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Short sequence-paper

Analysis of *PBX1* as a candidate gene for type 2 diabetes mellitus in Pima Indians[1]

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Abstract

The human proto-oncogene *PBX1* codes for a homeodomain containing protein that modulates expression of several genes, including those contributing to regulation of insulin action and glucose metabolism. *PBX1* is located on chromosome 1q22, a region linked with type 2 diabetes in Pima Indians, Caucasians, and an Old Order Amish population. We have investigated the *PBX1* genomic sequence to identify polymorphisms that may contribute to diabetes susceptibility in the Pimas. *PBX1* is composed of nine exons spanning approx. 117 kb and is located within 300 kb of microsatellite *D1S1677*, which marks the peak of linkage to diabetes susceptibility in the Pima Indians. We detected 16 single nucleotide polymorphisms in *PBX1* including one causing a glycine to serine substitution at residue 21. Comparison of the frequencies of the polymorphisms between affected and unaffected Pima Indians did not detect any significant differences, indicating that mutations in *PBX1* do not explain the linkage of 1q with type 2 diabetes in this population. The genomic structure of *PBX1* provides a basis for similar systematic examinations of this candidate locus in other populations in relation to both type 2 diabetes and other metabolic disorders.

Keywords: Pre-B-cell leukemia factor-1; Type 2 diabetes; Single nucleotide polymorphism; Candidate gene; Association analysis; Pima Indian

Type 2 diabetes is a multifactorial endocrine disorder characterized by defects in insulin action and insulin secretion [1]. It appears to be a heterogeneous syndrome resulting from interactions of environmental and genetic factors [2]. Studies in various populations including the Pima Indians of Arizona, a population with the highest reported prevalence of type 2 diabetes, indicate that genetic susceptibility plays a major role in the pathogenesis of type 2 diabetes [3–5]. A genome-wide scan in the Pima population revealed a suggestive evidence for linkage of diabetes to a 30 cM interval on 1q21-q23 with a maximum multipoint LOD score of 2.48 at the *D1S1677* marker [5]. Linkage of diabetes to the same region has been reported in Caucasian families from Utah [6], an Old Order Amish population [7] and in Northern Europeans living in England [8]. Evidence for linkage of type 2 diabetes with 1q21-23 obtained in diverse populations strongly indicates the presence of a diabetes susceptibility locus in this region.

To elucidate the basis for the linkage in the Pima Indians, we have investigated potential candidates among known genes previously mapped to 1q21-q23. *PBX1* (pre-B-cell leukemia factor), localized on 1q21-22, was originally identified by its involvement in a t(1;19) chromosomal translocation in acute pre-B-cell leukemias [9,10]. The PBX1 protein is a member of the PBC (*PBX*, *exd* and *Caenorhabditis elegans*) family of homeodomain containing proteins that fold into DNA-binding helix-turn-helix structures [11]. The PBX1 protein acts as a transcriptional regulator by forming dimers with other homeodomain proteins. One example is PDX1 (pancreas duodenum factor 1) [12–14], a homeodomain protein required for pancreatic development in mammals [15,16] and in the expression of the somatostatin [17] and insulin [18] genes. Furthermore, the heterodimeric complex of *PBX1* and Prep1 (PBX regulatory protein 1) has also been shown to restrict glucagon gene expression in a cell type-dependent manner [19]. PBX1 has two isoforms, PBX1a (46.5 kDa) and PBX1b (38.4 kDa), produced by alternative splicing that are widely expressed in a variety of tissues [20] including adult pancreas, where PBX1a and 1b are differentially expressed in endocrine versus exocrine compartments [21].

