A Common Variant in *PPP1R3* Associated With Insulin Resistance and Type 2 Diabetes

James Xia, Stephen W. Scherer, Patricia T.W. Cohen, Martin Majer, Tina Xi, Rodney A. Norman, William C. Knowler, Clifton Bogardus, and Michal Prochazka

Selected candidate genes have been analyzed in the Pima Indians of Arizona based on evidence that insulin resistance and type 2 diabetes have significant genetic determinants. An amino acid substitution at codon 905 of the glycogen-targeting subunit of type 1 protein phosphatase that regulates skeletal muscle glycogenesis was recently reported to be associated with changes in insulin action in Danish subjects. In addition to the variant at 905, we report here a novel substitution at codon 883 and common variant of an "ATTTA" element in the 3'-untranslated region (UTR) of the corresponding gene (*PPP1R3*). The 3'-UTR variant resembled the mRNA-destabilizing AT(AU)-rich elements (AREs) and resulted in a 10-fold difference in reporter mRNA half-life, was correlated with *PPP1R3* transcript and protein concentrations in vivo, and was associated with insulin resistance and type 2 diabetes in the Pimas. The variant is more common in Pimas (0.56) than in Caucasians (0.40). Because of its apparent effect on expression of *PPP1R3*, it may, in part, contribute to the higher prevalence of type 2 diabetes in this Native American population. *Diabetes* 47:1519–1524, 1998

I nsulin resistance (IR) is characterized by a reduced ability of insulin to mediate the uptake and metabolism of glucose by responsive tissues (1,2). IR is a risk factor for type 2 diabetes, and there is strong evidence that compensatory hyperinsulinemia contributes to the pathogenesis of hypertension and dyslipidemia, predisposing to atherosclerosis, coronary heart disease, and stroke (3–6). Studies in various populations—including the Pima Indians of Arizona, who have the world's highest reported prevalence of type 2 diabetes—demonstrate that type 2 diabetes and IR have significant heritable components (7–10). To identify the predisposing genes in the Pima Indians, we have analyzed selected candidate genes that regulate glucose storage as glycogen in skeletal muscle, because it is the main pathway of insulin-mediated glucose disposal in vivo (11,12). Insulin-resistant Pima Indians are characterized by decreased activity of skeletal muscle glycogen synthase as well as its regulatory enzyme, the glycogen-bound type 1 protein phosphatase (PP1G) (13–15). However, structural abnormalities have not been found in the gene encoding glycogen synthase (*GYS1*) (16) or the PP1 catalytic β-subunit (*PPP1CB*) (17). An amino acid polymorphism involving codon 905 (Asp905Tyr) of the glycogen-targeting subunit (G-subunit) of type 1 protein phosphatase was recently described to be associated with changes in insulin action in nondiabetic Danish Caucasians (18,19). We have now investigated the corresponding gene (*PPP1R3*) in the Pima Indians.

RESEARCH DESIGN AND METHODS

Subjects. The participants were full-blooded Pima/Papago Indians of the Gila River Indian Community in Arizona who have been participating in a longitudinal study of the development of type 2 diabetes. A subset of nondiabetic subjects was recruited for inpatient studies to determine their insulin action and secretion. Briefly, these subjects were admitted to the clinical research center for 10–12 days and fed a weight-maintaining diet (50% carbohydrates; 30% fat; 20% proteins). Three or more days after admission, fasting plasma insulin and insulin-mediated glucose uptake (IMGU) rates were determined in response to low-dose (plasma insulin concentration 845 ± 10 pmol/l) and high-dose (plasma insulin concentration 12,210 ± 185 pmol/l) insulin by the two-step hyperinsulinemic-euglycemic clamp, as described (20). Insulin secretory function was assessed by an intravenous glucose tolerance test after an injection of 25 g glucose and in response to a 75-g oral glucose tolerance test. All parameters were adjusted for age, sex, and percent body fat determined by underwater weighing (20). For analysis, the person with the lowest BMI was selected from each nuclear family. If a person was examined more than once, data from his or her first visit was used. Characteristics of the subjects are summarized in Table 1. All studies were approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases and the Tribal Council of the Gila River Community, and each individual signed an informed consent form.

Chromosomal localization of *PPP1R3*. The position of *PPP1R3* was initially determined by typing of somatic cell hybrids and subsequently refined by analysis of the radiation hybrid mapping set RH G3. Clones 701A8, 742F8, and 772H4 containing *PPP1R3* as identified by polymerase chain reaction (PCR) screening of the CEPH YAC human genomic library were used to determine its organization.

Transcript analysis. Transcript sizes and tissue distribution were determined with a human Multiple Tissue Northern blot from Clontech (Palo Alto, CA) hybridized with ^{32}P-labeled probe spanning *PPP1R3* coding sequence amplified by PCR from skeletal muscle cDNA. The alternatively spliced cDNA missing central part of exon 1 was PCR-amplified and sequenced in skeletal muscle cDNA from 24 Pima Indians and 1 Caucasian. The 3'-RACE experiment was performed using Marathon-Ready cDNA (Clontech) prepared from skeletal muscle poly-A$^+$ RNA. PCR products obtained by amplification with a *PPP1R3*-specific reverse primer and the AP1 primer provided with the Marathon-Ready cDNA were cloned into the pCR2.1 vector using the TA cloning kit from Invitrogen (Carlsbad, CA) for subsequent sequencing.

