Rapid Publication

Characterization of Allelic and Nucleotide Variation Between the RAGE Gene on Chromosome 6 and a Homologous Pseudogene Sequence to Its 5′ Regulatory Region on Chromosome 3

Implications for Polymorphic Studies in Diabetes

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Activation of the receptor for advanced glycation end products (RAGE) appears to be a key mechanism in the pathogenesis of diabetic vascular disease, making RAGE a candidate gene for investigation. RAGE is located in the major histocompatibility complex locus on chromosome 6, which contains a multitude of overlapping and duplicated genes involved predominantly in inflammatory and immune responses. The RAGE 5′ flanking region from −505 in a 5′ direction overlaps with PBX2, a gene that has a pseudogene copy on chromosome 3, making any studies of polymorphisms in this duplicated region potentially fraught with error. In this study we have addressed these issues by confirming RAGE as a predominantly single-copy gene and PBX2 to have two gene copies in the haploid human genome. We have characterized the gene:pseudogene differences between RAGE/PBX2 on chromosome 6 and Ψ*PBX2* on chromosome 3, which include a change from C to A at position −1139 RAGE/+2298 PBX2, previously reported as a polymorphism. Single chromosome–specific DNA amplification of the duplicated region has clarified five polymorphisms to be on chromosome 3 and one (at −1202 RAGE/+2234 PBX2) to be on chromosome 6. In conclusion, this study provides essential data for the study of RAGE and its genetics. *Diabetes* 50:2646–2651, 2001

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Received for publication 1 August 2001 and accepted in revised form 1 October 2001. Posted on the World Wide Web at http://www.diabetes.org/diabetes_rapids/ on 9 November 2001.

AGE, advanced glycation end product; DHPLC, denaturing high-performance liquid chromatography; FXIII, Factor XIII; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RAGE, receptor for AGEs; RFLP, restriction fragment–length polymorphism; SSCP, single-strand conformation polymorphism; UTR, untranslated region.

Advanced glycation end products (AGEs) exert their pathogenic effects by various mechanisms, most importantly via cellular receptors, in particular the receptor for AGEs (RAGE) (1). Activation of RAGE via AGEs increases receptor expression and activation of proinflammatory and procoagulatory pathways leading to vascular dysfunction (2). Evidence to implicate RAGE is provided by the beneficial effect soluble RAGE has on the development of vascular disease (3), as well as the demonstration of increased expression in diseased vascular tissue of diabetic animal models and human subjects (4,5). The role of allelic variation on changes in the regulation of the RAGE gene may therefore be important in the pathogenesis of diabetic vascular disease.

The RAGE gene is located on chromosome 6p21.3 in the major histocompatibility complex (MHC) locus in the class III region (6), a gene-rich region of the genome containing overlapping gene regions and an average of one gene per 10 kb of DNA (7). The 5′ flanking region of RAGE from around −500 in the 5′ direction overlaps with the 3′ untranslated region (UTR) of the PBX2 gene, a transcription factor implicated in the development of pre–B-cell leukemia (8). There also exists a pseudocopy of PBX2 on chromosome 3 (Ψ*PBX2*), making any studies of this region of RAGE a difficult task. We have identified variants of RAGE in the coding region resulting in amino acid changes (9) and, more recently, we identified eight novel polymorphisms in the 5′ regulatory region of RAGE, which we confirmed to be on chromosome 6 (10). A recent study identified a polymorphism causing a change from C to A in the duplicated region at −1139 (numbered as −1152 by Poirier et al. [11] from the translational start site); however, our own chromosome 6–specific data did not support −1139 C/A as a polymorphism of RAGE (10). Therefore, characterization of both this anomaly and the Ψ*PBX2* and RAGE/PBX2 loci are needed. In this study we have addressed these issues by completely characterizing the gene:pseudogene differences that occur and the allelic

variation of both the RAGE 5′ regulatory region and Ψ*PBX2*.