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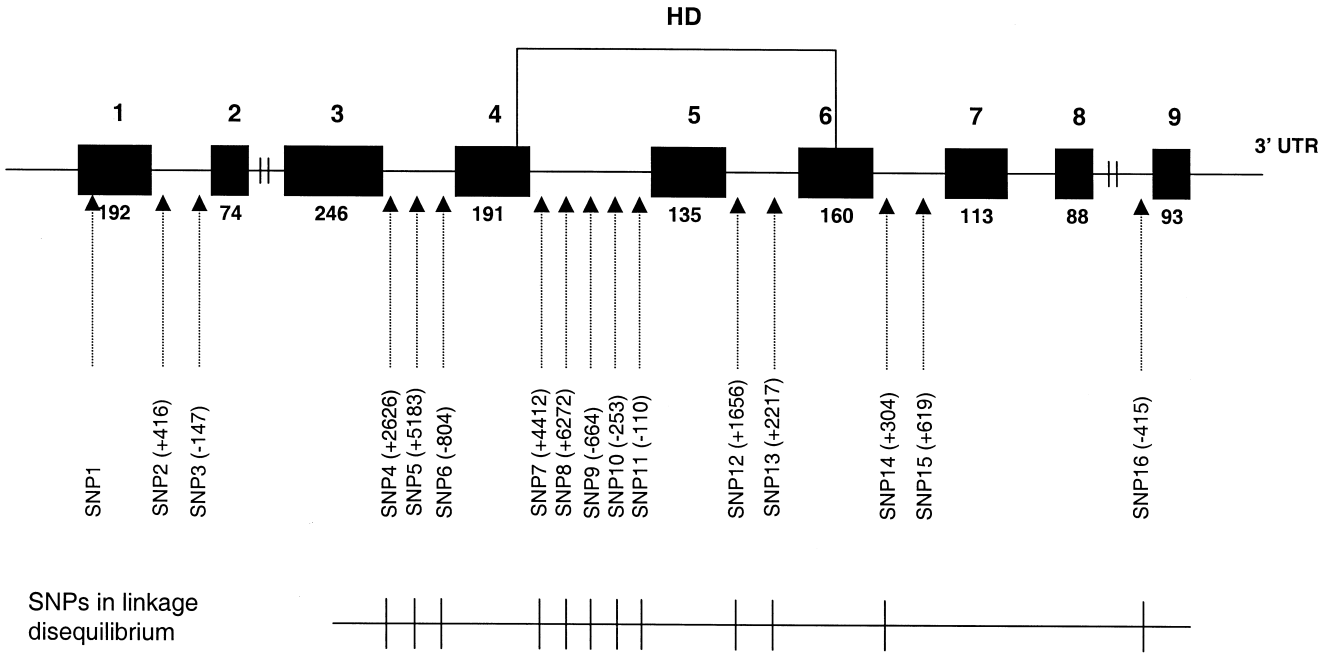

Fig. 1. Schematic diagram of the *PBX1* gene with exons shown as black boxes with their individual sizes (in base pairs) listed below. For the first and last exon, only the size of the coding sequence is given. The introns are presented as a thin horizontal line. The homeodomain region (HD) encoded by exons 4–6 is bracketed. SNP markers are indicated by vertical dotted arrows and the exact base positions are given in parentheses (positive numbers show distance from the first base of the intron; negative numbers correspond to the distance from the first base of the following exon). The appropriate sizes of introns 1, 2, 3, 4, 5, 6, 7, 8, and 9 are 3.2 kb, 63 kb, 7.0 kb, 7.7 kb, 4.3 kb, 7.9 kb, 1.4 kb and 22 kb, respectively. Figure is not drawn to scale.

PBX1 therefore represented a candidate gene for further analysis in the Pima population due to its localization within a region on 1q linked with diabetes and its regulation of genes that are important for glucose homeostasis. The aim of this study was to determine the genomic organization of *PBX1*, and to identify and analyze potential DNA polymorphisms that may impact on diabetes susceptibility.