TABLE 1
Association of *PPP1R3* polymorphisms with insulin action and diabetes

Genotypes	ATTTA			P	Adjusted P	883			P	Adjusted P	905			P	Adjusted P
	11	12	22			Ser/Ser	Ser/Arg	Arg/Arg			Tyr/Tyr	Asp/Tyr	Asp/Asp		
Insulin action															
M/W	14/6	44/17	22/11	—	—	6/1	35/15	38/19	—	—	13/6	46/18	22/10	—	—
Age (years)	29 ± 8	27 ± 6	27 ± 6	NS	NS	30 ± 7	28 ± 6	27 ± 6	NS	NS	28 ± 8	28 ± 6	27 ± 6	NS	NS
Body fat (%)	30 ± 9	30 ± 9	31 ± 9	NS	NS	30 ± 10	30 ± 9	30 ± 8	NS	NS	30 ± 9	30 ± 8	31 ± 9	NS	NS
Log_{10} fasting (pmol/l)	1.4 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	0.07	<0.01	1.3 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	0.05	<0.01	1.4 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	0.05	<0.02
IMGU to low-dose insulin (mg/kg EMBS · min)	3.46 ± 1.49	2.65 ± 0.99	2.60 ± 0.88	0.01	<0.01	4.24 ± 1.74	2.64 ± 0.89	2.72 ± 1.05	<0.01	<0.01	3.43 ± 1.47	2.61 ± 0.93	2.69 ± 0.98	<0.02	<0.01
IMGU to high-dose insulin (mg/kg EMBS · min)	9.71 ± 2.14	8.20 ± 2.27	8.65 ± 2.11	<0.04	0.01	11.01 ± 1.64	8.27 ± 2.24	8.52 ± 2.22	0.01	<0.01	9.60 ± 2.40	8.19 ± 2.13	8.85 ± 2.21	<0.04	<0.03
Diabetes															
M/W	84/98	189/261	123/175	—	—	29/40	170/212	197/282	—	—	87/102	189/259	120/173	—	—
Age (years)	44 ± 14	43 ± 14	46 ± 15	NS	NS	45 ± 14	43 ± 15	45 ± 15	NS	NS	44 ± 14	43 ± 14	46 ± 15	NS	NS
BMI (kg/m^2)	33.9 ± 8.4	34.1 ± 8.1	33.3 ± 7.7	NS	NS	33.9 ± 8.7	34.1 ± 8.2	33.6 ± 7.8	NS	NS	34.3 ± 8.5	33.9 ± 8.1	33.3 ± 7.7	NS	NS
Prevalance (%)	56	64	69	<0.02	<0.04	57	60	68	<0.02	<0.05	56	64	69	<0.01	<0.04

The data (means ± SD) of fasting insulin and of low-dose and high-dose IMGU rates were analyzed by multiple regression analysis. Differences in prevalence of type 2 diabetes with onset age <45 years in 930 people were analyzed by the χ^2 method. $P = P$ values for statistical comparison of unadjusted parameters. Adjusted $P = P$ values for comparison of parameters adjusted for sex, age, percent body fat, or BMI. EMBS, estimated metabolic body size (33).

Genomic DNA analysis. Genomic DNA was prepared from white blood cells or transformed lymphoblasts after signed informed consent was obtained from the participants. Southern blot analysis was performed with *Eco*RI-digested genomic DNA. Coding sequences, splicing sites, and the 5′- and 3′-untranslated regions (UTRs) were screened for mutations in 30 nondiabetic Pima Indians by PCR, and the products were sequenced with nested primers using the Dye terminator cycle sequencing FS kit and the ABI automated sequencer model 373A from ABI Division of Perkin Elmer (Foster City, CA). Primer information is available upon request.

Genotyping of polymorphisms at codon 883, 905, and ARE. The *Dde*I variant at codon 905 was genotyped on PCR products amplified with the forward primer 5′-TAGGTATTTGTAATGTACGTGTA-3′ and the reverse primer 5′-GTAACTGCATTCTCTACAGCAA-3′. The ARE variant was genotyped using primers 5′-CAGATAAAACATGGACAATGGC-3′ and 5′-TTGAAATATTTGAT-CAATGAATCC-3′. The variant at codon 883 was genotyped using the mismatch-PCR/RFLP assay (21). We designed a reverse primer that lies immediately adjacent to the variant site and incorporates a single base mismatch (indicated in lower case "g" instead of "T") at the second base preceding its 3′ end (5′-TTTCTTTGATAATTCTTGAACCgG-3′). PCR was performed in combination with a perfectly matched upstream forward primer (5′-TAGGTATTTGTAATGTACGT-GTA-3′) to produce a 254-bp fragment. In the presence of a "C" at the polymorphic site immediately adjacent to the 3′ end of the mismatched primer, the PCR product gained an artificial *Hae*III restriction site (gGCC), whereas DNA amplified from an allele containing an "A" at this position (gGAC) remained uncleaved. All polymorphisms were resolved by electrophoresis on a 4% agarose gel.

