Polymorphism in the Calsequestrin 1 (*CASQ1*) Gene on Chromosome 1q21 Is Associated With Type 2 Diabetes in the Old Order Amish

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Calsequestrin (CASQ)1 is involved in intracellular storage and release of calcium, a process that has been shown to mediate glucose transport in muscle. Its gene, *CASQ1*, is encoded on chromosome 1q21, a region that has been linked to type 2 diabetes in the Amish and several other populations. We screened all 11 exons, exon-intron junctions, and the proximal regulatory region of *CASQ1* for mutations. We detected four novel single nucleotide polymorphisms (SNPs) (−1470C→T, −1456delG, −1366insG, and 593C→T). Ten informative SNPs within *CASQ1* were genotyped in Amish subjects with type 2 diabetes (n = 145), impaired glucose tolerance (n = 148), and normal glucose tolerance (n = 358). Rs2275703 and rs617698 in introns 4 and 2 were significantly associated with type 2 diabetes (P = 0.008 and 0.04, respectively); three other SNPs showed borderline evidence for association to type 2 diabetes (P = 0.076–0.093). Furthermore, in nondiabetic subjects (n = 754), both rs2275703 and rs617698 were significantly associated with glucose area under the curve during an oral glucose tolerance test (P = 0.035 and 0.013, respectively). Haplotype analysis suggested that no haplotype could explain these associations better than rs2275703. These findings, coupled with similar findings in Utah Caucasians, suggest that sequence variation in *CASQ1* may influence risk of type 2 diabetes. *Diabetes* 53: 3292–3299, 2004

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AFDS, Amish Family Diabetes Study; AUC, area under the curve; CASQ, calsequestrin; HOMA, homeostasis model assessment; HOMA-IR, HOMA of insulin resistance; IGT, impaired glucose tolerance; LD, linkage disequilibrium; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; SNP, single nucleotide polymorphism.

Type 2 diabetes is a common heterogeneous disorder in which genetic and nongenetic factors (i.e., excess caloric intake and physical inactivity) potently influence risk (1,2). Genome-wide scans have led to the chromosomal localization of susceptibility loci for type 2 diabetes, with the most promising and replicated findings on chromosomes 1q21-q24 (3–10), 2q37 (11), 12q24 (12–14), and chromosome 20 (15–18). Consistent with current thinking, multiple linked regions suggest that several susceptibility genes contribute to the development of type 2 diabetes (2). To date, positional cloning has identified variants in calpain 10 (*CAPN10*) on chromosome 2q37 (19) and hepatocyte nuclear factor 4α (*HNF4A*) on chromosome 20q12-q13.1 (20,21) as type 2 diabetes susceptibility genes in some populations.

We previously reported evidence for linkage of type 2 diabetes and impaired glucose homeostasis to a locus on chromosome 1q21-q24 (logarithm of odds 2.35, P = 0.0008) in the Old Order Amish (3). Linkage of this region on chromosome 1 to type 2 diabetes has been reported in at least five other populations, including Pima Indians (4), Utah Caucasians (5), French Caucasians (6), U.K. Caucasians (7), and Chinese (8); linkages of diabetes-related traits to this region have also been reported (9,10). Such robust replication provides strong evidence for a shared type 2 diabetes susceptibility locus in this chromosomal interval. However, this region contains at least 450 genes that include several excellent positional candidate genes, introducing the possibility that more than one gene variant in the region may influence diabetes risk. A number of positional candidate genes within this region have been studied to date, with variation in potassium inwardly rectifying channel, subfamily J, members 9 and 10 (*KCNJ9/10*) (22,23), liver-specific pyruvate kinase (*PKLR*) (24), C-reactive protein (*CRP*) (25), lamin A/C (*LMNA*) (26), and omentin (*ITLN*) (27) showing modest evidence for association with type 2 diabetes or related traits.

To further refine this interval with the aim of positionally cloning the type 2 diabetes gene(s) in this region, we embarked upon linkage disequilibrium (LD) mapping by performing genotype and association analysis of single nucleotide polymorphisms (SNPs) in Amish type 2 diabetic cases and nondiabetic control subjects. To date, ~250 SNPs over the 28.5-Mb region from 148.5 to 170 Mb (NCBI [National Cancer for Biotechnology] build 34.3)

have been genotyped. Multiple SNPs associated with type 2 diabetes were identified within the interval between ~157 and 158.5 Mb, including rs617698, located within the calsequestrin 1 (*CASQ1*) gene. CASQ1 is a calcium storage protein localized in the sarcoplasmic reticulum of fast-twitch skeletal muscle cells. It is a constituent of the protein backbone of the luminal Ca^{2+} reservoir and plays a pivotal role in calcium flux through regulation of calcium channel activity and interaction with other proteins present in the sarcoplasmic reticulum (28). Calcium release from the sarcoplasmic reticulum into the cytosol has been shown to regulate GLUT4 expression (29) and glucose transport in muscle (30). Furthermore, Howarth et al. (31) reported that CASQ1 expression and calcium binding is increased in streptozotocin-induced diabetic rat skeletal muscle. We hypothesized that variation in *CASQ1* may affect insulin action on glucose uptake and glycogen synthesis in skeletal muscle and thus type 2 diabetes susceptibility. To examine this positional candidate gene further, we screened *CASQ1* for mutations and determined whether the observed sequence variation was associated with type 2 diabetes and related traits in the Old Order Amish.

