including increased fasting, 30, 60, and 120 min plasma glucose concentrations and increased fasting plasma insulin during an oral glucose tolerance test (all *P* < 0.01), as well as a decreased rate of insulin-stimulated glucose disposal at both physiologically and maximally stimulated insulin concentrations (both *P* < 0.002). Functional analysis of this variant showed that the nonrisk allele had a 2.3-fold higher expression compared with the risk allele.

**CONCLUSIONS**—A functional variant in *CACNA1E* contributes to type 2 diabetes susceptibility by affecting insulin action. This variant partially explains the linkage to type 2 diabetes on chromosome 1q21-25 in Pima Indians. *Diabetes* 56:3089–3094, 2007

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LOD, logarithm of odds; OGTT, oral glucose tolerance test; SNP, single nucleotide polymorphism; UTR, untranslated region.

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Voltage-gated Ca<sup>2+</sup> channels mediate Ca<sup>2+</sup> entry into cells in response to membrane depolarization (1). These channels are composed of a major pore-forming subunit (α1-subunit) and multiple auxiliary subunits. The human Ca<sub>v</sub>2.3 subunit is expressed in neuronal cells (2) and pancreatic β-cells (3). In β-cells, this subunit has an important role in coordinating Ca<sup>2+</sup>-dependent processes such as excitation secretion of insulin. Recent studies in Ca<sub>v</sub>2.3-deficient mice showed that disruption of Ca<sub>v</sub>2.3 Ca<sup>2+</sup> channels impaired glucose tolerance and insulin secretion (4,5), while insulin tolerance tests in Ca<sub>v</sub>2.3, knockout mice indicated reduced insulin sensitivity (5). Ca<sub>v</sub>2.3<sup>-/-</sup> mice were also significantly heavier than wild-type mice, and their basal levels of plasma glucose and insulin tended to be higher, suggesting that the cause of hyperglycemia in Ca<sub>v</sub>2.3<sup>-/-</sup> mice may be insulin resistance (5).

Human Ca<sub>v</sub>2.3 is encoded by the *CACNA1E* gene, which maps to a region on chromosome 1q21-25. Linkage to type 2 diabetes at chromosome 1q21-25 has been reported in diverse populations with European, East Asian, Native American, and African-American ancestries (6–12). In a genome-wide linkage scan in Pima Indians, this region was linked to young-onset type 2 diabetes (12), and follow-up studies in the Pima Indians included association mapping with single nucleotide polymorphisms (SNPs) at ~10 kb density spanning the 25 Mb region of linkage. Results from association mapping identified several variants in/near *CACNA1E* that were modestly associated with type 2 diabetes. Therefore, *CACNA1E* was directly analyzed as a positional candidate gene for linkage to type 2 diabetes in Pima Indians.

## RESEARCH DESIGN AND METHODS

A total of 24 non-first degree-related Pima Indians were selected for sequencing of the *CACNA1E* coding and promoter regions. Among these 24 subjects, 12 developed type 2 diabetes before the age of 25 years and 12 were nondiabetic after 45 years of age. Variants were genotyped for association analyses in 1,037 Pima Indians (from 332 nuclear families) who were participants in our prior genome-wide linkage study for determinants of type 2 diabetes (12). Among these 1,037 subjects, 680 contributed to the original linkage analysis on chromosome 1q and were analyzed in the present study for association with young-onset diabetes. These 680 subjects included 578 with type 2 diabetes with an age of onset of <45 years (mean ± SD age onset 31.0 ± 7.8 years; 62.1% female; maximum BMI 37.9 ± 8.0 kg/m<sup>2</sup>) and 102 who were nondiabetic and ≥45 years of age (mean age 53.7 ± 6.3 years; 60.8% female; maximum BMI 34.6 ± 6.3 kg/m<sup>2</sup>). The remaining genotyped subjects were analyzed for quantitative metabolic traits (see below). Diabetes was diagnosed by an oral glucose tolerance test (OGTT) according to World Health Organization criteria (13). Subjects provided written consent before partici-

pation. The study was approved by the Tribal Council of the Gila River Indian Community and the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases.