Analysis of RNA and protein from skeletal muscle biopsies. Collection of skeletal muscle biopsies and preparation of RNA and protein extracts were described previously (16). In Northern analysis, 20 mg total RNA was fractionated by electrophoresis through a 1.2% formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized with radiolabeled cDNA probes. Oligo-dT primed cDNA was synthesized from 2 µg of total RNA using the Superscript cDNA Synthesis Kit from GIBCO BRL (Gaithersburg, MD). All primer combinations for mRNA/cDNA quantification were designed to span at least one intron in the gene to avoid co-amplification of genomic DNA that may contaminate RNA preparations. β-Actin served as an internal control, as described (16). Differences in expression between transcripts containing ARE alleles 1 and 2 in heterozygotes were assessed by simultaneously comparing the relative allelic ratio in genomic DNA and skeletal muscle cDNA from each individual. PCR was performed using ^{32}P-labeled forward primer (5′-CAGATAAAACATGGACAATGGC-3′) and unlabeled reverse primer (5′-TTGAAATATTTGATCAATGAATCC-3′), and products were resolved on a 6% sequencing gel. After 24 cycles, aliquots were collected every two to three subsequent cycles to determine when the amplification was in an exponential phase. G-Subunit protein was detected by Western blotting using a polyclonal rabbit antibody (diluted 1:400) developed against a synthetic

peptide encompassing amino acids 391–407 from Research Genetics (Huntsville, AL), followed by treatment with a secondary anti-rabbit Ig heavy chain antibody (diluted 1:15,000) from Pierce (Rockford, IL). Variation in loading between lanes was normalized by immunostaining of the blots with a mouse monoclonal antibody against human α-actin (diluted 1:500) from Sigma (St. Louis, MO), followed by a secondary anti-mouse antibody (diluted 1:15,000; Pierce). The blots were stained with the SuperSignal chemiluminescent substrate (Pierce), and films were exposed for different time periods.

Analysis of reporter mRNA. The reporter constructs were created by inserting a 860-bp fragment of *PPP1R3* 3′-UTR encompassing ARE allele 1 or allele 2 between the stop codon and the first polyadenylation site of the rabbit β-globin gene in vector pNEORβG^{GC} (22). The allelic fragments were PCR-amplified with primers introducing an artificial *Xba*I site at both ends, and the products were ligated into a unique *Xba*I site within the 3′-UTR of the β-globin reporter gene. Constructs containing allele ARE 1 and ARE 2 in the forward (F) and reverse (R) orientations designated pR3ARE1F, pR3ARE2F, pR3ARE1R, and pR3ARE2R, respectively, were identified by PCR and verified by sequencing. NIH3T3 cells were grown in Dulbecco's modified eagle medium (GIBCO BRL) supplemented with 10% fetal bovine serum, 50 U/ml penicillin G and 50 µg/ml streptomycin. Cells were seeded at density 2×10^6 into 60 mm Petri dishes and incubated for 24 h, reaching approximately 50% confluency. Transfections were performed using 4-µg plasmid DNA and CalPhos Maximizer Transfection Kit (Clontech), and poly-A$^+$ RNA was isolated with Oligotex Direct mRNA Kit from QIAGEN (Valencia, CA) after 24 and 48 h. To determine the half-life of the reporter RNA, cells were treated with actinomycin D (5 µg/ml), and RNA was extracted at different time points over the 24-h period after transfection. Reporter transcripts were quantified by reverse transcription (RT)-PCR using *neo* mRNA/cDNA as an internal control.

All measurements of *PPP1R3* transcript, G-subunit protein, and reporter mRNA were performed in triplicate. The coefficients of variation were 15, 11, and 6%, respectively.

Statistical analysis. Statistical comparison of quantitative traits were performed using the general linear modeling program of the SAS Institute (Cary, NC). In these analyses, genotype was treated as a class variable. Plasma insulin concentrations were log_{10} transformed before analysis to approximate a normal distribution. Repeated measures of analysis of variance and t tests were also performed for quantitative comparisons of *PPP1R3* and reporter mRNA levels.

RESULTS

We have determined that *PPP1R3* is located on chromosome 7q31.1-q31.2 and consists of four exons (Fig. 1*A*). Northern blot hybridization revealed two transcripts (~5 kb
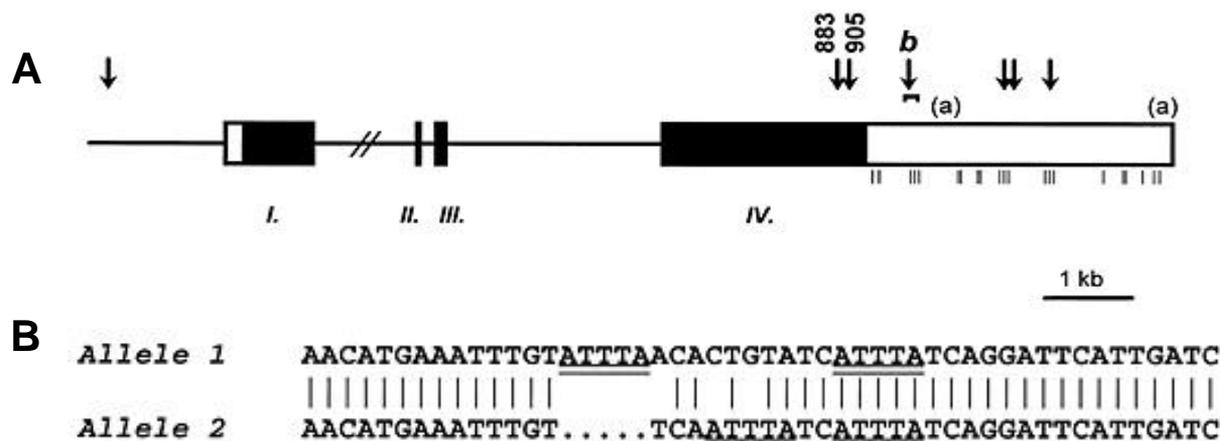
FIG. 1. Genomic structure of *PPP1R3*. *A*: Coding sequences are shown as solid boxes, 5'- and 3'-UTR as open boxes, and introns as thin lines. Two polyadenylation sites (each preceded by the characteristic AATAAA signal) are marked by (a), and ARE motifs in 3'-UTR by vertical tick marks. All variants are indicated by arrows. The Arg883Ser and Asp905Tyr variants are determined by an AGG AGT and GAC TAC substitution, respectively. The polymorphic ARE is indicated by *b*. *B*: Alignment of ARE allele 1 and 2 encompassing the third and fourth ATTTA element (double underlined) in 3'-UTR. Due to a small rearrangement, the distance between both elements is 10 bp in allele 1 and 2 bp in allele 2. GenBank accession numbers: AF024576-AF024579.