RESEARCH DESIGN AND METHODS

The Old Order Amish are a genetically well-defined Caucasian founder population who live in a relatively homogeneous environment and have large sibships. Nearly all of these individuals share common ancestors; >95% of the chromosomal material of the Amish community of Lancaster County (now numbering ~30,000 individuals) appears to have descended from <100 founders who emigrated to this area in the mid 1700s (12–14 generations) (32,33). The Amish Family Diabetes Study (AFDS) was begun in 1995 to identify susceptibility genes for type 2 diabetes and related traits. Probands with diabetes onset between 35 and 65 years and all willing first- and second-degree family members ≥18 years of age were invited to participate. DNA from 18 non–first-degree relatives with type 2 diabetes (36 alleles) from families providing evidence for linkage of diabetes to chromosome 1q21-q24 was used for sequence analysis of *CASQ1*. To define LD and haplotype structure of *CASQ1*, all SNPs were genotyped in a limited set of 258 Amish subjects chosen to be relatively unrelated (non–first-degree relatives) to each other. Next, to examine the relationship between haplotype-tagging polymorphisms in *CASQ1* and diabetes, we genotyped DNA from subjects with type 2 diabetes (n = 145), impaired glucose tolerance (IGT) (n = 148), and normal glucose tolerance (NGT) (n = 358) for case-control association analysis. NGT subjects included in this analysis were required to be at least 38 years of age at the time of examination. For association analysis of quantitative traits (e.g., glucose and insulin), a larger set of 754 nondiabetic subjects (including the 506 NGT and IGT subjects described above) was studied. Informed consent was obtained from all AFDS participants, and the study protocol was approved by the institutional review board at the University of Maryland School of Medicine.

Trait measurements. Details of methods for phenotyping of AFDS subjects have been previously described (3,34). Briefly, body height and weight were measured in all subjects using standard protocols. After a 12-h overnight fast, a 75-g oral glucose tolerance test (OGTT) was administered, with venous blood samples obtained at 0, 30, 60, 90, 120, 150, and 180 min for glucose and insulin measurements. The total glucose and insulin areas under the curve (AUCs) during the 3-h OGTT were calculated using the trapezoid method. Insulin resistance was estimated from fasting blood glucose and insulin levels with the homeostasis model assessment (HOMA) (HOMA of insulin resistance [HOMA-IR] = [fasting insulin {μU/ml} × fasting glucose {mmol/l}]/22.5). Diabetes was defined by fasting plasma glucose level (≥7 mmol/l), 2-h OGTT plasma glucose level (≥11.1 mmol/l), random plasma glucose level (≥11.1 mmol/l), the use of insulin or oral glucose-lowering agents, or a diagnosis of diabetes, with age of onset between 35 and 65 years, documented by a physician. IGT was diagnosed based on OGTT plasma glucose levels (2-h OGTT plasma glucose level between 7.8 and 11.1 mmol/l). NGT was defined based on fasting plasma glucose level (<6.1 mmol/l) and 2-h OGTT plasma glucose level (<7.8 mmol/l).

Mutation screening. Primers were designed to specifically amplify *CASQ1* exons, splice junctions, and 1,000 bp of the 5′ flanking sequence using primer

TABLE 1
Frequencies of *CASQ1* SNPs in subjects with type 2 diabetes, IGT, and NGT

SNP name	Location	Position†	Major/minor alele	Minor allele frequency			Type 2 diabetes vs. NGT*		Type 2 diabetes + IGT vs. NGT*	
				Type 2 diabetes (n = 145)	IGT (n = 148)	NGT (n = 358)	OR (95% CI)	P	OR (95% CI)	P
CasqSNP1	5′ flank	−1470	C/T	0.471	0.463	0.421	1.33 (0.89–1.98)	0.15	1.24 (0.92–1.68)	0.17
Rs1186694	5′ flank	−1404	C/T	0.265	0.250	0.263	1.14 (0.78–1.66)	0.47	0.98 (0.74–1.32)	0.92
Rs3838216	Intron 1	+1074	−/ggcattcagataggcct	0.092	0.101	0.139	0.73 (0.41–1.31)	0.31	0.70 (0.44–1.08)	0.091
Rs617698	Intron 2	+2312	A/G	0.279	0.327	0.355	0.65 (0.42–0.99)	**0.040**	0.72 (0.52–1.00)	**0.047**
Rs617599	Intron 2	+2399	G/A	0.281	0.339	0.368	0.58 (0.32–1.07)	0.076	0.77 (0.47–1.26)	0.42
Rs3747622	Intron 2	+2889	G/C	0.071	0.087	0.124	0.64 (0.36–1.15)	0.18	0.56 (0.33–0.94)	**0.026**
CasqSNP4	Exon 3	+3059	C/T	0.071	0.059	0.030	1.94 (0.87–4.31)	0.093	1.82 (0.93–3.59)	0.064
Rs2275703	Intron 4	+4535	C/A	0.312	0.371	0.426	0.57 (0.41–0.79)	**0.008**	0.64 (0.47–0.85)	**0.005**
Rs822450	Intron 7	+7808	G/A	0.280	0.311	0.341	0.70 (0.45–1.07)	0.092	0.75 (0.50–1.14)	0.10
Rs3827532	Intron 10	+10171	G/A	0.034	0.026	0.067	0.35 (0.10–1.32)	0.13	0.26 (0.08–0.88)	**0.030**