We initially determined the genomic structure of *PBX1*. Using sequence database searches, we identified several BAC clones containing partial sequences of the *PBX1* locus. These included 259I12, 26N3, 503N16 and 506O24, which were used to elucidate the genomic structure of *PBX1* either by long distance PCR or utilizing available BAC sequences. The reported cDNA encoding the larger isoform PBX1a has a 1292 bp open reading frame encoding a protein of 416 amino acids [20]. We determined that *PBX1* consists of nine exons spanning approx. 117 kb (Fig. 1). All exon-intron boundaries are in agreement with the consensus motif of splice donor and acceptor sites in eukaryotic genes [22]. The homeodomain box, comprised of 64 amino acids, is encoded by exons 4–6 (Fig. 1). To identify *cis*-acting elements/factors potentially involved in the regulation of *PBX1* expression, we determined 780 bp of sequence upstream from the predicted translation start site (ATG). By analysis with the TransFac program [23], we detected several motifs matching the binding sites for the transcription factors Oct1, MZF1, AP2, SP1, C/EBFα, and TF-IID. A single TATA box consensus motif was identified 665 bases upstream from the translation start site (Fig. 2).

Diabetic and non-diabetic Pima Indians selected for genomic screening of *PBX1* are participants of ongoing longitudinal studies of diabetes conducted among members of the Gila River Indian Community since 1965 [24]. For the present study, association analyses were conducted using groups of unrelated individuals (50 cases and 50 controls), as well as 59 discordant sibling pairs who contributed most significantly to the linkage. This sample size has 80–90% power to detect a difference of at least 30% in the prevalence of the at-risk genotype between the case and control groups at $P < 0.05$ [25]. Differences in allelic frequencies between the affected and unaffected groups were analyzed by Chi-square test and the strength of the association between prevalence of at risk genotype and affected status was assessed by the odds ratio (SAS Institute, Cary, USA).

Primers used for variant screening and sequencing are given in Table 1. All *PBX1* exons, introns 1, 3, 4, 5, the 5′ and 3′ ends of the remaining introns, approx. 700 bp upstream from the initial ATG codon, and the 3′-UTR were PCR amplified and the products scanned for sequence variants by denaturing high performance liquid chromatography (DHPLC) using the WAVE DNA fragment analysis system (Transgenomic, Omaha, NE, USA) as described [26]. For the initial screening, pooled samples com-