RESEARCH DESIGN AND METHODS

Identification of the Ψ*PBX2* sequence. Ψ*PBX2* was identified by homology searches to PBX2/RAGE with BLAST (www2.ncbi.nlm.gov) searches and aligned to establish regions of homology using ClustalW. The RAGE 5′ regulatory sequence was numbered from the transcriptional start site (12), and the PBX2 sequence (both chromosome 6 and the chromosome 3 pseudogene) was numbered from the translational start site using Genbank entry NM_002586.

Real-time polymerase chain reaction for quantification of RAGE and PBX2 gene copy number. Taqman probe/primer sets were designed for three regions: RAGE/PBX2 duplicated region (−1204 to −507), RAGE promoter (−506 to −247), and the control single-copy Factor XIII (FXIII) exon 2. The sequence of the primers and probes are available from the authors. After primer and probe optimization, efficiencies of primer amplification were corrected using a linearized control plasmid containing segments of RAGE/PBX2 and FXIII. This construct contained the RAGE promoter (−1204 to −247) and exon 2 of the FXIIIA gene. Standard curves were generated for each primer probe set using the target plasmid and four genomic DNA samples that were subsequently used with each primer/probe set along with controls containing either no DNA or only pGEM-3Zf(+) DNA. All reactions were performed in triplicate using an ABI Sequence Detection System 7700 (Applied Biosystems).

Single chromosome polymerase chain reaction amplification to determine gene:pseudogene nucleotide differences. Polymerase chain reaction (PCR) was performed specifically from chromosomes 6 and 3 using primers with a common 5′ forward sequence (−1350/+2086 5′ TGGGGAAGTAGCTT GTTTTTT 3′) in the duplicated region and a specific 3′ reverse primer in each single chromosome copy (RAGE/PBX2 5′ CAGAGCCCCCGATCCTATTT 3′ and Ψ*PBX2* 5′ ATTTATCCTGTTGCCTTTCCC 3′). PCR fragments were gel-purified using a NucleoSpin kit (Macherey Nagel), and DNA was sequenced using an automated ABI310 (Applied Biosystems).

Polymorphism characterization. Polymorphisms within the −1350 to −507 RAGE/+2086 to +2930 PBX2 duplicated region were detected by denaturing high-performance liquid chromatography (DHPLC) and single-strand conformation polymorphism (SSCP) as previously described (10). Regions −1350 to −1156, −1196 to −968, −896 to −646, and −719 to −546 previously demonstrated polymorphic patterns using primers that amplified within the duplicated region; however, chromosome 6–specific PCRs demonstrated these polymorphisms to originate from another locus (10). These regions correspond to Ψ*PBX2* +2086 to +2281 (region A), +2240 to +2470 (region B), +2541 to +2770 (region C), and +2718 to +2891 (region D). Allelic differences determined by SSCP/DHPLC were reamplified individually from chromosome 3– and 6–specific PCR fragments for regions A–D and sequenced.

Restriction fragment–length polymorphism studies to confirm polymorphic and nucleotide differences. To verify the SSCP/DHPLC results for RAGE/PBX2 and Ψ*PBX2*, PCR–restriction fragment–length polymorphism (PCR-RFLP) was used. Fragments A–D were amplified from chromosome 3– and 6–specific PCR products, endonuclease-digested, and electrophoresed. To completely verify our results for the −1139/+2298 C/A variation as a gene:pseudogene difference, PCR-RFLP with *Dde*I was used to analyze a fragment from −1204 to −247 (chromosome 6) and from +2232 of PBX2 to 246 bp of the 3′ end of the pseudogene insert (chromosome 3) from DNA isolated from 100 previously described anonymous blood donors (10). In addition, the chromosome 6 target was PCR-amplified from DNA obtained from 200 previously described type 2 diabetic subjects (10).