*P values are based on genotype frequencies and ORs reflect the odds of disease associated with having two copies of the minor allele versus the odds of disease associated with having two copies of the major allele. For rs2275703 and rs617698, the ORs for type 2 diabetes of the major alleles are 1.75 (95% CI 1.27–2.43) and 1.54 (1.01–2.38), respectively. P values <0.05 are shown in bold. †The nucleotide position of each polymorphism is counted from the A of the ATG start codon, which was designated as position +1.

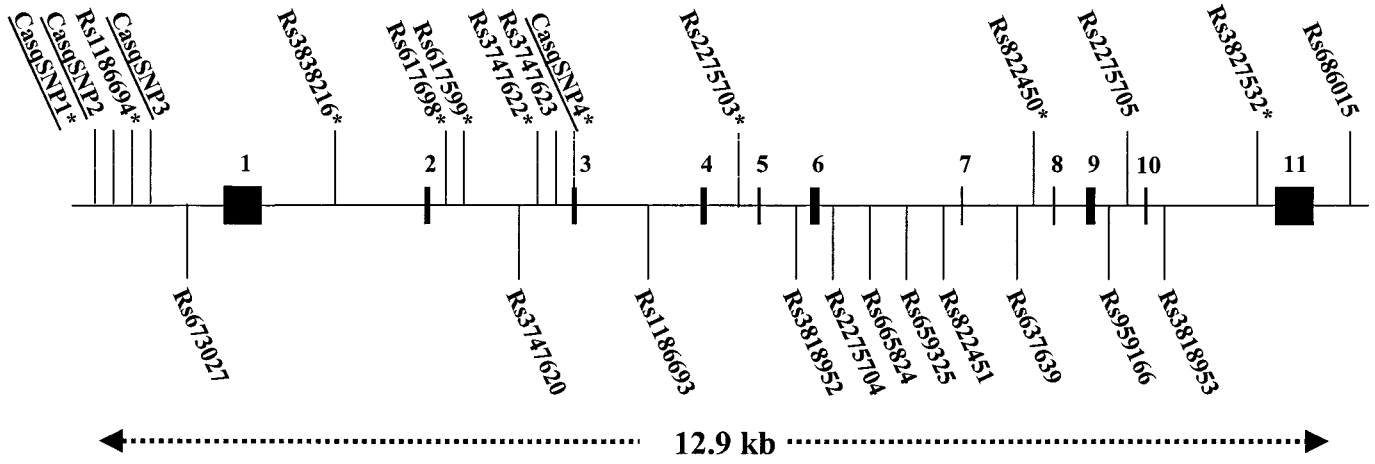

FIG. 1. Schematic of *CASQ1* and SNPs. Exons are shown as boxes and horizontal lines as introns, regulatory region or flanking segments. Novel SNPs discovered by sequence analysis are underlined, and dbSNPs are designated by the "Rs " numbers; those that were informative are above, while those that were not polymorphic in the Amish are below. SNPs with asterisks next to them were designated as informative haplotype-tagging polymorphisms and genotyped in the full sample set (see text for details). Note exon and intron sizes are not strictly drawn to scale.

3 (available at http//www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (see Table 1 of online appendix [available at http://diabetes.diabetesjournals.org]). PCR conditions were 95°C for 2 min, followed by 35 cycles of 95°C for 45 s, 56°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. Both strands were sequenced from DNA of the 18 subjects on an ABI 3700 DNA sequencer and analyzed with Sequence Analysis 3.2 software (Applied Biosystems Division, PerkinElmer, Foster City, CA).

Genotyping. The 593C→T polymorphism in exon 3 (casqSNP4) was amplified and detected by sequence analysis on an ABI 3700 DNA sequencer (A of ATG start codon of Genbank accession no. NM_001231, designated as position +1). CasqSNP1 (−1470 C→T), rs1186694, rs617698, and rs617599 were typed using the AcycloPrime-FP SNP detection system (PerkinElmer Life Sciences, Boston, MA), which determines the base at a SNP location by template-directed dye-terminator incorporation followed by detection with fluorescence polarization (35). Rs3747622, rs3747623, rs2275703, rs822450, rs2275705, and rs686015 were typed using the SNPstream UHT Genotyping System (Beckman Coulter, Fullerton, CA) (36). SNP −1456delG (CasqSNP2), rs3838216, and rs3827532 were genotyped by pyrosequencing using a PSQ HS 96A Pyrosequencer according to the manufacturer's protocol (Pyrosequencing, Uppsala, Sweden). In addition to SNPs within *CASQ1*, we also genotyped SNPs flanking the gene to determine whether associated SNPs within *CASQ1* were in LD with extragenic SNPs. These included rs860294, rs1408664, rs881291, rs625129, rs7545350, rs6427504, and rs2820553, which are located 1.9–145.7 kb upstream of *CASQ1*, and rs680083, rs1041066, and rs2147472, which are located 8.9–169.9 kb downstream of *CASQ1*, all performed by SNPstream UHT genotyping. All typing included at least 5% duplicate samples to determine mistyping rates, which were 0–2%.

