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Analysis of an exon 1 polymorphism of the B2 bradykinin receptor gene and its transcript in normal subjects and patients with C1 inhibitor deficiency

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Sections

- [Abstract](#)
- [Methods](#)
- [Results](#)
- [Discussion](#)
- [References](#)
- [Publishing and Reprint Information](#)
- [Articles with References to this Article](#)

Abstract

TOP

The B2 bradykinin receptor (B2BKR) mediates most of the inflammatory actions of bradykinin. To evaluate its potential role in allergic diseases, we assessed the structure of the human B2BKR gene. Screening a human placenta genomic DNA library identified only clones containing exons 2 and 3. Human placenta and colon tissues were used for 5' rapid amplification of complementary DNA ends to identify nine exon 1 clones, each containing one 9 bp and two 1 bp deletions compared with published sequences. Exon 1 genomic polymerase chain reaction of human

leukocyte DNA revealed two distinct products, which were shown to differ by the presence or absence of the 9 bp deletion. Alleles with the 9 bp deletion were designated as (-)²¹⁻²⁹, whereas alleles without the deletion were designated as (+)²¹⁻²⁹. Genomic polymerase chain reaction in 39 Caucasian, 31 African-American, and 32 Asian normal subjects revealed a highly significant difference in the allelic frequency of the two genotypes, primarily because of an absence of the (+)²¹⁻²⁹ allele in Asian subjects. Analysis of steady-state B2BKR messenger RNA levels by reverse-transcription polymerase chain reaction in heterozygous normal subjects revealed consistently higher expression of (-)²¹⁻²⁹ transcripts. To investigate the potential clinical significance of the exon 1 polymorphism, 21 patients with angioedema and C1 inhibitor deficiency were genotyped. None were homozygous for the (+)²¹⁻²⁹ allele ($p = 0.0088$ compared with normal subjects). In contrast, two patients with immunochemical evidence of hereditary angioedema without history of clinical angioedema were (+)²¹⁻²⁹ homozygous. These results suggest that the B2BKR genotype may influence clinical status in diseases characterized by involvement of bradykinin. (J Allergy Clin Immunol 1997;99:134-46.)

df	Degrees of freedom
HAE	Hereditary angioedema
hB2BKR	Human B2 bradykinin receptor
PCR	Polymerase chain reaction
5' RACE	5' Rapid amplification of cDNA ends
RT-PCR	Reverse-transcription polymerase chain reaction
TBE	Tris-borate ethylenediaminetetraacetic acid

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Key Words: [B2 bradykinin receptor](#), [polymorphism](#), [mRNA](#), [C1 inhibitor deficiency](#), [hereditary angioedema](#), [genomic PCR](#), [5' RACE](#), [bradykinin](#)

Bradykinin, a nonapeptide released from kininogen by kallikrein, can cause vascular dilation, edema, inflammation, and pain.^{1,3} Bradykinin may mediate tissue injury in a diverse group of diseases including asthma, rhinitis, arthritis, sepsis, coronary artery disease, hypertension, and angioedema.^{1,4,5} Pharmacologic characterization of bradykinin receptors has revealed two subtypes, the B1 and B2 subtype receptors.^{6,7} B1 subtype receptors have high affinity to [des-Arg⁹] bradykinin and low affinity to bradykinin.^{6,7} In contrast, B2 subtype receptors have high affinity to bradykinin but low affinity to [des-Arg⁹] bradykinin.^{6,8} Farmer et al.⁹ have proposed an additional bradykinin receptor subtype, which has high affinity for bradykinin but is not antagonized by B2 antagonists; however, the existence of the putative B3 subtype receptors remains to be proven. B2 subtype receptors appear to mediate the inflammatory actions of kinins and are widely distributed through most tissues.^{10,12}

The human complementary DNA and genomic B2 bradykinin receptor (hB2BKR) sequences have been recently elucidated.^{13 15} It is now clear that the hB2BKR is a member of the seven transmembrane G protein–coupled receptor superfamily. Analyses of the hB2BKR gene structure, however, have yielded conflicting results. Powell et al.¹⁶ isolated the hB2BKR gene from a *HindIII*-digested human DNA cosmid library and described an intronless gene. Other investigators, however, have suggested that at least one intron exists in the hB2BKR gene.^{14 15 17} Ma et al.¹⁸ reported the hB2BKR genomic DNA sequence and demonstrated that hB2BKR is composed of three exons with an intron 1 size greater than 20 kb.