**Metabolic phenotyping.** Among the 1,037 subjects genotyped, 372 were nondiabetic full-heritage Pima Indians who had also undergone metabolic testing. Glucose tolerance was determined by a 75-g OGTT with measurements of fasting, 30, 60, 120, and 180 min plasma glucose and insulin concentrations (14). To measure the acute insulin response, blood samples were collected before a 25-g glucose bolus infusion and at 3, 4, 5, 6, 8, and 10 min after infusion. The acute insulin response was calculated as the mean increment in plasma insulin concentrations from 3 to 5 min (15). Insulin action in vivo was assessed using a two-step hyperinsulinemic-euglycemic clamp (14,15). Body composition was estimated by underwater weighing until January 1996 and by dual-energy X-ray absorptiometry (DPX-1; Lunar Radiation) thereafter.

**Sequencing and genotyping.** Sequencing was done using Big Dye terminator (Applied Biosystems) on an automated DNA capillary sequencer (model 3730; Applied Biosystems). Variants were genotyped using the TaqMan Allelic Discrimination Assay (Applied Biosystems) on an ABI Prism 7700 (Applied Biosystems) or SNPlex genotyping System 48-plex (Applied Biosystems) on an automated DNA capillary sequencer.

**Allelic-specific gene expression.** Subcutaneous abdominal adipose tissue biopsies were obtained from 11 subjects known to be heterozygous (G/A) for the 3' untranslated region (UTR) SNP +8130G/A. Biopsies were performed in our clinical research center as previously described (16). Total RNA was extracted from the adipose tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized by reverse transcription (Clontech). Allelic-specific gene expression was assessed by real-time PCR using TaqMan allele-specific probes (TGCAGCTGGTGTGGG-VIC and TGCAGCTGA TGTGGG-FAM; Applied Biosystems) on an ABI Prism 7700 (Applied Biosystems) (17). Triplicate experiments were performed. Data were presented as a ratio of allelic expression (A/G). Background was calculated as allelic ratio of genomic DNA (assumed ratio = 1).

**Statistical analysis.** Associations were assessed using SAS programs (SAS Institute, Cary, NC). The relationship between trait and marker was generally assessed via a numeric variable representing genotype (i.e., an additive model). For continuous variables, linear regression was used to adjust for covariates. The association with young-onset diabetes was assessed by logistic regression and was controlled for sex and heritage. Both linear and logistic models were fit with generalized estimating equations to account for the correlation among siblings (18). This method provides a general test of association that accounts for the nonindependence among siblings, but as it is not a test of within-family association, it is not robust to population stratification. Therefore, to control for potential population stratification, the association with diabetes was also analyzed with a within-family association test using the method of Abecasis et al. (19). To assess the impact of the observed association on the linkage, logarithm of odds (LOD) scores were recalculated with and without adjustment for the marker and the statistical significance of the difference assessed with a modified bivariate Haseman-Elston model (20). Haplotype frequencies for pairs of SNPs were calculated in all 1,037 individuals with the Estimating Haplotypes (EH) program.  $D'$  was calculated as a measure of allelic association, and  $r^2$  as a measure of concordance. For haplotype analyses, SNP were placed in "blocks," which were defined as consecutive SNPs with  $D' > 0.9$ . By this definition, blocks can overlap; for the sake of thoroughness, we analyzed all blocks, regardless of overlap, and made no attempt to place each SNP in a unique block. Association between traits and individual haplotypes were examined with a modification of the zero-recombinant haplotyping procedure, as described previously (21).

## RESULTS AND DISCUSSION

Sequencing of 49 exons and ~2 kb of the upstream region of *CACNA1E* in 24 Pima Indians identified 14 variants. Two variants were novel, a -1039G/T (1,039 bp upstream of the ATG site) and a +8130G/A in the 3'-UTR (8,130 bp downstream from ATG in the cDNA), while the remaining 12 SNPs could be found in public databases. Sixteen additional SNPs, positioned within introns and more distal regions flanking the *CACNA1E* locus that were not sequenced, were selected from public databases to provide a denser coverage of genetic information across this region. All 28 SNPs (supplemental Fig. 1 [available in an online appendix at <http://dx.doi.org/10.2337/db07-0587>] and Table 1) were genotyped in 1,037 Pima Indians for association analyses.