Statistical analysis. Before analysis, all SNPs were checked for Mendel errors using the PedCheck software program (37) in the extended Amish pedigree. A small number of Mendel errors were either resolved or genotypes removed before analysis. Allele frequencies of polymorphisms were determined by gene counting. Because of the extensive relatedness of study subjects in our Amish pedigrees, we tested for associations of SNP genotypes while accounting for the pedigree structure. Briefly, we used a variance component approach in which we modeled the probability that the subject was a case or control as a function of the individual's age, sex, and genotype, conditional on the correlations in phenotype among relative pairs. For the primary analysis, we considered an additive genetic model in which the genotype was coded as 0, 1, or 2, depending on whether the subject was homozygous for the minor allele (genotype = 2), heterozygous (genotype = 1), or homozygous for the major allele (genotype = 0). Statistical testing was performed by testing the likelihood of the data under a model in which the genotype effect was estimated against the likelihood of a nested model in which the genotype effect was constrained to be zero. Under the likelihood ratio test, two times the difference in minus $\log_{10}$ base likelihoods is distributed as a χ^2 statistic with degrees of freedom equal to the difference in number of parameters between the models (in this instance, one). Secondary analyses were carried out assuming dominant and recessive genetic models, in which instance the genotype was coded as 0 or 1 depending on whether the subject carried the minor allele (genotype = 1 if minor allele carrier, 0 if not [dominant model]; genotype = 1 if homozygous for minor allele, 0 if not, [recessive model]). Parameter estimates (i.e., β-coefficients) were obtained by maximum likelihood, and odds ratios (ORs) were obtained by taking the inverse log of the β-coefficient. The OR for the additive model was scaled to reflect the odds that a case was homozygous for the minor allele versus the odds that the case was homozygous for the major allele. The variance components analysis was carried out using the SOLAR software program (38).

In similar fashion, we also evaluated the effect of genotype on levels of quantitative traits (e.g., glucose and insulin levels) by comparing mean trait levels across genotypes. We compared the likelihood of a model in which the trait values were allowed to vary by genotype (unconstrained model) with that in which the genotype effects were constrained to be zero. As before, the model likelihoods and parameter estimates were computed conditional on the pedigree structure, and likelihoods obtained from competing models were compared by likelihoods ratio test. Again, the additive model was used as our primary analysis, and dominant and recessive models were used as secondary analyses. The quantitative trait analyses were conducted in nondiabetic (NGT and IGT) individuals only.

The disequilibrium statistics r^2 and $|D'|$ between each pair of SNPs were calculated using ZAPLO (39). For SNP pairs with $r^2 > 0.9$, only one of the two SNPs was genotyped in the full sample set. To determine the haplotype structure of SNPs with *CASQ1*, we used Haploview (40). We used Haploscore (41), which uses efficient score statistics within a general linear model framework, to test for haplotype associations between case and control subjects. Haploscore computes empirical *P* values by simulation. For all analyses, *P* values ≤0.05 were considered statistically significant, while *P* values between 0.05 and 0.1 were considered of borderline statistical significance.

RESULTS

Sequencing of all exons, exon-intron boundaries, and the proximal regulatory region of *CASQ1* revealed a total of six polymorphisms. These include three novel SNPs upstream of the ATG start codon, −1470T→C (CasqSNP1), −1456delG (CasqSNP2), and −1366insG (CasqSNP3), and one novel SNP in exon 3, 593C→T (CasqSNP4), which was silent (Genbank accession no. NM_001231) (Fig. 1). In addition, we detected two SNPs previously reported in the dbSNP database (rs1186694 and rs3747623), located at 1,404 bp upstream of the ATG start codon and in intron 2, respectively. Of the 22 SNPs in and immediately flanking this gene reported in the public databases at the time of this study (including rs1186694 and rs3747623), we confirmed 11 by genotype analysis; the other 11 were not polymorphic (or had very low allele frequency) in the Amish (Fig. 1). In total, there were 15 SNPs in *CASQ1*; one occurred in the coding region and did not predict alter-