To study the structure of the hB2BKR gene, we screened a human placenta genomic library by restriction mapping, Southern blot analysis, and partial DNA sequencing. These studies identified exons 2 and 3 of the hB2BKR gene but failed to detect exon 1. Using 5' rapid amplification of cDNA ends (5' RACE), we were able to clone exon 1 of the hB2BKR gene. Analysis of the exon 1 clones and exon 1 genomic polymerase chain reaction (PCR) products revealed a polymorphism that was identical to the hB2BKR exon 1 polymorphism recently reported by Braun et al.¹⁹ We subsequently characterized the distribution of the hB2BKR exon 1 genotype in different ethnic populations and found a highly significant difference in the allelic frequency between the ethnic groups. On the basis of these findings, we investigated the functional consequence of the polymorphism. By studying heterozygous subjects, we found a consistent difference in the steady-state messenger RNA levels of each of the alleles. The potential clinical significance of the polymorphism was suggested by the observation that the hB2BKR exon 1 genotype had a statistically significant correlation with clinical manifestation of angioedema in patients with C1 inhibitor deficiency.

Methods

[TOP](#)

Cell culture

Human T24, MCF-7, T47D, A431, Hela S3, and WI38 cells were obtained from the American Type Culture Collection (ATCC HTB4, HTB 22, HTB 133, CRL 1555, CCL 2.2, and CCL 75; Rockville, Md.) and were maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal bovine serum (BioWhittaker), 2 mmol/L L-glutamine (Sigma Chemical Co., St Louis, Mo.), 1× nonessential amino acids (Irvine Scientific Inc., Santa Ana, Calif.), 1 mmol/L sodium pyruvate (Irvine Scientific Inc.), and 50 µg/ml gentamicin (Sigma Chemical Co.) in a humidified 5% CO₂ incubator at 37° C.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared by the phenol/guanidine thiocyanate procedures with RNAzol B solution (Biotechx, Houston, Texas).²⁰ First-strand cDNA was generated by reverse transcription with Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Gaithersburg, Md.) and random hexamer primers (Pharmacia Biotech, Piscataway, N.J.). "Hot-start" facilitated PCR²¹ was performed by using an Ericomp twin block easy cycler (Ericomp, San Diego, Calif.). The thermal cycle profile of PCR was as follows: 1 cycle of 96° C for 3 minutes and 80° C for 1 minute

with the addition of Taq DNA polymerase (Gibco BRL, Grand Island, N.Y.); 28 cycles of 96° C for 30 seconds, 60° C for 30 seconds, and 90 seconds at 72° C; 1 cycle of 72° C for 7 minutes. The primer sequences used in the hB2BKR PCR amplification were as follows: complete open reading frame forward primer-5'-AAAGGGATCCCGAGTCCAAATGTTCTCTCCCTGGAA-3' (nt 134-158), complete open reading frame reverse primer-5'-CCGCCTCGAGTTTATTCTCACTGTCTGCTCCCTGCCCA-3' (nt 1297-1318); exon 1 cDNA forward primer-5'-CTGGCTTCTGGGCTCCGA-3' (genomic nt 562-579), and exon 2 cDNA reverse primer-5'-GGATGTGAGTGAGGCCAC-3' (nt 110-127). Unless stated otherwise, all nucleotide numbering is based on the cDNA sequence of Hess et al.¹⁴

Human genomic DNA library screening

The hB2BKR gene was screened in a human placenta genomic DNA library constructed in λ FIX II (Stratagene, La Jolla, Calif.). This library was plated on the MagnaGraph nylon transfer membrane (MSI Inc., Westboro, Mass.) at a density of approximately 50,000 per plate. Replicas of master plates were lysed and processed for hybridization.²² DNA was then oven-dried at 80° C for 2 hours, prehybridized, and hybridized in hybridization buffer (50% formamide, 20 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 7.4, 3× standard sodium citrate [1× standard sodium citrate: 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0], 0.4 µg/ml transfer RNA, 3× Denhart's solution, 1% sodium dodecylsulfate, 60 µg herring sperm DNA) at 42° C overnight.