The frequencies of the 28 variants in subjects with young-onset type 2 diabetes compared with the older (aged >45 years) nondiabetic subjects, are given in Table 1. All SNPs were in Hardy-Weinberg equilibrium. Five of the variants, namely, rs625226, rs3753737, rs798209, rs473200, and +8130G/A, were nominally associated with young-onset type 2 diabetes in a general association test. Among these SNPs, rs3753737 and +8130G/A were also nominally associated with type 2 diabetes using a within-family test. In simulation studies, we estimate that the power of the present sample is >70% to detect a significant ( $P < 0.05$ ) association with a causal variant with minor allele frequency >0.05, accounting for 2% of the variance in age of onset of diabetes. Given the previous physiological implication of *CACNA1E* in diabetes in mice, we chose to preserve statistical power and did not correct for multiple comparisons. Therefore, it remains possible that these nominal associations may represent false-positive findings.

To help address whether these associations may be false-positives, we further investigated whether any of the 28 variants were associated with pre-diabetic traits among 372 nondiabetic, full-heritage Pima Indians who had undergone metabolic testing. Among these nondiabetic individuals, subjects with the G/G genotype at +8130G/A, which was the risk genotype for young-onset type 2 diabetes, had significantly higher mean fasting, 30, 60, and 120 min plasma glucose concentrations and higher mean fasting plasma insulin concentrations during an OGTT, as well as a lower mean rate of insulin-stimulated glucose disposal at both physiologically and maximally stimulated insulin concentrations during a hyperinsulinemic-euglycemic clamp (Table 2). These results in humans are consistent with the finding of increased postload plasma glucose levels and decreased insulin sensitivity in *CACNA1E* knockout mice (5). Prior in vitro studies using the insulinoma cell line INS-1 suggested a role of *CACNA1E* in insulin secretion (22), but our in vivo study did not detect an association between SNPs in this gene and acute insulin secretion using a 25-g glucose bolus infusion.

The degree of linkage disequilibrium (summarized by  $D'$  and  $r^2$ ) between the 28 variants in 1,037 subjects is shown in Fig. 1. The common variants (minor allele frequency >0.05) fell into 13 haplotype "blocks." Each block, ranging in size from 0.6 to 93.6 kb, contained on average two to three SNPs, except for one block located in 3'-UTR, which extended across five SNPs. Five haplotypes were associated with type 2 diabetes ( $P \leq 0.05$ ) (Table 3). The strongest association was with haplotype AA for +8130G/A and rs16858150, where its frequency was lower in case than in control subjects (frequency 0.09 in diabetic vs. 0.17 in nondiabetic subjects, odds ratio [OR] 0.44 [95% CI 0.28–0.70],  $P = 0.001$ ). Conversely, the frequency of haplotype GA for +8130G/A and rs16858150 was higher in the case than in the control subjects (frequency 0.85 in diabetic vs. 0.74 in nondiabetic subjects; 1.82 [1.23–2.69],  $P = 0.005$ ). This finding largely reflects the single-marker associations of +8130G/A. Additional analyses examined pairs of SNPs or haplotypes such that +8130G/A was analyzed in conjunction with each additional significant variant to determine their potential independent contributions to the association. The effect of +8130G/A was statistically significant ( $P < 0.05$ ) conditional on each of these other variants, but none of the other variants was significantly conditional on +8130G/A; the association of the GAG haplotype in block 2 was of marginal significance con-

TABLE 1  
Association of SNPs in *CACNA1E* with young-onset type 2 diabetes in Pima Indians