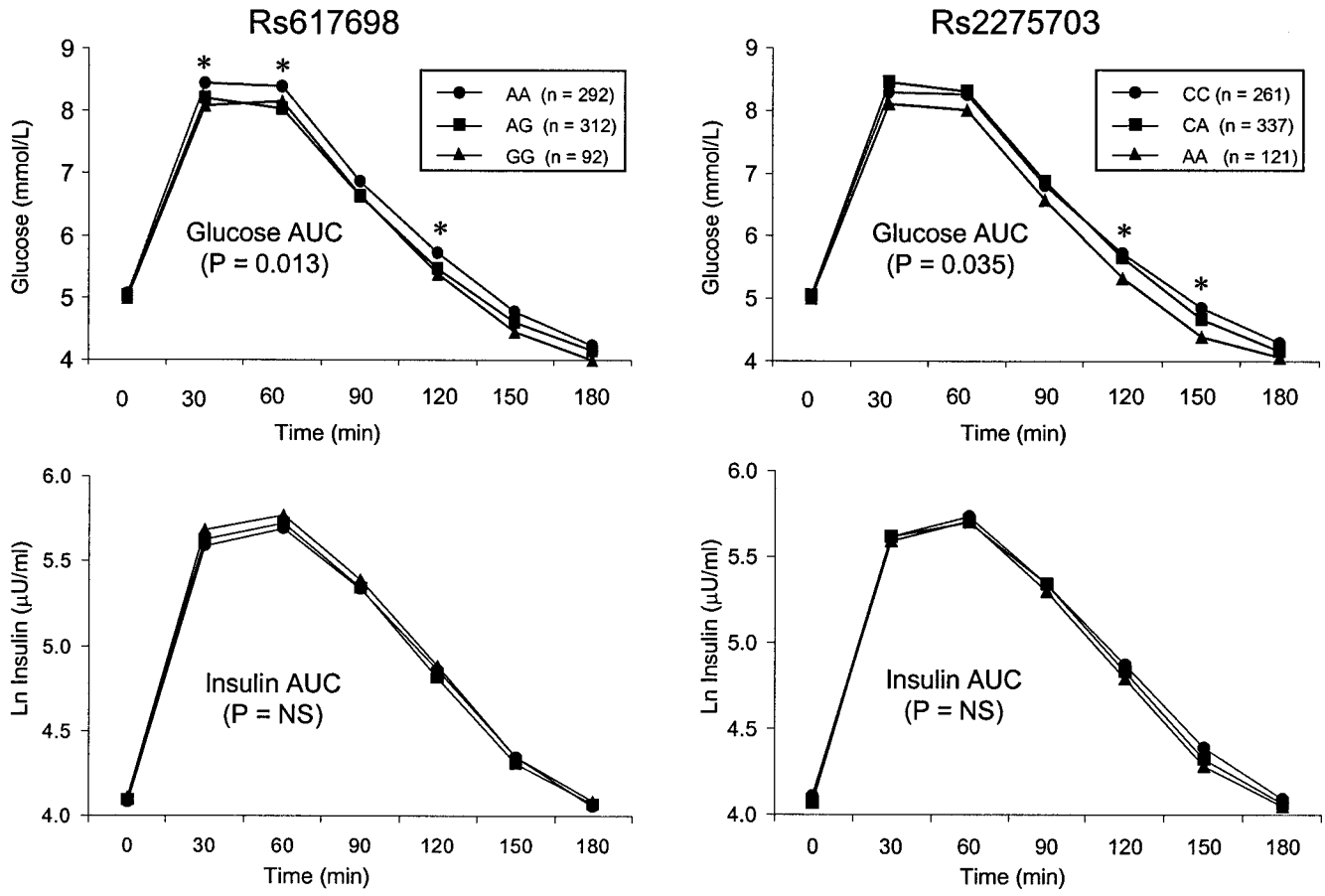

FIG. 2. Association of *CASQ1* SNPs rs617698 and rs2275703 with glucose and insulin levels during a 3-h OGTT in nondiabetic individuals. Both SNPs were associated with total OGTT glucose AUC ($P = 0.013$ and 0.035, addidtive model for rs617698 and rs2275703, respectively). The A allele of rs617698 appeared to better fit a recessive genetic model, with AA homozygotes having significantly higher glucose AUC than heterozygotes and GG homozygotes ($P = 0.013$). By contrast, the C allele of 2275703 appeared to better fit a dominant genetic model, with CC homozygotes and heteroyzgotes having higher glucose AUC than AA homozygotes ($P = 0.03$). *$P < 0.05$ between genotypes for a given time point. Rs822450 was also significantly associated with glucose AUC ($P = 0.042$) (data not shown). There was no evidence for association of rs617698 or rs2275703 with OGTT insulin levels.

ations in the amino acid sequence, four were in the 5′ flanking region, one was in the 3′ flanking region, and all others were in introns and did not predict any obvious alterations in RNA splicing.

Based on our initial genotype and LD analysis performed in 258 relatively unrelated Amish subjects, CasqSNP1 and CasqSNP2, located only 14 bp apart in the 5′ flanking region, were in complete LD with one another; therefore, only CasqSNP1 was genotyped in the full sample. Similarly, strong LD was observed between rs617698 and rs374723 ($r^2 = 0.94$) and rs822450 and rs686015 ($r^2 = 0.93$); thus, only rs617698 and rs822450 are reported in the full sample. In addition, CasqSNP3 and rs2275705 were found to be rare (allele frequencies 0.034 and 0.002, respectively) in the Amish and not pursued further. We typed the remaining 10 informative haplotype-tagging polymorphisms in 145 type 2 diabetic individuals, 148 individuals with IGT, and 358 individuals with NGT and tested for association between *CASQ1* polymorphism and diabetes. Using an additive model, genotype frequencies of rs2275703 and rs617698 in introns 4 and 2 of *CASQ1* differed significantly between subjects with type 2 diabetes and those with NGT ($P = 0.008$ and 0.04, respectively), with the more common allele being the at-risk allele for type 2 diabetes (OR for common alleles 1.75 [95% CI 1.27–2.43] and 1.54 [1.01–2.38], respectively) (Table 1). Three other SNPs spanning from intron 2 to the immediate 3′ flanking region showed borderline evidence for association to type 2 diabetes ($P = 0.076–0.093$). Rs2275703 was also associated with the combined trait type 2 diabetes/IGT (OR for common allele 1.56 [1.18–2.23], $P = 0.005$), as were the common alleles of rs3747622 (1.78 [1.06–3.03], $P = 0.026$) and rs617698 (1.39 [1.00–1.92], $P = 0.047$), both in intron 2, and rs3827532 in intron 10 (3.85 [1.14 –12.50], $P = 0.030$). Three other polymorphisms showed borderline association with type 2 diabetes/IGT ($P = 0.064–0.10$). Genotypic associations estimated under recessive and dominant genetic models did not provide a better fit to the data than that estimated under the additive model (data not shown). Eight SNPs lying 1.9–217 kb upstream and three SNPs lying 8.9–169.9 kb downstream of *CASQ1* were not significantly associated with type 2 diabetes (data not shown).