RESULTS

Identification of the duplicated region of RAGE/PBX2 on chromosome 3. A homologous sequence of RAGE on chromosome 6 was identified on chromosome 3; however, a number of crucial differences were observed. It was found that it contained the sequence in the 5′ direction of −507 of RAGE, with none of the −506 to +1 and coding regions of RAGE. For PBX2, the entire mRNA sequence, including 233 bp of 5′ UTR and the entire 3′ UTR (minus the last two T nucleotides, +2931 and +2932), matched the chromosome 3 sequence, with no intronic chromosome 6 sequence of PBX2 being present. This indicated that the sequence was a retrotransposed pseudogene for PBX2, which was further confirmed by the presence of a 3′ polyadenosine tract at the end of the 3′ UTR.

TABLE 1
Real-time quantification of genomic DNA for the RAGE gene promoter, the adjacent PBX2 gene, and a target in exon 2 for coagulation factor FXIII A subunit.

DNA sample	RAGE/PBX2	RAGE	FXIIIA
1	332 (210)	158 (100)	154 (97)
2	441 (191)	231 (100)	243 (105)
3	461 (215)	214 (100)	249 (116)
4	347 (238)	146 (100)	191 (131)
Mean (%)	395 (211)	187 (100)	209 (112)

Data are amount of target (standard curve). The amount of target for four different genomic DNA samples is given in fentogram equivalents of plasmid containing all three targets, used to make a standard curve for each primer/probe set. The standard curve was generated using 50–800 pg of target plasmid. The mean is shown for each target with the equivalent percent to RAGE shown in brackets normalized to RAGE set to 100%.

Genomic copy number of the RAGE gene. Table 1 shows the values of gene copy number for four genomic DNA samples for RAGE and PBX2. For all samples, RAGE (−506 to −247) and FXIII had an equal copy number, whereas the region of the RAGE promoter that overlaps with the PBX2 gene (−1204 to −507) is present in twice the amount of the other two targets, indicating that there are two loci for this target.

Identification of gene:pseudogene sequence differences. All identified nucleotide differences between the chromosome 6–overlapping RAGE/PBX2 sequence and the Ψ*PBX2* chromosome 3 sequence are shown in Fig. 1. These include a −1139 RAGE/+2298 PBX2 C/A change, previously identified as a polymorphism on RAGE by Poirier et al. (11) (numbered as −1152 C/A in their study).

Identification of gene:pseudogene polymorphisms. Within the RAGE/PBX2 chromosome 6 sequence from −1350 to −507, only one rare polymorphism was detected at position −1202 of RAGE/+2234 PBX2 (Fig. 2), as previously reported (10). Chromosome-specific PCR demonstrated that Ψ*PBX2* on chromosome 3 contained at least four common polymorphisms from the SSCP/DHPLC results. Polymorphisms were identified on chromosome 3 at position +2114 (A insertion), +2345 (T/C), +2609 (T insertion), and +2862 (C/T) (homologous to RAGE −1324, −1092, −828, and −575, respectively), with an additional polymorphism detected toward the end of the pseudogene at +2920 (G/C) (homologous to RAGE −517) when sequenced. The insertion polymorphism at +2114 of Ψ*PBX2* created an extra adenosine insertion (AAA) in addition to the A insertion nucleotide sequence difference between −1334 of RAGE/+2112 chromosome 6 (A) and +2112/+2113 of Ψ*PBX2* chromosome 3 (AA) at this locus.

PCR-RFLP studies of chromosome 3 and 6 to verify pseudogene polymorphisms and the −1139/+2298 C/A variation as a gene:pseudogene difference. Polymorphisms on chromosome 3, which altered a restriction site, were identified for the A insertion at +2114 (*Dra*I), the T-to-C substitution at +2345 (*Mwo* I), and the C-to-T substitution at +2609 (*Ear* I). PCR-RFLP subsequently confirmed these polymorphisms on the chromosome 3 Ψ*PBX2* but not the chromosome 6 RAGE/PBX2 PCRs,