and ~7.5 kb) in human skeletal muscle and heart that differ by the length of the 3'-UTR as detected by 3'-RACE. In skeletal muscle, the 5-kb transcript was about 2.5-fold more abundant than the 7.5-kb transcript. We also found a shorter transcript missing 521 bp of the coding sequence between bases 179 and 699 of the published cDNA (23). The beginning and end of the excluded segment are demarcated by the characteristic splice donor (GT) and splice acceptor (AG) sites, respectively, and we conclude that the shorter mRNA is produced by alternative splicing. This form, representing 8–24% of the full-length transcript, has a change in reading frame and predicts a truncated protein consisting of 74 amino acids. However, it is not yet known whether this shorter transcript is translated.

Southern blot analysis of *Eco*RI-digested DNA from 52 nondiabetic Pimas did not show any major structural alterations in *PPP1R3*, regardless of whether the subjects were insulin sensitive or insulin resistant. Screening of the gene by PCR and sequencing in 30 nondiabetic individuals revealed two single-base substitutions within the last exon that predict an aspartic acid–to–tyrosine variant at codon 905 (Asp905Tyr), as previously described in Caucasians (18), and a novel arginine-to-serine variant at codon 883 (Arg883Ser). Four single-base substitutions were identified in noncoding regions, including one approximately 1.6 kb upstream from the starting methionine codon, and three variants within a 600-bp region downstream from the first polyadenylation signal (Fig. 1*A*). There was also a 5-bp length polymorphism in 3'-UTR affecting the distance between two ATTTA pentanucleotides (alleles ARE1 and ARE2; Fig. 1*B*) that are the smallest consensus motifs of mRNA-destabilizing AREs (24). All polymorphisms were in linkage disequilibrium, and we have genotyped the di-allelic variants at codons 883 and 905, as well as ARE, in 930 Pima Indians. The frequencies of Ser883, Tyr905, and ARE1 were 0.27, 0.44, and 0.44, respectively; each was associated with a lower fasting plasma insulin, higher IMGU in response to low-dose and high-dose insulin infusions, and lower prevalence of type 2 diabetes than alleles Arg883, Asp905, and ARE2 (Table 1). The differences

remained statistically significant (<0.05) after adjusting for sex, age, and BMI or percent body fat individually, as well as for all covariates combined.

By analysis of DNA from 56 CEPH Caucasians, we found that the ARE polymorphism was also common in this population. However, the frequency of the ARE2 allele (0.4) was significantly lower than in the Pimas ($P < 0.001$). Furthermore, codon 883 polymorphism was not observed in this Caucasian sample.

In skeletal muscle biopsies from 20 nondiabetic Pima Indians, there was a sixfold interindividual variation of *PPP1R3* mRNA concentration that positively correlated with differences in G-subunit protein concentration ($r = 0.73$; $P < 0.01$; Fig. 2*A*). Moreover, the mRNA and protein correlated positively with IMGU in response to high-dose insulin ($r = 0.51$, $P = 0.02$; Fig. 2*B* and *C*). ARE motifs have been implicated in posttranscriptional regulation of mRNA stability in some genes (24); and the mean *PPP1R3* mRNA concentration in skeletal muscle from nine ARE2/ARE2 homozygous individuals was 44% lower compared with that from four ARE1/ARE1 homozygotes ($P = 0.01$; Fig. 2*D*). The quantitative differences between the ARE1- and ARE2-containing *PPP1R3* mRNA were further confirmed in 12 ARE1/ARE2 heterozygotes in whom the ARE2-containing mRNA had an approximately 20% lower concentration relative to the ARE1-containing mRNA ($P < 0.001$; not shown).