SNP	Major/ minor allele	Location	Risk allele frequency (genotype distribution as %)		General test		Within-family test ( <i>n</i> = 680)		Genotype call rate (%)
			Diabetes ( <i>n</i> = 578)	Nondiabetic ( <i>n</i> = 102)	OR (95% CI)	<i>P</i> additive/ recessive*	OR (95% CI)	<i>P</i> additive	
rs10752856	<u>T</u> /C	Upstream	0.78 (61, 34, 5)	0.73 (54, 39, 7)	1.19 (0.78–1.82)	0.41	0.97 (0.52–1.81)	0.91	86
rs11582261	<u>G</u> /T	Upstream	0.59 (34, 50, 16)	0.54 (26, 55, 19)	1.29 (0.94–1.79)	0.11	1.72 (1.12–2.64)	0.01	85
rs10752858	<u>A</u> /G	Upstream	0.78 (60, 37, 3)	0.74 (54, 40, 6)	1.30 (0.85–1.99)	0.21	1.38 (0.74–2.56)	0.3	89
rs2877622	<u>C</u> /A	Upstream	0.78 (60, 37, 3)	0.73 (53, 40, 7)	1.40 (0.91–2.16)	0.12	1.30 (0.69–2.46)	0.42	88
rs2877651	<u>G</u> /A	Upstream	0.79 (63, 32, 5)	0.75 (56, 38, 6)	1.15 (0.77–1.73)	0.47	0.97 (0.49–1.95)	0.93	82
rs541886	<u>T</u> /C	Upstream	0.76 (57, 37, 6)	0.70 (47, 46, 7)	1.31 (0.91–1.87)	0.13	1.17 (0.64–2.13)	0.61	89
-1039G/T	<u>G</u> /T	Upstream	0.78 (61, 34, 5)	0.74 (53, 41, 6)	1.15 (0.79–1.66)	0.45	0.84 (0.49–1.45)	0.53	89
rs679931	A/ <u>T</u>	Intron	0.47 (19, 55, 26)	0.45 (16, 58, 26)	1.01 (0.71–1.44)	0.93	1.46 (0.84–2.51)	0.17	84
rs625226	<u>A</u> /G	Intron	0.73 (62, 31, 7)	0.68 (45, 46, 8)	1.43 (1.02–2.03)	0.03	0.94 (0.48–1.84)	0.85	86
rs10910955	G/ <u>A</u>	Intron	0.27 (7, 41, 52)	0.24 (2, 43, 55)	1.09 (0.74–1.60)	0.63	0.54 (0.29–1.02)	0.05	86
rs3856091	<u>T</u> /A	Intron	0.72 (55, 34, 11)	0.66 (43, 45, 12)	1.37 (0.96–1.95)	0.07	2.44 (1.28–4.63)	0.006	85
rs4651109	C/ <u>A</u>	Intron	0.25 (7, 37, 56)	0.20 (1, 37, 61)	1.37 (0.93–2.04)	0.1	1.83 (0.95–3.51)	0.07	84
rs199939	C/ <u>T</u>	intron	0.31 (9, 43, 48)	0.27 (4, 47, 49)	1.23 (0.86–1.76)	0.23	1.69 (0.96–2.98)	0.06	87
rs3753737	<u>A</u> /G	Intron	0.55 (34, 42, 24)	0.46 (20, 51, 29)	1.41 (1.04–1.91)	0.02	2.05 (1.20–3.49)	0.008	90
rs17743547	<u>A</u> /G	Intron	0.25 (7, 35, 58)	0.20 (4, 31, 65)	1.28 (0.88–1.87)	0.18	1.08 (0.58–2.01)	0.8	90
rs3766999	<u>C</u> /G	Intron	0.63 (41, 44, 15)	0.58 (33, 51, 16)	1.12 (0.80–1.57)	0.47	1.15 (0.66–2.03)	0.62	86
rs4652678	T/C	Hisl317His	0.30 (9, 42, 49)	0.29 (8, 42, 50)	1.00 (0.71–1.41)	0.95	0.77 (0.45–1.31)	0.33	90
rs199930	<u>C</u> /T	Asnl672Asn	0.75 (55, 39, 6)	0.74 (55, 38, 7)	1.05 (0.72–1.52)	0.79	1.04 (0.58–1.89)	0.88	89
rs798209	<u>T</u> /G	Intron	0.88 (77, 21, 2)	0.82 (65, 34, 1)	1.59 (1.06–2.38)	0.02/0.007	1.54 (0.90–2.61)	0.11	89
rs546191	C/ <u>T</u>	Intron	0.25 (56, 38, 6)	0.23 (60, 34, 6)	1.06 (0.70–1.59)	0.76	1.07 (0.54–2.11)	0.83	88
rs473200	<u>C</u> /T	intron	0.88 (77, 21, 2)	0.82 (66, 33, 1)	1.53 (1.03–2.27)	0.03/0.009	1.32 (0.83–2.09)	0.24	89
rs704326	G/ <u>A</u>	Ala1936Thr	0.33 (9, 48, 43)	0.28 (7, 41, 52)	1.35 (0.93–1.96)	0.1	1.86 (1.09–3.17)	0.02	89
rs2280869	<u>T</u> /C	3'-UTR	0.64 (41, 44, 15)	0.56 (30, 51, 19)	1.31 (0.94–1.82)	0.1	1.57 (0.91–2.71)	0.1	89
rs3831945	del/ <u>T</u>	3'-UTR	0.96 (92, 8, 0)	0.95 (90, 10, 0)	1.16 (0.47–2.83)	0.73/0.72	0.33 (0.08–1.38)	0.12	89
rs10632577	<u>AG</u> /del	3'-UTR	0.85 (63, 32, 5)	0.83 (70, 25, 5)	1.24 (0.76–2.02)	0.37/0.61	1.27 (0.50–3.21)	0.61	89
rs638132	<u>A</u> /G	3'-UTR	0.80 (63, 34, 3)	0.79 (63, 32, 5)	1.17 (0.72–1.89)	0.51	0.91 (0.40–2.07)	0.81	90
+8130G/A	<u>G</u> /A	3'-UTR	0.90 (82, 17, 1)	0.83 (68, 31, 1)	2.09 (1.31–3.33)	0.001/0.0006	2.44 (1.27–4.67)	0.007	89
rs16858150	<u>A</u> /C	3'-UTR	0.94 (88, 11, 1)	0.92 (85, 14, 1)	1.16 (0.54–2.47)	0.69/0.64	0.30 (0.09–0.96)	0.04	90