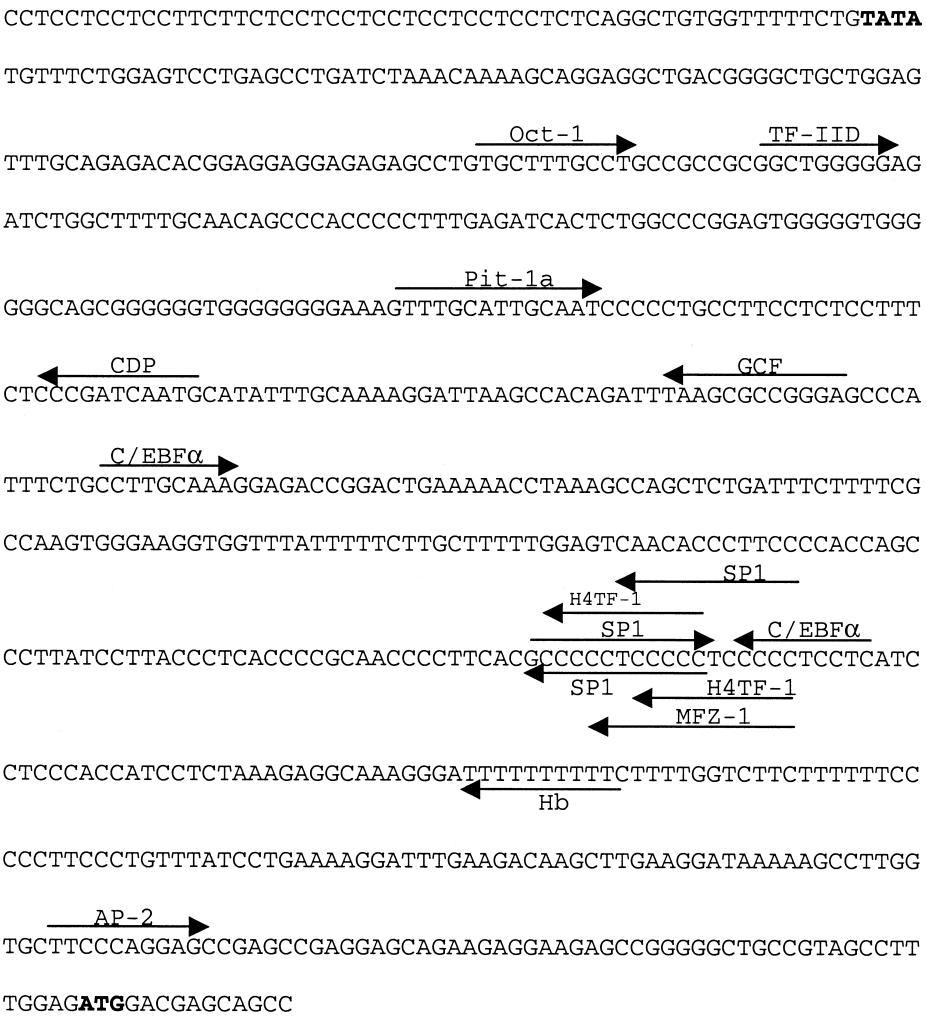

Fig. 2. Genomic sequence of the 5′ flanking region of *PBX1*. The start codon (ATG) and the putative TATA box are in boldface. Arrows indicate the predicted binding sites for transcription factors and the forward and reverse orientation corresponds to the sense and complementary sequences, respectively. Oct-1a, octamer factor-1a; TF-IID, transcription factor-IID; Pit-1a, pituitary factor-1a; CDP, CCAAT displacement protein; GCF, GC rich sequence binding factor; C/EBFα, CCAAT enhancer binding protein α; SP1, stimulating protein 1; H4TF-1, histone 4 promoter transcription factor 1; MZF-1, myeloid zinc finger protein 1; AP-2, activator protein 2.

prised of a 50/50 mix of DNA from diabetic and non-diabetic Pimas were used for PCR followed by DHPLC analysis. Single nucleotide polymorphisms (SNPs) were validated by sequencing of PCR products from individual DNA samples with an ABI Automated Sequencer model 377 using the ABI Prism BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, USA). We detected 16 polymorphisms designated PBX-SNP1 through PBX-SNP16 (Fig. 1), including one in exon 1 producing a glycine to serine substitution at codon 21 (Gly21Ser), two in intron 1, three in intron 3, five in intron 4, two in each intron 5 as well as 6, and one in intron 8 (Fig. 1). All detected polymorphisms, allelic frequencies, and the results of the association analyses are summarized in Table 2.

The *PBX1* variant (SNP1) causing an amino acid substitution at codon 21 in exon 1 (Gly21Ser) was genotyped by allelic discrimination-PCR (AD-PCR) with the TaqMan universal PCR master mix and ABI Prism 7700 sequence detector (PE Applied Biosystems) using the forward primer 5′-CCCAGGCTGATGCATTCC-3′ and reverse primer 5′-TGTGATGGTCATAATTTGCTGTAAAA-3′. The wild type and mutant probes were CGGACACCCCGGCCTGT and CGGACACCCCAGCCTGT, respectively (the variant nucleotides are underlined).