To determine whether the *PPP1R3* mRNA differences observed in skeletal muscle biopsies could be attributed to the polymorphic 3'-UTR, we constructed plasmids in which 860 bp between the stop codon and the first polyadenylation site of *PPP1R3* (encompassing ARE1 and ARE2 alleles, respectively) were inserted in either orientation downstream of the stop codon in the 3'-UTR of rabbit β-globin reporter gene in the vector pNEORβG[GC] (22) (Fig. 3*A*). The abundance of the chimeric β-globin transcript was measured 24 h and 48 h after transfection, and no significant differences in β-globin mRNA levels were observed between cells transfected with pNEORβG[GC] vector alone and those transfected with constructs containing the 860-bp *PPP1R3* fragment in reverse ori-
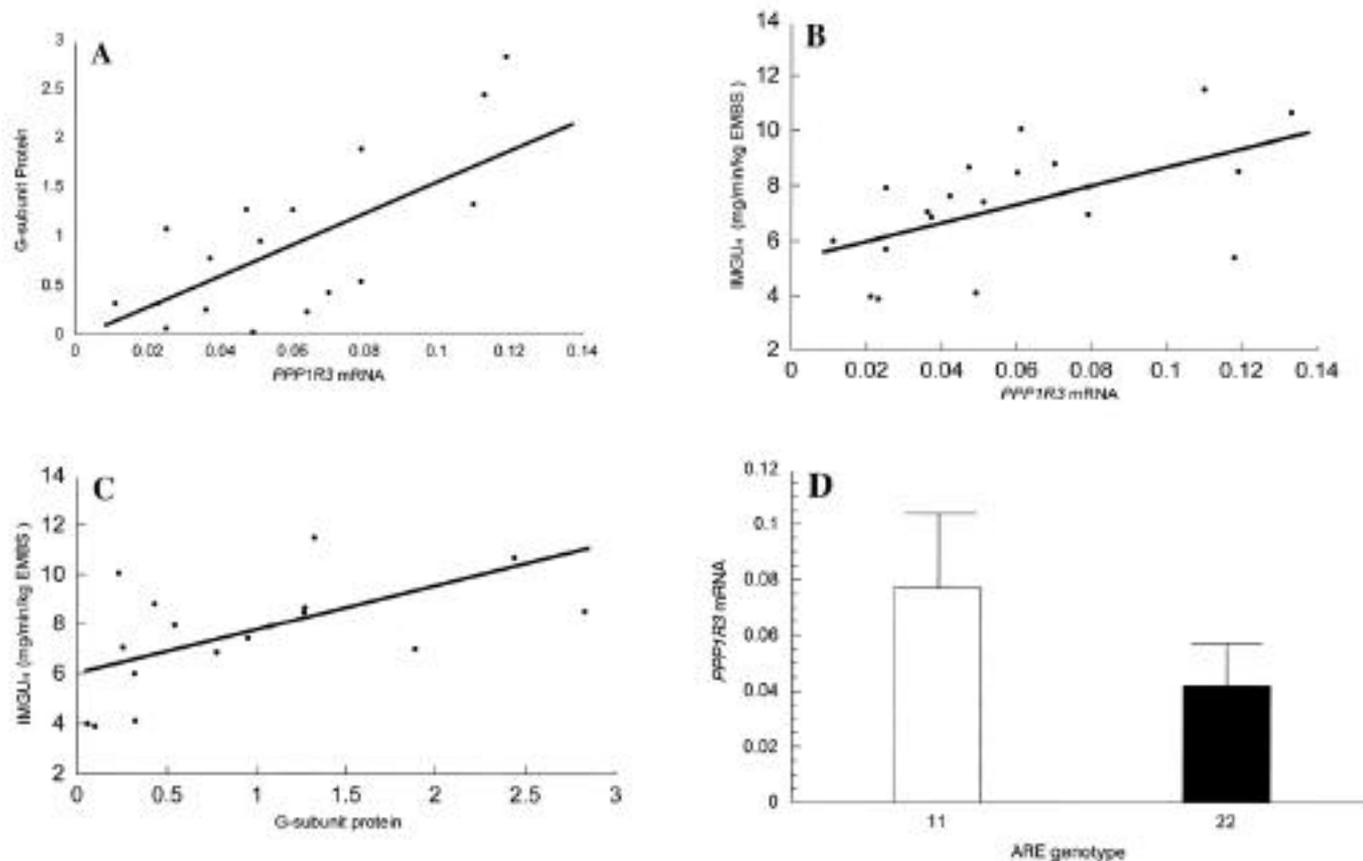
FIG. 2. *PPP1R3* mRNA and G-subunit protein levels in skeletal muscle. *A*: Correlation between levels of *PPP1R3* mRNA and G-subunit protein ($r = 0.73$, $P < 0.01$). Correlation between *PPP1R3* mRNA (*B*) ($r = 0.51$, $P = 0.02$) or G-subunit protein (*C*) ($r = 0.51$, $P = 0.02$) and IMGU in response to a high dose of insulin (15,744 ± 1,482 pmol/l) measured by the hyperinsulinemic-euglycemic clamp (20). *D*: Concentration of mRNA in homozygotes for ARE allele 1 versus allele 2 ($P = 0.01$). The relative mRNA concentration (*PPP1R3*/β-actin mRNA ratio) determined by RT-PCR is the mean of triplicate measurements, and protein levels were measured by Western blots.

entation (pR3ARE1R and pR3ARE2R; Fig. 3*B*). However, the relative abundance of reporter mRNA encoded by constructs containing the 860-bp fragment in forward orientation (pR3ARE1F and pR3ARE2F) was approximately sevenfold lower than that observed with pR3ARE1R, pR3ARE2R, and pNEORβG^GC alone. Furthermore, the reporter mRNA encoded by pR3ARE2F was 20–40% lower than that observed for pR3ARE1F 24 h and 48 h after transfection ($P < 0.01$; Fig. 3*B*). The decay rates of all transcripts were also measured during a 24-h period after treatment with the transcription inhibitor actinomycin D. No significant differences in β-globin mRNA were observed with pR3ARE1R, pR3ARE2R, and pNEORβG^GC, whereas a 47 and 84% decrease was observed with pR3ARE1F and pR3ARE2F, respectively ($P < 0.001$; Fig. 3*B*). The estimated apparent half-life of the chimeric β-globin mRNA containing ARE2 was at least 10-fold shorter than that observed for mRNA with ARE1 (~2 h vs. >24 h, respectively; $P < 0.001$; Fig. 3*C*).

DISCUSSION

We have investigated *PPP1R3* as a functional candidate gene that could contribute to IR and type 2 diabetes in the Pima Indians. We describe here several variants in this gene that are associated with parameters of insulin action and with differences in diabetes prevalence. During the progress of this study, we have completed a genome-wide linkage scan of microsatellite markers in this population. Analysis of affected sib-pairs revealed that the location of markers showing an evidence for potential linkage with type 2 diabetes on chromosome 7 coincides with the genetic location of *PPP1R3* (25,26). The finding of linkage with diabetes in this region lends additional support to the hypothesis that variants in the *PPP1R3* gene contribute to the etiology of type 2 diabetes.