RAGE	−1350	TGGGGAAGTA	GCTTGTTTTT	TTTTTTA−TAA	ATATGTTGAT	TTCTTGTCTT	−1301
Pseudogene	1	TGGGGAAGTA	GCTTGTTTTT	TTTTTTA**A**TAA	ATATGTTGAT	TTCTTGTCTT	51
RAGE	−1300	TTTTTT**TT**AT	TTCTTACTTT	CCCATATTAG	GGGTGATAGC	CAAAGGGGTT	−1251
Pseudogene	52	TTTTTT−−AT	TTCTTACTTT	CCCATATTAG	GGGTGATAGC	CAAAGGGGTT	99
RAGE	−1250	CTGGTAAGAG	AAAGGGGGAC	AAACAGAACT	GGTAAAGAGG	CCCCCCTGGC	−1201
Pseudogene	100	CTGGTAAGAG	AAAGGGGGAC	AAACAGAACT	GGTAAAGAGG	CCCCCCTGGC	149
RAGE	−1200	TCCAGGCCTG	TCCATCAGGA	AGTAAATTTT	ACAGGGCACC	AAGCTTTGCC	−1151
Pseudogene	150	TCCAGGCCTG	TCCATCAGGA	AGTAAATTTT	ACAGGGCACC	AAGCTTTGCC	199
RAGE	−1150	CCCTAAAATC	C**C**TTAGGTGT	TCTTTGTTCA	TGCAGGCAGG	TTTCTGCCGC	−1101
Pseudogene	200	CCCTAAAATC	C**A**TTAGGTGT	TCTTTGTTCA	TGCAGGCAGG	TTTCTGCCGC	249
RAGE	−1100	ATTTGATGTG	GAGGCAGTGA	AGGGCTTGCC	CTGCTGGCCT	CTCATCCCCC	−1051
Pseudogene	250	ATTTGATGTG	GAGGCAGTGA	AGGGCTTGCC	CTGCTGGCCT	CTCATCCCCC	299
RAGE	−1050	TTCTTCCCAC	AACCCTTGGG	CAGGGCTGGA	CTCAGTAATT	TTGAGGAAAT	−1001
Pseudogene	300	TTCTTCCCAC	AACCCTTGGG	CAGGGCTGGA	CTCAGTAATT	TTGAGGAAAT	349
RAGE	−1000	TGAAGATGCC	ATCTTCCCCT	GTGAGTGACA	TGTCTTTAAT	TTTTTAAAAA	−951
Pseudogene	350	TGAAGATGCC	ATCTTCCCCT	GTGAGTGACA	TGTCTTTAAT	TTTTTAAAAA	399
RAGE	−950	ACT**A**CTATTT	GAAAATTGGA	GGGGGAAGAA	TGGGAAGGGA	GTTATTGCCA	−901
Pseudogene	400	ACT−CTATTT	GAAAATTGGA	GGGGGAAGAA	TGGGAAGGGA	GTTATTGCCA	448
RAGE	−900	AATATGTTAA	**A**TATGGGTTG	GGGTGCTTGT	ATATGTATCT	TCCTCAATTT	−851
Pseudogene	449	AATATGTTAA	**T**TATGGGTTG	GGGTGCTTGT	ATATGTATCT	TCCTCAATTT	498
RAGE	−850	CC**C**CATAAAT	GAGGTATCTT	TTTGTCACAC	CAAAATCAAG	GGGTAGGGAG	−801
Pseudogene	499	CC**G**CATAAAT	GAGGTATCTT	TTTGTCACAC	CAAAATCAAG	GGGTAGGGAG	548
RAGE	−800	AGGGAGGAGG	TTGCAAAAAG	CCAGATGTGG	GGGAAAAGTA	ACATCAACAC	−751
Pseudogene	549	AGGGAGGAGG	TTGCAAAAAG	CCAGATGTGG	GGGAAAAGTA	ACATCAACAC	598
RAGE	−750	TGTCCCATCC	TCAGCCCTGA	AC**TAG**CTACC	ATCTGATCCC	CTCAGACATT	−701
Pseudogene	599	TGTCCCATCC	TCAGCCCTGA	AC**C**−−CTACC	ATCTGATCCC	CTCAGACATT	646
RAGE	−700	CTCAGGATTT	TACAAGACTG	TCAGAGTGGG	GAACCCCTCC	CATTAAAGAT	−651
Pseudogene	647	CTCAGGATTT	TACAAGACTG	TCAGAGTGGG	GAACCCCTCC	CATTAAAGAT	696
RAGE	−650	CCGGGCAGGA	CTGGGGACAG	GTTGGAAGT**G**	TGATGGGT**G**G	GGGGGTGGGA	−601
Pseudogene	697	CCGGGCAGGA	CTGGGGACAG	GTTGGAAGT**A**	TGATGGGT**T**G	GGGGGTGGGA	746
RAGE	−650	CCGGGCAGGA	CTGGGGACAG	GTTGGAAGT**G**	TGATGGGT**G**G	GGGGGTGGGA	−601
Pseudogene	697	CCGGGCAGGA	CTGGGGACAG	GTTGGAAGT**A**	TGATGGGT**T**G	GGGGGTGGGA	746
RAGE	−600	GGCATGGGCC	GGGGGCAGTT	CTCTCCTCAC	TTGTAAACTT	GTGTAGTTTC	−551
Pseudogene	747	GGCATGGGCC	GGGGGCAGTT	CTCTCCTCAC	TTGTAAACTT	GTGTAGTTTC	796
RAGE	−550	ACAGAAAAAA	AA**CAA**AATGC	AGTTTTAAAT	AAAGAAATTT	CTTT**TTTCCC**	−501
Pseudogene	797	ACAGAAAAAA	AA−−−AATGC	AGTTTTAAAT	AAAGAAATTT	CTTT**AAAAAA**	843
RAGE	−500	**TGGGTTTAGT**	**TGAGAATTTT**	**TTTCAAAAAA**	**CATGAGAAAC**	**CCCAGAAAAA**	−451
Pseudogene	844	**AAAAAAAATG**	**TCAATGTGTC**	**CCTAGGCCTG**	**GAGACCATGT**	**GTGATTAGGC**	893
RAGE	−450	**AAATGATTTT**	**CTTTCACGAA**	**GTTCCAAACA**	**GGTTTCTCTC**	**CTGTTCCCCA**	−401
Pseudogene	894	**TAATATTTTC**	**CCATAAAATA**	**TTCCAAACTG**	**AACAATCTCT**	**CATTTCTACA**	943