We assessed the relationship between the SNPs and quantitative traits in an expanded set of 754 nondiabetic members of the AFDS. Three SNPs (rs617698, rs2275703, and rs822450) were significantly associated with glucose AUC during the OGTT ($P < 0.05$) (Fig. 2). These findings in nondiabetic subjects provide additional support for an effect of *CASQ1* polymorphism on glucose homeostasis.

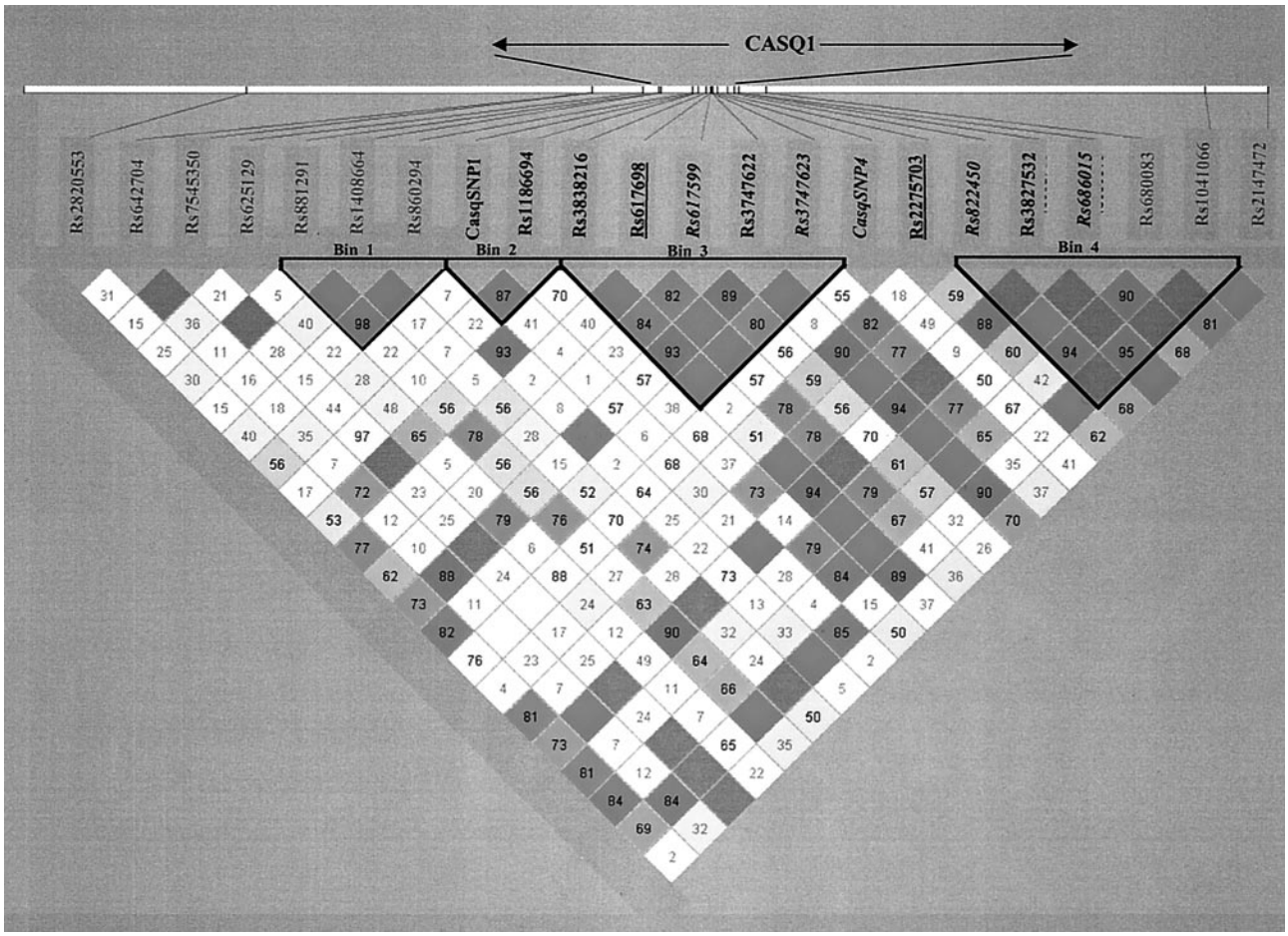

FIG. 3. Haplotype structure of CASQ1 and flanking regions. Shown is the haplotype structure of *CASQ1*, as estimated by Haploview (40), on genotypes from 258 Amish subjects. |D′| values ×100 are shown in the boxes, with empty boxes being 100 (or |D′| = 1). Not shown are CasqSNP1, which was in complete LD with CasqSNP2, and CasqSNP3 and rs2275705, which were both rare in the Amish (allele frequency 0.034 and 0.002, respectively). SNPs showing significant association to type 2 diabetes are underlined, while SNPs showing borderline significant association to type 2 diabetes are shown in italics.