The associated polymorphisms we detected in *PPP1R3* include a potential mRNA destabilizing ARE motif in the 3′-UTR. ARE motifs in 3′-UTR have been implicated in mRNA degradation (22,24,27,28). The 7.5-kb transcript of *PPP1R3* contains 21 ARE motifs that may explain the ~2.5-fold lower abundance in muscle compared with the 5-kb mRNA, which has 5 motifs. In addition to their number, the relative spacing of AREs and base composition of the surrounding sequences play an important role in establishing their function as destabilizing elements (24). For example, it has been demonstrated that a transcript containing four clustered ATTTA elements was rapidly degraded, whereas an analogous transcript with four scattered AREs was stable (29). The ARE polymorphism of *PPP1R3* changes spacing between the third and fourth motif and may affect mRNA stability. The half-life of the natural *PPP1R3* mRNA has not been reported yet, and we attempted to measure it in skeletal muscle–derived
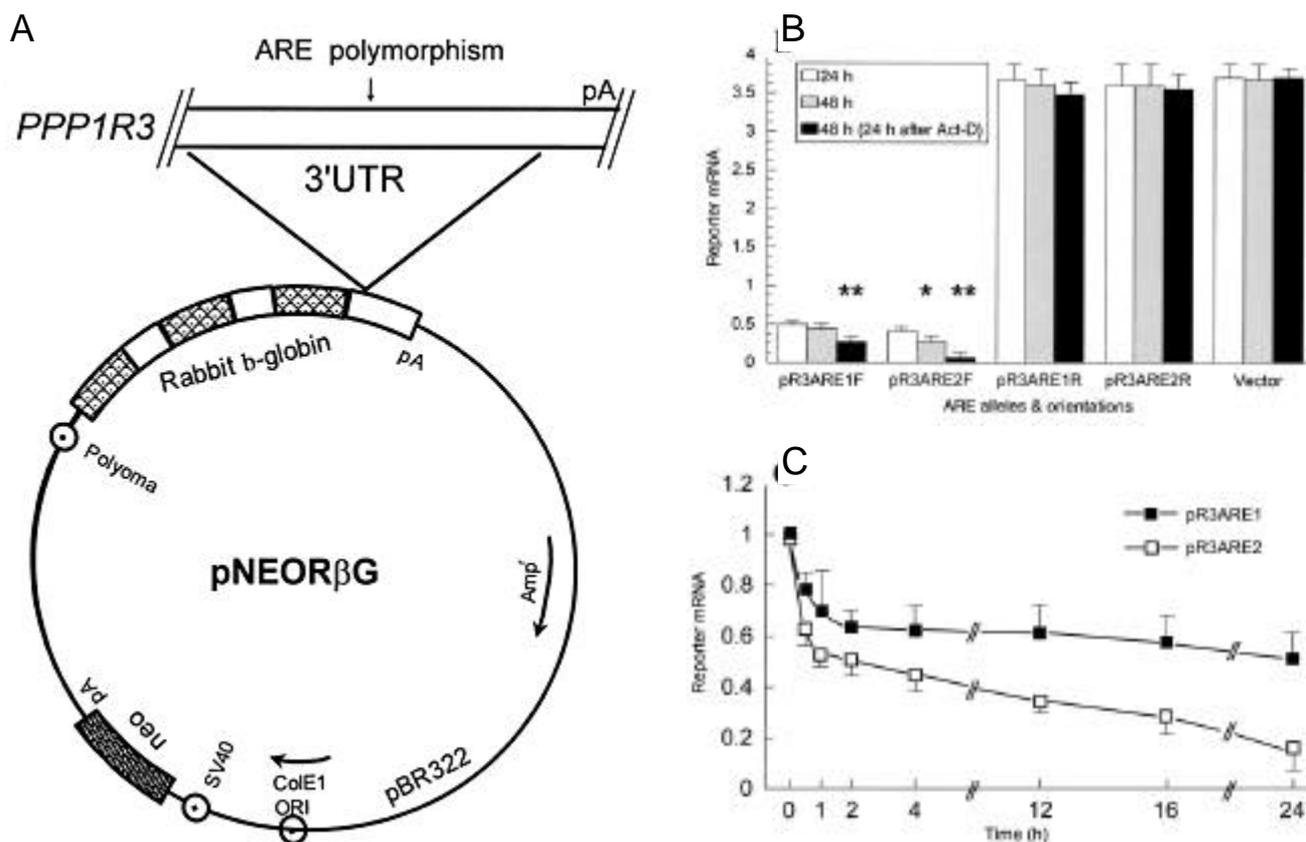
FIG. 3. Expression of ARE-containing reporter constructs in NIH3T3 cells. *A*: Reporter constructs were created by inserting 860 bp of 3'-UTR segments encompassing *PPP1R3* ARE allele 1 or allele 2 in either the forward (F) or reverse (R) orientation in the 3'-UTR of rabbit β-globin reporter gene in pNEORβG^{GC}. The constructs were designated pR3ARE1F, pR3ARE1R, pR3ARE2F, and pR3ARE2R, respectively. *B*: Reporter mRNA expression in NIH3T3 cells was measured 24 and 48 h after transfection without or with actinomycin D (Act-D) added 24 h after transfection. Statistically significant differences of the measurements (mean ± SD) between pR3ARE1F and pR3ARE2F determined by the *t* test are indicated (*P < 0.01, **P < 0.001). *C*: Time-dependent degradation of reporter mRNA in cells treated with actinomycin D 24 h after transfection. Data (mean ± SD) from three different experiments were used to test differences between ARE1- and ARE2-containing reporter transcripts by repeated measures of analysis of variance (P < 0.001).

human cell lines. However, we were unable to detect the transcript by RT-PCR in RNA extracted from three different primary myoblast cell cultures derived from nondiabetic Pima Indians (30) or in the rhabdomyosarcoma cell line A204. In contrast, the control β-actin transcript was abundantly expressed in these cells (not shown). Nevertheless, our data obtained with the chimeric β-globin constructs demonstrate that the 3'-UTR segment encompasses mRNA destabilizing signals, which act in an orientation-dependent manner, and that the polymorphic ARE further modulates the steady-state level of chimeric β-globin reporter transcripts. The 20–44% reduction in the abundance of ARE2-containing mRNA found in skeletal muscle of both homozygotes and heterozygotes for ARE1 and ARE2 alleles is consistent with the differences in the steady-state reporter mRNA concentration observed between pR3ARE1F and pR3ARE2F.