FIG. 1. Nucleotide sequence alignment of the chromosome 6 RAGE 5′ regulatory sequence, with the homologous chromosome 3 Ψ*PBX2* sequence. Differences between the sequences are shown and highlighted in bold type.

with matching allelic patterning obtained from SSCP/DHPLC (data not shown). The PCR-RFLP obtained for the −1139/+2298 C/A variation for both chromosome 3 and 6 is shown in Fig. 3, which includes samples of all allelic pattern variations obtained by SSCP/DHPLC for the B region. These experiments demonstrated the RAGE −1139/PBX2 +2298 nucleotide on chromosome 6 to be a C (n = 300) and the Ψ*PBX2* nucleotide on chromosome 3 to be an A (n = 100) and not polymorphic.

DISCUSSION

Pseudogenes are a common feature of the genome and are defined as a nonfunctional copy of a gene that can arise by

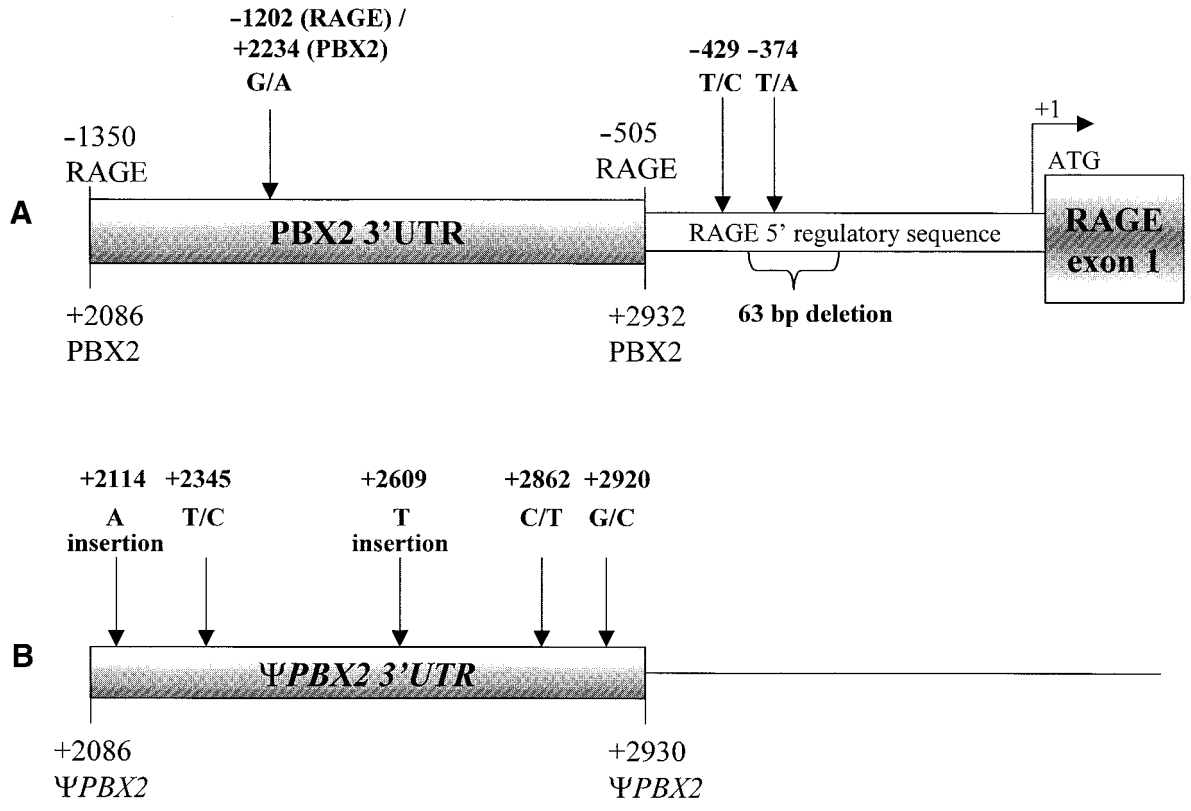

FIG. 2. Map of the polymorphisms of the RAGE 5′ regulatory sequence/PBX2 3′ UTR on chromosome 6 (*A*) and the Ψ*PBX2* 3′ UTR on chromosome 3 (*B*). Polymorphisms are indicated by arrows and in bold type.

two major mechanisms: the entire duplication of a region of genomic DNA or the retrotransposition of a double-stranded sequence generated from the single-stranded RNA copy of a gene (13). Ψ*PBX2* is of the latter category because of its lack of intronic structure and the presence of a 3′ polyadenylation tract. The implications of the PBX2 gene duplication is that any studies of RAGE polymorphisms within the overlapping region could either spuriously identify a polymorphism of chromosome 3 or identify a gene:pseudogene nucleotide difference as an allelic variant. In our previous study, we clarified which detectable polymorphisms were located on chromosome 6 of the RAGE gene (10), but we were unable to establish the polymorphisms that occurred on Ψ*PBX2*, the exact overlap of the homologous chromosome 3 and 6 regions, and the gene:pseudogene differences. A recent report has emphasized the need for this study with the description of a polymorphism in the duplicated region of RAGE at position −1139 (numbered as −1152, taken from the translational start site) (11), which we had not detected using a more stringent SSCP/DHPLC combined mutation detection methodology (10). The completion of the human genome project has created a volume of new gene data, including the sequence for the Ψ*PBX2*, which has allowed us to clarify any discrepancies.

Pseudogenes are by no means a rare event, as exemplified by the complete mapping and sequencing of chromosome 21, which revealed that of 225 identified genes, 57 were found to be pseudogenes (14). The MHC locus alone on chromosome 6p21.3 containing RAGE has perhaps more genes than the whole of chromosome 21 and therefore should make researchers studying this region more cautious for the occurrence of duplication. Current estimates are not available for the level of pseudogenes present for the MHC class III locus but, significantly, in the class II locus, ≥10% of the genes have pseudogene copies (7).