The risk allele (major or minor) is underlined, and del = deleted nucleotide(s). Genotype distribution (in parentheses) is given as the percent of subjects homozygous for the risk allele and heterozygous and homozygous for the nonrisk allele, respectively. Subjects with diabetes had age of onset <45 years. Nondiabetic subjects were all >45 years of age. *P* values and ORs were adjusted for age, sex, birth year, heritage, and family membership. \*Recessive *P* value (rare homozygotes combined with heterozygotes) is also given for SNPs with minor allele frequency <0.2. In the general test, OR is expressed as per copy of the underlined risk allele (and, thus, by definition is  $\geq 1$ ). In the within-family test, OR is expressed as the same allele as in the general test; therefore, those <1 indicate that the risk alleles are in the opposite directions in two tests.

TABLE 2  
Metabolic characteristics of nondiabetic full-heritage subjects grouped by +8130G/A genotype

	G/G	G/A + A/A	<i>P</i>
<i>N</i> (men/women)	291 (163/128)	81 (52/29)	
Age (year)	26 ± 0.3	27 ± 0.8	
% Fat	34 ± 0.5	31 ± 1.1	0.24
Fasting plasma glucose (mg/dl)	91 ± 0.6	86 ± 1.1	0.002
30 min plasma glucose (mg/dl)	147 ± 1.4	138 ± 2.8	0.003
60 min plasma glucose (mg/dl)	152 ± 2.1	133 ± 3.4	0.00001
2 h plasma glucose (mg/dl)	125 ± 1.7	114 ± 3.2	0.01
Fasting insulin (μU/ml)	38 (36–40)	30 (27–33)	0.001
2 h insulin (μU/ml)	166 (151–182)	126 (104–151)	0.06
Glucose disposal low-dose insulin clamp (mg · kg EMBS <sup>-1</sup> · min <sup>-1</sup> )	3.4 (3.3–3.5)	3.9 (3.6–4.2)	0.002
Glucose disposal high-dose insulin clamp (mg · kg EMBS <sup>-1</sup> · min <sup>-1</sup> )	8.2 ± 0.12	9.2 ± 0.23	0.003
Acute insulin response (μU/ml)	224 (204–245)	229 (199–257)	0.73