A 1.2 kb PCR product containing the complete hB2BKR open reading frame was generated from isolated human T24 cell total RNA by using the RT-PCR techniques and further subcloned into the pCR-Script SK(+) vector (Stratagene). The plasmid construct was designated as phB2BKR, and the DNA sequence was determined in the 373A DNA sequencer (Applied Biosystems Inc., Foster City, Calif.). The phB2BKR cDNA was labeled with a phosphorous 32-labeled deoxyadenosine triphosphate (3000 Ci/mmol; ICN Pharmaceuticals Inc., Costa Mesa, Calif.) by using PRIME-IT II kits (Stratagene) for screening the genomic DNA library.

To identify the first exon of the hB2BKR gene, a 51 bp oligonucleotide primer (genomic nucleotides 574-624) that corresponded to the known exon 1 sequence identified by Ma et al.¹⁸ was synthesized. The 51 bp exon 1 oligonucleotide was labeled with γ -³²P deoxyadenosine triphosphate (6000 Ci/mmol; ICN Pharmaceuticals Inc.) by T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and used for probing the DNA library for the first exon of the hB2BKR gene.

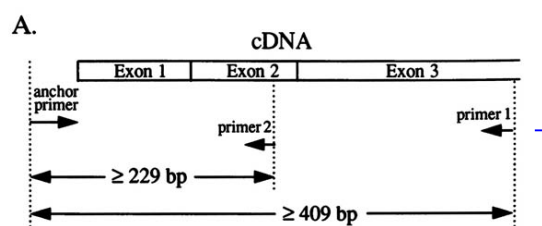
Filters were washed in 0.1× standard sodium citrate–0.1% sodium dodecylsulfate three times at 51° C and exposed to Kodak XAR 5 x-ray film (Eastman Kodak, Rochester, N.Y.) at -70° C. Positive phage clones were isolated and re-screened until single-phage colonies were isolated. Appropriate restriction enzyme–digested DNA fragments were electrophoresed in 1% agarose gels, isolated by GeneClean kits (Bio101 Inc., San Diego, Calif.) and subcloned into the pCR-Script SK(+) vector. Restriction mapping, Southern blot analysis, and partial genomic DNA sequencing were performed as described above to identify the isolated clones.

Determining the 5' end sequences of hB2BKR mRNA

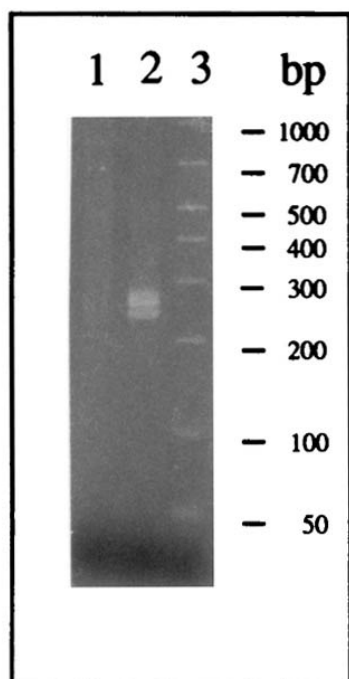
The 5' end of hB2BKR nucleotide sequence was determined by anchored PCR, with 5' RACE-

Ready kits (Clontech, Palo Alto, Calif.) according to the manufacturer's protocols. The template DNA was commercial anchored cDNA, prepared by ligating an anchor oligonucleotide to the 3' end of first-strand cDNA. The anchored cDNA was amplified by using a forward primer complementary to the anchor sequence and a reverse primer complementary to the known downstream hB2BKR sequence. To obtain sufficient specificity, a second nested PCR was performed with the same forward primer and a different reverse primer. The 5' RACE strategy is summarized in Fig. 1, A.