We set out to confirm the copy number of RAGE and PBX2 because of the Aguado and Campbell (15) study showing that PBX2 had perhaps two or three copies in the genome. Using real-time PCR, we demonstrated RAGE is a single-copy gene, as previously demonstrated (6,16), and the PBX2 gene has two copies, supporting previous studies showing that a functional copy of PBX2 was present on chromosome 6 and that a possible pseudogene copy was on chromosome 3 (15). It was therefore unsurprising that when we characterized Ψ*PBX2*, we found many gene:pseudogene nucleotide differences, and the majority of the polymorphisms detected in the RAGE/PBX2 duplicated region were located in the pseudogene on chromosome 3. The results confirm that polymorphisms are more frequent on the PBX2 pseudogene as opposed to RAGE/PBX2, in line with the theory of the nonselective nature of pseudogenes to undergo more sequence changes than the gene of origin.

The nucleotide changes between chromosome 6 and 3 included a C (chromosome 6) to A (chromosome 3) change at −1139/+2298, reported as a polymorphism by Poirier et al. (11). To verify our results and confirm the −1139/+2298 C/A gene:pseudogene difference, we performed a PCR-RFLP study on the pseudogene polymorphisms we detected. We demonstrated the PCR-RFLP genotypes of the polymorphisms matched their corresponding SSCP/DHPLC allelic patterning. This included a