Only three subjects were homozygous for the A-allele of +8130 G/A; therefore, these individuals were combined with the G/A heterozygotes for statistical analyses. Plasma insulin concentrations (fasting and 2 h), rates of glucose disappearance during the low-dose insulin infusion clamp, and the acute insulin response were log transformed before analyses to approximate a normal distribution. Data for these variables are presented as the geometric mean (95% high and low CIs). The *P* value for % fat was adjusted for age, sex, and family membership. The *P* value for the acute insulin response was adjusted for age, sex, % fat, and rate of glucose disappearance during the low-dose insulin infusion clamp. All remaining *P* values (except for age) were adjusted for age, sex, % fat, and family membership. The acute insulin response was analyzed only in normal glucose-tolerant nondiabetic subjects (*n* = 203 with genotype G/G and *n* = 69 with genotype G/A or A/A). EMBS, estimated metabolic body size.

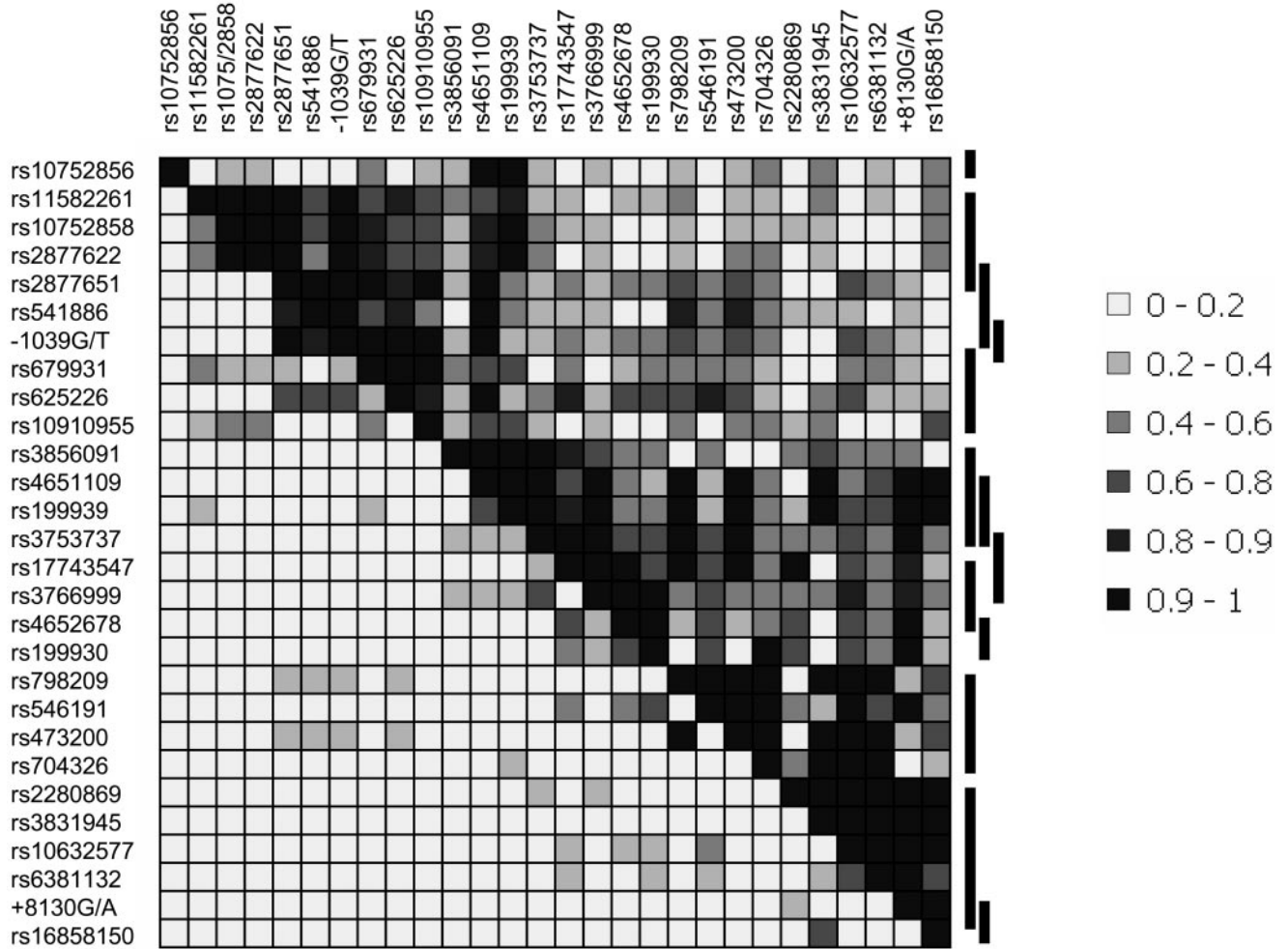


FIG. 1. Pairwise linkage disequilibrium among 28 SNPs genotyped across CACNA1E in 1,037 Pima Indians. Values in the upper right represent D', while values in the bottom left represent r<sup>2</sup>. The bars at the right indicate 13 linkage disequilibrium "blocks" defined as consecutive SNPs among whom all pairs had D' > 0.9.

trolled for +8130G/A (P = 0.07). Thus, +8130G/A represents the major associated variant in the present study.

Since SNP +8130G/A provided the strongest evidence for disease association using either individual SNP or

haplotype analysis, this variant was functionally analyzed. The location of this variant in the 3'-UTR led to the hypothesis that it could affect mRNA expression/stability. Allelic-specific expression in adipose tissue mRNA iso-