Chen et al. (23) also reported up to fivefold variation in the abundance of *PPP1R3* mRNA in Danish Caucasians, but no significant differences were found between 15 diabetic and 14 control subjects. We have found that the frequency of the ARE1 and ARE2 alleles in a group of 56 CEPH Caucasians was 0.60 and 0.40, respectively, which demonstrates that this ARE polymorphism is not unique to the Pima Indian population. Genotyping of this polymorphism in a large number of subjects will be necessary to assess its cor-

relation with mRNA differences and with type 2 diabetes in the Danish and in other populations. In our study, we limited the quantitative analysis of *PPP1R3* mRNA to nondiabetic subjects to avoid potentially misleading secondary changes in the expression in skeletal muscle of diabetic subjects. Furthermore, based on our data, we do not believe that the large interindividual variability of the transcript concentration is attributable only to the ARE variant. Rather, this polymorphism modulates the mRNA level, and other genetic as well as nongenetic factors are likely to contribute to the variation between subjects.

Similar to our findings of differences in insulin action in vivo second to changes in PP1G levels, Ragolia and Begum (31) recently reported that the overexpression of this subunit in rat skeletal muscle cells resulted in increased insulin-stimulated glucose uptake rates, glycogen synthase activation, and rates of glycogen synthesis. Also, the Tyr905 encoding allele has been recently associated with an increased insulin sensitivity in obese Caucasian males, although no effect on type 2 diabetes prevalence was observed (19). From our current data, we cannot determine whether the ARE polymorphism alone accounts for the observed associations in the Pimas. The functional consequences of the Asp905Tyr and Arg883Ser substitutions, if any, are yet unknown, and relevant studies are still in progress to investigate their biological

effects. Because we found an association of insulin resistance and diabetes with a polymorphism of an mRNA-destabilizing element that genetically and functionally correlates with quantitative differences in *PPP1R3* expression, it appears more likely that this variant largely contributes to the association of *PPP1R3* with insulin resistance and diabetes. Nevertheless, it is also formally possible that the polymorphisms in *PPP1R3* represent markers for yet another susceptibility gene in this region that could be the basis for the observed associations.

Insulin-mediated glucose disposal rate varies considerably, even among subjects with normal glucose tolerance (1,3). It has been estimated that 38–49% of the variance in insulin action in the Pima Indians is heritable (32), and the polymorphisms at *PPP1R3* therefore do not account for all of the heritability in this population. We anticipate that additional genes contribute to the manifestation of IR that predisposes to type 2 diabetes in the Pima Indians. The discovery that the ARE2 allele contributes to impaired insulin action and hyperinsulinemia, and the high frequency of this allele in Caucasians, indicates that this common variant may have a broader significance, contributing to IR and type 2 diabetes in various populations.