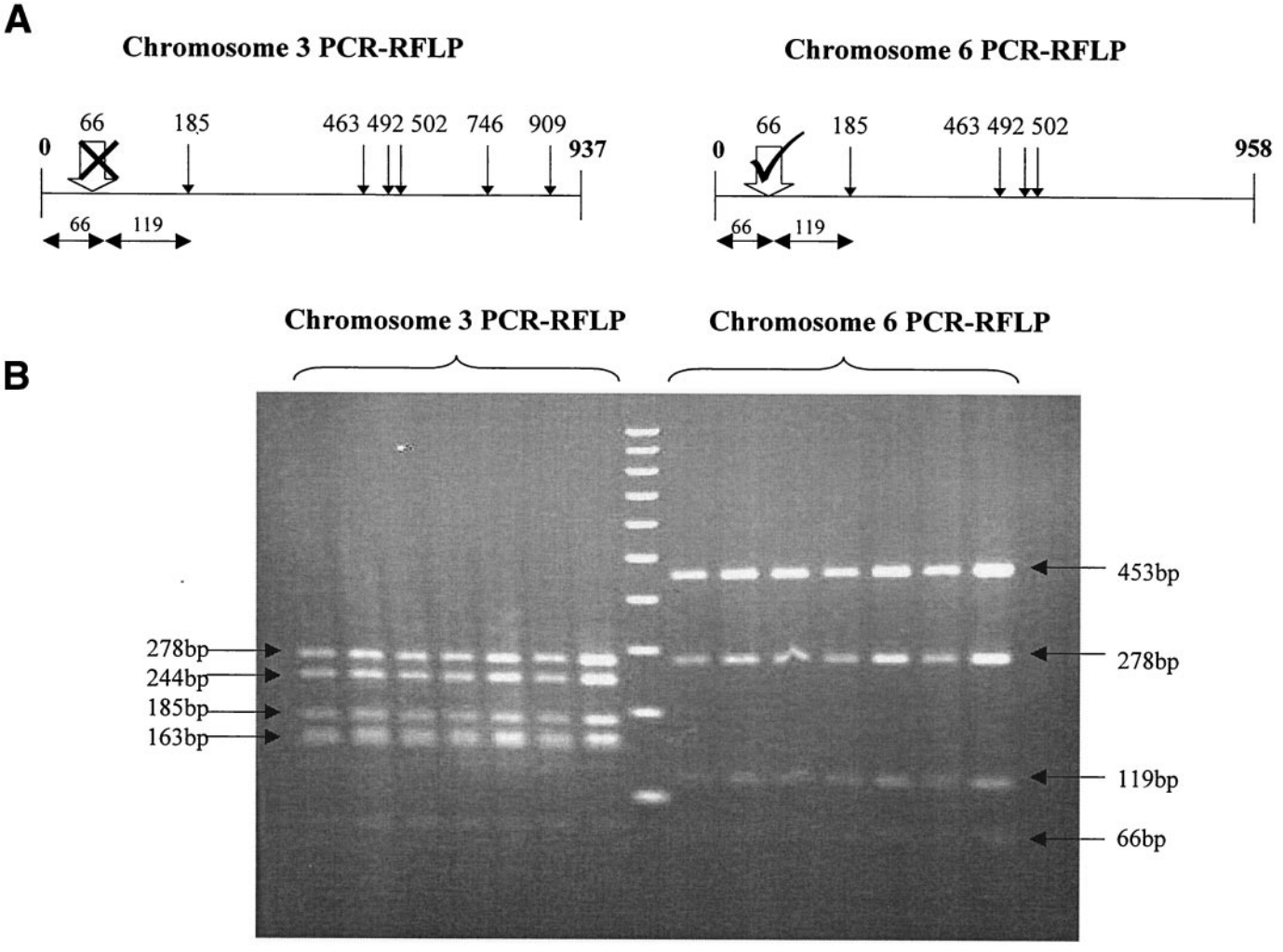

FIG. 3. Chromosome-specific genotyping of the −1139 RAGE/+2298 PBX2 nucleotide. *A*: Restriction endonuclease map for *Dde*I of the chromosomes 3 and 6 PCR-amplified regions. Both maps indicate with a large arrow the −1139 RAGE/+2298 PBX2 *Dde*I restriction site. *Dde*I does not cut the −1139/+2298 A nucleotide, but does cut the −1139/+2298 C nucleotide. *B*: Agarose gel electrophoresis of *Dde*I-restricted products demonstrating that chromosome 3 Ψ*PBX2* +2298 is an A nucleotide because of the presence of the uncut 185-bp band. In contrast, chromosome 6 1139 RAGE/+2298 PBX2 is a constant C nucleotide because of the absence of the 185-bp band and the presence of both the 119- and 66-bp bands.

+2345 T/C polymorphism in the −1196 to −968 RAGE/+2240 to +2470 PBX2 region, which indicated that for the pattern differences detected, the +2345 T/C polymorphism—not variation at the −1139/+2298 locus—was responsible. Sequencing of samples for any SSCP/DHPLC patterns of this region demonstrated the RAGE nucleotide on chromosome 6 as −1139C and the Ψ*PBX2* nucleotide on chromosome 3 to be an A. Further investigation of the −1139/+2298 C/A variation as a gene:pseudogene nucleotide difference and not a polymorphism by PCR-RFLP confirmed our results. Poirier et al. (11) reported that the −1139A allele occurred with a frequency of 4–7% in 392 Danish Caucasian subjects, with 8.5–15% being C/A heterozygous. We therefore studied 100 Caucasian random blood donors based on the expectation that at least eight samples would be heterozygous if this were a polymorphism. We investigated both chromosomes 3 and 6 individually by chromosome-specific PCR-RFLP, which demonstrated not one individual to be polymorphic for the −1139/+2298 polymorphism for either locus. To verify this and to demonstrate that this is not a diabetes-specific polymorphism, we screened 200 Caucasian type 2 diabetic subjects, finding no detectable variation for RAGE at −1139, which therefore suggests that position −1139 is monomorphic.

In conclusion, this study has identified the overlap and copy number of RAGE and has shown that on chromosome 3, a pseudogene of PBX2 does exist. We identified the gene:pseudogene differences that occur and the allelic variation of the two loci. Finally, our data suggest the −1139 RAGE/+2298 PBX2 C/A variation is a gene:pseudogene difference and not a polymorphism of the RAGE gene. This new data should aid future studies of allelic variation of RAGE in its relevant biological settings.

ACKNOWLEDGMENTS

This study was supported by the British Heart Foundation (Junior Research Fellowship FS/2000007).

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