TABLE 3  
CACNA1E haplotypes associated with early-onset type 2 diabetes in Pima Indians

Haplotype block	Haplotype							Haplotype frequency			P (corrected)	Bonferroni correction							
	rs11582261	rs10752858	rs2877651	rs679931	rs625226	rs10910955	rs546191	rs473200	rs704326	rs2280869			rs3831945	rs10632577	rs638132	+8130G/A	rs16858150	Diabetic	Nondiabetic
2	G	A	G												0.38	0.25	1.94 (1.27-2.98)	0.006	3
5			A	G	G										0.22	0.31	0.63 (0.43-0.92)	0.04	3
11						C	T	G							0.13	0.19	0.61 (0.40-0.92)	0.05	3
12									Cdel(AG)		A	A			0.10	0.17	0.49 (0.31-0.78)	0.009	4
13												G	A		0.85	0.74	1.82 (1.23-2.69)	0.005	2
13												A	A		0.09	0.17	0.44 (0.28-0.70)	0.001	2

Haplotype analysis was carried out in 13 "blocks" defined as consecutive SNPs among whom all pairs had D' > 0.9. All haplotypes with frequency >0.05 were analyzed within each block. ORs were expressed as per copy of the haplotype of interest (additive model). The corrected P values reflect a Bonferroni correction for the number of "independent" haplotypes in the same block (total number of haplotypes analyzed = 1). Only haplotypes with P value <0.05 are shown in the table, where brackets indicate the specific SNPs used in constructing each haplotype. Haplotype C-del-(AG)-A-A contains a deletion at the second position (rs3831945 = del/T) and an AG insertion at the third position (rs10632577 = AG/del).

lated from 11 subjects heterozygous for the G/A showed that the ratio of A (nonrisk) allele versus G (risk) allele (A/G) was  $2.37 \pm 0.30$  (mean  $\pm$  SEM), which was significantly different from the background ratio of  $0.97 \pm 0.09$  (*t* test  $P = 0.008$ ).