Ten variants (SNPs 3, 4, 5, 6, 8, 9, 10, 11, 12, and 16) caused a gain or loss of restriction sites and were genotyped by PCR-RFLP in the cases and controls ($n = 100$) using appropriate endonucleases (Table 2). SNPs 2, 7, 13, 14 and 15 did not create or destroy any restriction enzyme recognition site sequences and were initially screened by sequencing in a subset of 20 subjects, including ten from the sib pair group and ten from the case/control group. With the exception of SNP1, 2, 3 and 15, the genotypes of the remaining markers showed complete concordance in all subjects consistent with a linkage disequilibrium be-

Table 1
PBX1 primers for genomic PCR and comparative sequencing

Designation	Gene segment	PCR primer (5′→3)	Size (bp)
PBX-Ex-1F	Exon 1	CGA GGA GCA GAA GAG GAA G	179
PBX-Ex-1R		CTC CAA TGT CCT GCT TCC TG	
PBX-Ex-2F	Exon 2	TTA GGT TAG TTC TGT TCT GTA G	432
PBX-Ex-2R		TGA GGT CAA CCT TCA TAG TAC	
PBX-Ex-3F	Exon 3	GAA AGG TGA GCC GCT CCT C	441
PBX-Ex-3R		ACT TGG CAG CTT ATG TAG CC	
PBX-Ex-4F	Exon 4	GTC CTC CTT GCT GCT ACC C	520
PBX-Ex-4R		GCT GAA AGG GAC GTC TAC C	
PBX-Ex-5F	Exon 5	AAG ACC TCT AAG AGC CTG CC	460
PBX-Ex-5R		TTA TAC TCC TCA CTA CTC TGC	
PBX-Ex-6F	Exon 6	GGA TAA GAC CAA TTA GCT TAC C	348
PBX-Ex-6R		CCC AAT GTA GGA ACA GCC AG	
PBX-Ex-7F	Exon 7	GGG TTG CTT TGC ATG TCA TTC	398
PBX-Ex-7R		TGC ATT CAC AAA GTG TGG ACG	
PBX-Ex-8F	Exon 8	GAT GGC ATT TAA TAT GGC ATG C	363
PBX-Ex-8R		AGA ATG CTG CCG ATG GCA TG	
PBX-EX-9F	Exon 9	CAT ATC CAG AGG CTC ACT GG	344
PBX-Ex-9R		AAC TGG GGC ACA GGG TCA GC	

Primer direction is indicated by F (forward) and R (reverse), respectively, according to the transcriptional orientation of *PBX1*. Primers were designed from intronic sequences flanking the 5′ and 3′ ends of each exon. Primers PBX-Ex-1F and PBX-Ex-9R were designed from the 5′-UTR and 3′-UTR, respectively.

tween these variants. Therefore, only SNP16 was chosen for genotyping as a representative marker for this cluster of associated SNPs. In addition to the cases and controls, SNP1, SNP3 and SNP16 were also analyzed in sibling pairs. Based on comparison of the frequencies of these polymorphisms between affected and unaffected Pima Indians, we conclude that none of these markers is significantly associated with type 2 diabetes (Table 2). SNPs 2 and 15 were identified only in one subject and were not investigated further because of their low frequency.

PBX1 is widely expressed in both fetal and adult tissues [20,21]. As there was no published information about *PBX1* expression in insulin responsive tissues, we assessed the presence of the transcripts by PCR in selected cDNA samples using a primer set that amplifies a segment common to both alternatively spliced forms. Adult human QUICK-Clone cDNAs from various tissues were purchased from Clontech (Palo Alto, CA, USA) and PCR was performed with a forward primer (5′-GAGGAAG-CAGGACATTGGAG-3′) designed from exon 1 and a

Table 2
Association analysis of *PBX1* SNPs with diabetes[a]