There was no evidence for association of any of the SNPs with BMI, insulin levels (Fig. 2), or HOMA-IR.

The two SNPs associated with type 2 diabetes, rs617698 and rs2275703, are in introns 2 and 4, and are located only 2,223 bp apart. We thus sought to further define the haplotype structure of *CASQ1* to determine whether these two SNPs might be tagging a single haplotype that is associated with type 2 diabetes or whether they may be defining distinct haplotypes, both of which are associated with type 2 diabetes. Using the Hapview program (40), the *CASQ1* region formed a complex pattern of haplotype structure. There were four main blocks (or bins): bin 1 (rs881291, rs1408664, and rs860294; promoter), bin 2 (CasqSNP1 and rs1186694; promoter), bin 3 (rs3838216, rs617698, rs617599, rs3747622, and rs3747623; intron 1–2), and bin 4 (rs822450, rs3827532, 686015, rs680083, and rs1041066; intron 7 to 3′ flank). Rs2275703 in intron 4 and several SNPs in bin 4 (e.g., rs822450, rs3827532, rs686015, and rs680083 from intron 7 to the 3′ flanking region) appeared to be in partial LD with bin 3 SNPs (Fig. 3) (pairwise r^2 and |D′| for all SNP pairs also reported in Table 2 in the online appendix).

Although the two SNPs most significantly associated with type 2 diabetes, rs617698 and rs2275703, are not assigned to the same bin, they are moderately correlated (|D′| = 0.78, r^2 = 0.49). In fact, the borderline-significant *P* value of 0.04 for association of rs617698 with type 2 diabetes can be accounted for by this correlation by examining the haplotype analysis results in Table 2. These results indicate that the more common C allele at rs2275703 is associated with increased risk of diabetes (as indicated by a positive Haploscore) and that the A allele at rs617698 is also associated with increased risk primarily because it tracks with the rs2275703 C allele. In other words, the rs617698 at-risk A allele association with type 2 diabetes is derived from its association with the common A–C haplotype containing the rs2275703 at-risk C allele. Furthermore, examination of three-, four-, and five-SNP haplotypes did not provide evidence for association with

TABLE 2
Haploscore results comparing type 2 diabetic cases with NGT control subjects using SNPs rs617698 and rs2275703

Rs617698	Rs2275703	Frequency	Haploscore	*P**
G	A	0.286	−2.29	0.022
A	A	0.105	−1.79	0.073
G	C	0.0466	0.357	0.72
A	C	0.563	2.95	0.0032

*Global *P* value = 0.016.

type 2 diabetes that was any greater than that of rs2275703 alone (data not shown).

DISCUSSION

Chromosome 1q21-q24 has perhaps the best replicated linkage to type 2 diabetes in diverse populations, including Caucasians, Native Americans, and Chinese (3–10). Thus, there is a high likelihood that at least one type 2 diabetes susceptibility gene resides in this region. We and others have embarked upon a systematic LD mapping and positional candidate gene effort toward the positional cloning of the gene(s). Our LD mapping in Amish samples, as well as finer linkage analysis in Utah Caucasians (42), suggest at least two distinct type 2 diabetes loci within the 1q21-q24 interval. One of these regions, at ~157–158.5 Mb, contains a number of good positional candidate genes, including *CASQ1*.

Calcium release from the sarcoplasmic reticulum into the cytosol has been shown to regulate GLUT4 expression (29) and glucose transport in muscle (30). Hayashi et al. (43) showed that an increase in cytoplasmic calcium is a key step in the activation of intracellular signaling cascades that mediate the immediate and prolonged effect of exercise on glucose transport. CASQ is present in muscle cells and located in the lumen of the sarcoplasmic reticulum. The major CASQ isoforms in skeletal (CASQ1) and cardiac (CASQ2) muscle exist as predominantly fast and slow isoforms, respectively (44). CASQ1 binds and releases large quantities of Ca^{2+} rapidly through its high capacity (40–50 Ca^{2+} per CASQ molecule) and relatively low-affinity interactions with Ca^{2+} (28,45). Howarth et al. (31) investigated the expression of CASQ and Ca^{2+} binding in cardiac and skeletal muscle from the streptozotocin-induced diabetic rat and found no significant changes in heart but an increase in the relative abundance of CASQ and CASQ-like proteins in skeletal muscle. Together, these data suggest that CASQ1 may play an important role in insulin- and/or contraction-stimulated glucose transport. We thus hypothesized that sequence variants in *CASQ1* that alter expression or function may change intracellular Ca^{2+} flux and decrease glucose transport leading to glucose intolerance and/or diabetes.