To assess whether the +8130G/A contributed to the evidence for linkage to young-onset type 2 diabetes on chromosome 1 in Pima Indians, LOD scores were recalculated with and without adjustment for this marker. A reduction in linkage of 0.6 LOD (from 2.6 to 2.0,  $P = 0.004$ ) was observed after adjustment for +8130G/A. No reduction, or a smaller reduction, was observed when the linkage was adjusted for the other SNPs genotyped in/near this gene.

Although the +8130G/A variant appears to underlie a portion of the linkage on chromosome 1q21-25 among families selected for having a high prevalence of young-onset type 2 diabetes, the effect of this variant on diabetes risk in the general Pima population appears to be minimal. The association of this variant with type 2 diabetes in a population-based sample of Pima Indian subjects (after excluding all participants of the linkage study,  $n = 2,427$ : 862 with diabetes and 1,565 without diabetes) was marginal (OR 1.17, one-sided  $P = 0.08$ ). Thus, it appears that this variant has its greatest impact in families with a high prevalence of young-onset diabetes, such as those selected for the linkage study and the present follow-up study.

Linkage to type 2 diabetes on chromosome 1q21-25 has also been observed in seven other populations in the International Type 2 Diabetes Chromosome 1q Consortium (6-14). As part of this consortium, SNPs are being genotyped in subjects from all of these populations across a region that spans 148-170 Mb on chromosome 1 (23). *CACNA1E* is positioned slightly telomeric to this region at 179.5 Mb. However, SNP +8130G/A was specifically selected to attempt replication in the consortium samples (1,540 case subjects with type 2 diabetes and 1,512 control subjects without diabetes). The frequency of the G-allele was  $\sim 0.70$  among the Chinese and  $\sim 0.95$  for most of the European-derived populations and for African Americans; the one exception was the Amish, in whom the frequency was 0.83. This SNP was not associated with type 2 diabetes in the combined samples from the consortium (OR 1.06 per copy of the G-allele [95% CI 0.90-1.27],  $P = 0.46$ , adjusted for population), nor was this SNP significantly associated ( $P < 0.05$ ) with diabetes among any of the individual populations. Two additional SNPs in *CACNA1E* (rs473200 and rs704326) were also genotyped in the consortium samples, but neither replicated. Further detailed mapping in the consortium samples or other populations with a high prevalence of young-onset type 2 diabetes is required to determine the role of other variants in or near *CACNA1E* in susceptibility to type 2 diabetes.

It is possible that the present findings represent a false-positive; however, this SNP has the second strongest evidence for association with young-onset type 2 diabetes in Pima Indians among the 5,150 SNPs typed to date across the region of linkage. The SNP with the strongest evidence for association maps to an intergenic region, and this SNP is similarly not associated with type 2 diabetes in the combined consortium samples. The third strongest association maps to *ARHGEF11*, where variants in this gene are also associated with type 2 diabetes in the Amish population but not in the combined consortium samples (24,25). Therefore, an alternative explanation for the lack of replication in other ethnic groups is that multiple

variants underlie the various linkage observations on chromosome 1q21-25, and these variants have different effect sizes among the different ethnic groups. The observed difference between populations for the effect of this variant could also be explained by clinical heterogeneity. Pima Indians, in particular those selected for the linkage study and the current follow-up study, have a much earlier age of onset of diabetes than other populations. Pima Indians, on average, also have a much higher BMI. Studies of *CACNA1E* in other groups of subjects with young-onset diabetes and/or high BMI are required to examine this possibility.

Our allele-specific expression study suggests that +8130G/A may affect diabetes susceptibility via reduction of *CACNA1E* expression. Since the risk allele was the common allele (frequency 0.89), reduced *CACNA1E* expression, reduced in vivo insulin action, and a modest increase in diabetes risk is the common phenotype among Pima Indians.

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