ACKNOWLEDGMENTS

REFERENCES

1. Reaven GM: Pathophysiology of insulin resistance in human disease. *Physiol Rev* 75:473–486, 1995
2. Reaven GM: Role of insulin resistance in human disease. *Diabetes* 37:1959–1967, 1988
3. Reaven GM, Lithell H, Landsberg L: Hypertension and associated metabolic abnormalities: the role of insulin resistance and the sympathoadrenal system. *N Engl J Med* 334:374–381, 1996
4. Bressler P, Baile SR, Matsuda M, DeFronzo RA: Insulin resistance and coronary artery disease. *Diabetologia* 39:1345–1350, 1996
5. Hong Y, Pedersen NL, Brismar K, de Faire U: Genetic and environmental architecture of the features of the insulin-resistance syndrome. *Am J Hum Genet* 60:143–152, 1997
6. Bogardus C: Insulin resistance in the pathogenesis of NIDDM in Pima Indians. *Diabetes Care* 16:228–231, 1993
7. Bogardus C, Lillioja S: Pima Indians as a model to study the genetics of NIDDM. *J Cell Biochem* 48:337–343, 1992
8. Schumacher MC, Hasstedt SJ, Hunt SC, Williams RR, Elbein SC: Major gene effect for insulin levels in familial NIDDM pedigrees. *Diabetes* 41:416–423, 1992
9. Hanis CL, Boerwinkle E, Chakraborty R, Ellsworth DL, Concannon P, Stirling B, Morrison VA, Wapelhorst B, Spielman RS, Gogolin-Ewens KJ, Shepard JM, Williams SR, Risch N, Hinds D, Iwasaki N, Ogata M, Omari Y, Petzold C, Rietzch H, Schroder HE, Schulze J, Cos NJ, Menzel S, Boriraj VV, Chen X: A genome-wide search for human non-insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. *Nat Genet* 13:161–166, 1996
10. Mahtani MM, Widen E, Lehto M, Thomas J, McCarthy M, Brayer J, Bryant B, Chan G, Daly M, Forsblom C, Kanninen T, Kirby A, Kruglyak L, Munnelly K, Parkkonen M, Reeve-Daly MP, Weaver A, Brettin T, Duyk G, Lander ES, Groop LC: Mapping of a gene for type 2 diabetes associated with an insulin secretion defect by a genome scan in Finnish families. *Nat Genet* 14:90–94, 1996
11. Young AA, Bogardus C, Wolfe-Lopez D, Mott DM: Muscle glycogen synthesis and disposition of infused glucose in humans with reduced rates of insulin-mediated carbohydrate storage. *Diabetes* 37:303–308, 1988
12. Bogardus C, Lillioja S: Where all the glucose doesn't go in non-insulin-dependent diabetes mellitus. *N Engl J Med* 322:262–263, 1990
13. Freymond D, Bogardus C, Okubo M, Stone K, Mott DM: Impaired insulin-stimulated muscle glycogen synthase activation in vivo in man is related to low fasting glycogen synthase phosphatase activity. *J Clin Invest* 82:1503–1509, 1988
14. Kida Y, Esposito-Del Puente A, Bogardus C, Mott DM: Insulin resistance is associated with reduced fasting and insulin-stimulated glycogen synthase phosphatase activity in human skeletal muscle. *J Clin Invest* 85:476–481, 1990
15. Mott DM, Kida Y, Nyomba BL: Human skeletal muscle, type-1 protein phosphatase and insulin resistance. *Adv Prot Phosph* 7:413–427, 1993
16. Majer M, Mott DM, Mochizuki H, Rowles JC, Pedersen O, Knowler WC, Bogardus C, Prochazke M: Association of the glycogen synthase locus on 19q13 with NIDDM in Pima Indians. *Diabetologia* 39:314–321, 1996
17. Prochazka M, Mochizuki H, Baier LJ, Cohen PTW, Bogardus C: Molecular and linkage analysis of type-1 protein phosphatase catalytic β-subunit gene: lack of evidence for its major role in insulin resistance in Pima Indians. *Diabetologia* 38:461–466, 1995
18. Hansen L, Hansen T, Vestergaard H, Bjorbaek C, Echwald SM, Clausen JO, Chen YH, Chen MX, Cohen PTW, Pedersen O: A widespread amino acid polymorphism at codon 905 of the glycogen-associated regulatory subunit of protein phosphatase-1 is associated with insulin resistance and hypersecretion of insulin. *Hum Mol Genet* 4:1313–1320, 1995
19. Hansen L, Reneland R, Hansen T, Berglund L, Rasmussen SK, Busch K, Lishell H, Pedersen O: Increased insulin stimulated glucose disposal rate in 1161 Caucasian males: interaction between obesity and the Asp905Tyr variant of the glycogen associated subunit of type 1 protein phosphatase (Abstract). *Diabetologia* 40 (Suppl. 1):A173, 1997
20. Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, Bogardus C: Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima Indians. *N Engl J Med* 329:1988–1992, 1993
21. Hingorani AD, Brown MJ: A single molecular assay for the C1166 variant of the angiotensin II type 1 receptor gene. *Biochem Biophys Res Com* 213:725–729, 1995
22. Shaw G, Kamen R: A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46:659–667, 1986
23. Chen YH, Hansen L, Chen MX, Bjorbaek C, Vestergaard H, Hansen T, Cohen PTW, Pedersen O: Sequence of the human glycogen-associated regulatory subunit of type-1 protein phosphatase and analysis of its coding region and mRNA level in muscle from patients with NIDDM. *Diabetes* 43:1234–1241, 1994
24. Ross J: mRNA stability in mammalian cells. *Microbiol Rev* 59:423–450, 1995
25. Hanson RL, Pima Diabetes Gene Group: Genomic scan for markers linked to type II diabetes in Pima Indians (Abstract). *Diabetes* 46 (Suppl. 1):51A, 1997
26. Knowler WC, Hanson RL, Pima Diabetes Genes Group: Genomic scan for genetic markers linked to diabetes in Pima Indians (Abstract). *Diabetologia* 40 (Suppl. 1):A8, 1997
27. Chen CY, Shyu AB: Selective degradation of early-response-gene mRNAs: functional analyses of sequence features of the AU-rich elements. *Mol Cell Biol* 14:8471–8482, 1994
28. Kabnick KS, Housman DE: Determinants that contribute to cytoplasmic stability of human *c-fos* and β-globin mRNAs are located at several sites in each mRNA. *Mol Cell Biol* 8:3244–3250, 1988
29. Gorospe M, Baglioni C: Degradation of unstable *interleukin-1α* mRNA in a rabbit reticulocyte cell-free system: localization of an instability determinant to a cluster of AUUUA motifs. *J Biol Chem* 269:11845–11851, 1994
30. Thompson DB, Pratley R, Ossowski V: Human primary myoblast cell cultures from non-diabetic insulin resistant subjects retain defects in insulin action. *J Clin Invest* 98:2346–2350, 1996
31. Ragolia L, Begum N: The effect of modulating the glycogen-associated regulatory subunit of protein phosphatase-1 on insulin action in rat skeletal muscle cells. *Endocrinology* 138:2398–2404, 1997
32. Sakul H, Pratley R, Cardon L, Ravussin E, Mott DM, Bogardus C: Familiality of physical and metabolic characteristics that predict the development of non-insulin-dependent diabetes mellitus in Pima Indians. *Am J Hum Genet* 60:651–656, 1997
33. Lillioja S, Bogardus C: Obesity and insulin resistance: lessons learned from the Pima Indians. *Diabetes Metab Rev* 4:517–540, 1988