We have extensively screened *CASQ1* for variants in Amish subjects with type 2 diabetes. We identified four novel SNPs (three in the promoter and one in exon 3), none of which encode variants that would be expected to alter the protein's structure. Although it is possible that the SNPs in the promoter may alter *CASQ1* expression, none were associated with type 2 diabetes. Genotyping and association analysis of 10 informative polymorphisms revealed that two were significantly associated with type 2 diabetes and three others showed borderline evidence for association with type 2 diabetes. The two most significantly associated SNPs, rs2275703 and rs617698, are located in introns 4 and 2, respectively. Interestingly, the more common alleles of rs2275703 and rs617698 were the at-risk alleles, which is in line with the common disease, common variant hypothesis and with findings for other type 2 diabetes susceptibility genes, e.g., calpain 10 and Pro12Ala persoxisome proliferator–activated receptor γ2. Supportive evidence that one or more of these variants influence glucose homeostasis is that allele frequencies of type 2 diabetes–associated SNPs were intermediate between type 2 diabetes and NGT in subjects with IGT (Table 1). Furthermore, rs2275703 and rs617698 were associated with glucose levels in nondiabetic subjects (Fig. 2).

Unfortunately, since our original linkage was obtained from large multiplex Amish families, determining how much of the original linkage can be accounted for by the *CASQ1* SNPs is not straightforward. However, given the relatively modest evidence for association of *CASQ1* SNPs with type 2 diabetes, it is unlikely that these SNPs (or haplotypes) can explain our original linkage result. Indeed, two-point linkage analysis with *CASQ1* SNPs showed little evidence for linkage to type 2 diabetes or type 2 diabetes/IGT.

Might these associations represent false-positive results due to multiple comparisons? For association studies such as these, this is always a possibility. Indeed a full Bonferroni correction for multiple comparisons would render our associations nonsignificant (at the $P < 0.05$ level). However, we believe that correction for multiple comparisons is overly conservative, since some of our traits are correlated with each other. Furthermore, we have evidence for linkage in this region not only in the Amish but also in several other populations, which led to this hypothesis-driven LD mapping and positional candidate gene effort. Further reducing concern regarding false-positive results are two internal consistencies. First, the same SNPs showed association to type 2 diabetes and glucose traits in nondiabetic subjects. Second, for 8 of the 10 SNPs, the allele frequencies for the IGT cases were intermediate between those for the type 2 diabetes cases and the NGT control subjects.

Perhaps most compelling is that our findings are similar to studies conducted in an independent study of Utah Caucasians showing association of *CASQ1* polymorphism with type 2 diabetes (46). The region of association with type 2 diabetes that overlaps in the Amish and the more outbred Utah Caucasian population appears to be within intron 2, specifically rs617599 and rs617598 in Utah Caucasians and rs617598 in the Amish (although this SNP was not the most significantly associated SNP in the Amish). Rs617599 and rs617598 are only 87 bp from one another and in moderate LD ($d' = 0.82$, $r^2 = 0.62$) in the Amish. However, contrary to our findings in the Amish, in which the rs617598 A allele was the at-risk allele for type 2 diabetes, the frequency of the G allele was significantly higher in Utah Caucasian subjects with type 2 diabetes. This discrepancy between the two populations may indicate that this SNP is not the functional SNP but is marking an at-risk haplotype that differs between the Amish and Utah Caucasians. Alternatively, these discrepancies between populations could represent false-positive or false-negative results.

The findings reported here do not provide insights into possible functional consequences of the variants in *CASQ1*. It is possible that variants in introns may affect mRNA splicing and/or expression. However, we cannot rule out the possibility that other variants in *CASQ1* or other nearby genes in LD with *CASQ1* SNPs might be the responsible functional variants.

We have observed evidence for association of other 1q21-q24 positional candidate genes with type 2 diabetes

or related traits, namely lamin A (*LMNA*) (26), guanine exchange factor-11 (*ARHGEF11*) (M.S., A.R.S., unpublished observations), and omentin 2 (*ITLN2*) (27). While all of these genes reside under our linkage peak, they are 4.0, 3.2, and 0.75 Mb from *CASQ1*, respectively. There does not appear to be any significant LD for the two *CASQ1* type 2 diabetes–associated SNPs, rs617598 and rs2275703, with SNPs in *LMNA*, *ARHGEF11*, or *ITLN2*, suggesting that the associations of variation in these genes with type 2 diabetes and related traits are independent of one another. These findings, coupled with more than one association signal in Pima Indians and Utah Caucasians, suggest that there are likely to be more than one gene (possibly several genes) responsible for the linkage at 1q21-q24.

In summary, we have reported a systematic search for polymorphism in *CASQ1* on chromosome 1q21 and identified four novel SNPs. None predict alterations in the protein sequence. We describe for the first time a significant association between *CASQ1* polymorphism and type 2 diabetes, possibly suggesting a new susceptibility gene for type 2 diabetes. Further analysis in other populations, as well as functional studies, will be necessary to further elucidate the role of polymorphism in *CASQ1* in the pathogenesis of type 2 diabetes.

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