SNPs	Location[b]	Alleles	Assay[c]	Allele frequency	Case-control		Discordant sib pairs	
					OR	P	OR	P
SNP1	Exon 1	G→A	Seq	0.77/0.23	1.30	0.51	4.00	0.25
SNP2	Intron 1	G→A	Seq	0.95/0.05	–	–	–	–
SNP3	Intron 1	T→G	*Rsa*I	0.57/0.43	0.60	0.08	0.75	0.23
SNP4	Intron 3	C→A	*Xba*I	0.10/0.90				
SNP5	Intron 3	T→C	*Bsa*JI	0.10/0.90				
SNP6	Intron 3	C→A	*Hin*dIII	0.10/0.90				
SNP7	Intron 4	A→G	Seq	0.10/0.90				
SNP8	Intron 4	T→C	*Blp*I	0.10/0.90				
SNP9	Intron 4	A→C	*Bsl*I	0.10/0.90				
SNP10	Intron 4	A→C	*Fok*I	0.10/0.90				
SNP11	Intron 4	T→C	*Msl*I	0.10/0.90				
SNP12	Intron 5	G→C	*Msl*I	0.10/0.90				
SNP13	Intron 5	T→C	Seq	0.10/0.90				
SNP14	Intron 6	G→A	Seq	0.10/0.90				
SNP15	Intron 6	A→G	Seq	0.95/0.05	–	–	–	–
SNP16	Intron 8	G→A	*Taq*I	0.10/0.90	0.70	0.52	2.30	0.20

[a]Diabetes was defined according to the WHO criteria as described in [5].
[b]See Fig. 1 for the location of each polymorphism.
[c]Listed are endonucleases used for genotyping of SNPs causing a gain or loss of the corresponding restriction site. SNPs typed by sequencing are indicated by 'Seq'. SNP16 was in complete linkage disequilibrium with SNPs4–14 and represents data obtained for all markers within this cluster. SNPs 2 and 15 were not analyzed because of the low frequency of the minor allele.

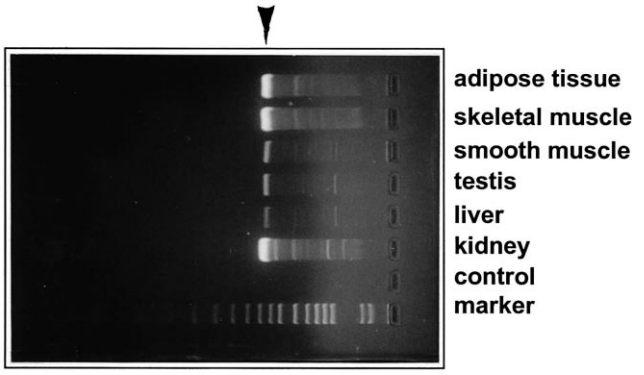

Fig. 3. Tissue distribution of *PBX1* transcript. Equal amounts of cDNA from each tissue were used for PCR as described in the text and products were run on a 2.4% agarose gel. The expected product size is 418 bp (indicated by an arrowhead). The negative control contained all reaction components except of the template cDNA. The size marker is a 50 bp ladder.

reverse primer (5′-GGTGGTGAACTCGTTGCAG-3′) designed from exon 4. We found that the expected 418 bp DNA fragment was amplified in insulin-responsive tissues such as skeletal muscle, and adipose tissue (Fig. 3).

While no association of the Gly21Ser substitution with diabetes was found in the Pima Indians, Gly21 is conserved in the mouse and chicken PBX1, consistent with a potential functional significance. Any effect of the Gly21Ser substitution on the function of the protein therefore remains to be elucidated. Our sequence analysis of the 5′ region upstream from the initial ATG codon revealed clustering of several motifs matching the consensus binding sites for various common transcription factors. Functional analyses of these elements will be required to determine which ones are important for the regulation of *PBX1* transcription.

In summary, we investigated the *PBX1* candidate gene for sequence variants in selected diabetic and non-diabetic Pima Indians and found no evidence of an association of the detected SNPs with the disease. We conclude that mutations in *PBX1* are unlikely to play a significant role in diabetes susceptibility in this Native American tribe. However, information on the organization of the gene and identification of SNPs will make it possible to explore this locus for its pathogenic significance in other populations showing linkage of 1q21-q23 with type 2 diabetes.

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