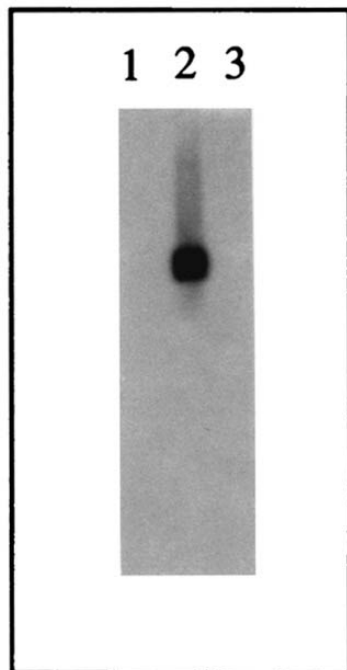
Fig. 1. 5' RACE of the hB2BKR gene. **A**, Schematic map of relative locations of anchor primer, reverse primers 1 and 2, and expected sizes of 5' RACE PCR products on the basis of the published hB2BKR cDNA sequence. 5' RACE PCR products in the ethidium bromide–stained agarose gel are shown in **B** with the corresponding Southern blot shown in **C**. In both **B** and **C**, *lane 1*: amplified PCR products after the first PCR; *lane 2*: amplified PCR products after the second PCR; *lane 3*: Bio-Rad DNA size standards.



B.



C.



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The sources of RNA were from both human placenta and colon tissue. Sequence-specific reverse primer 1 located within exon 3 (5'-AAGATGTTCTCTAGGGTGGCCAGC-3') and anchor primer (5'-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3') were used for the first PCR. For further amplification, sequence-specific reverse primer 2 (5'-GAGTCCTCACGAACAGACAGAAAC-3') located within exon 2 was used in combination with the anchor primer for the second PCR. The reverse primers were designed to have a high annealing temperature, but to not have either significant secondary structure or form primer dimers with the anchor primer. PCR was performed for 30 cycles as described above. PCR buffer composition was as follows: 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3; 2 mmol/L MgCl₂; 0.2 mmol/L concentrations of each deoxyribonucleoside triphosphate (Pharmacia Biotech); 10 μmol/L concentrations of each primer; and 2.5 U of Taq DNA polymerase (Gibco BRL) per 50 μl. Amplified PCR products were size-fractionated in 4% agarose gels, and their identity was confirmed by Southern blot analysis with the α³²P-labeled 1.2 kb cDNA probe. The desired PCR products were excised from agarose gels, isolated by GeneClean kits, and cloned into the pCR II vector (Invitrogen Co., San Diego, Calif.) by using T/A cloning kits according to the manufacturer's protocol. The Taq DyeDeoxy terminator cycle sequencing kit from Applied Biosystems Inc. was used for automated fluorescent sequencing. After the cycling reaction, the reaction mixture was subjected to 6% acrylamide Tris-borate ethylenediaminetetraacetic acid (TBE) gels containing 8.3 mol/L urea, and the nucleotide sequence was analyzed as described above.

Genomic DNA PCR and sequence analysis

Genomic DNA PCR was performed to further characterize hB2BKR exon 1 polymorphisms identified by 5' RACE. The forward primer (5'-CTGTTCCCGCCGCGCACTCCA-3', genomic nucleotides 539-558¹⁸) was designed to anneal within exon 1, whereas the reverse primer (5'-

CAGAGGTGAGGCGGCTGGAG-3', genomic nucleotides 697-716¹⁸) was designed to anneal within intron 1. Genomic DNA from both human peripheral blood leukocytes and cultured human cell lines was isolated by using the 340A DNA extractor (Applied Biosystems Inc.). PCR was performed with Ampliwax (Perkin Elmer) facilitated "hot-start" process²¹ in the Ericomp twin block easy cycler. The bottom mixture (7.5 µl) consisted of: 13.3 mmol/L MgCl₂, 16.7 mmol/L Tris-HCl, pH 8.3; 1.33 mmol/L concentrations of each deoxyribonucleoside triphosphate (Pharmacia Biotech); and 10 µmol/L concentrations of each forward and reverse primer. The top mixture (30 µl) consisted of: 12.5 mmol/L Tris, pH 8.3; 83.3 mmol/L KCl; 1.5 U of Taq DNA polymerase (Gibco BRL); and 300 ng of genomic DNA. The thermal cycle profile of PCR consisted of 28 cycles of 96.5° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and of 1 cycle of 72° C for 7 minutes. Amplified PCR products were further analyzed in 10% acrylamide TBE gels in 0.5× TBE buffer.

Amplified PCR products were purified by using QIAquick PCR purification kits (Qiagen Inc., Chatsworth, Calif.). For direct manual sequencing of the PCR products, the TAQuence cycle-sequencing kit (United States Biochemical Co., Cleveland, Ohio) was used according to the supplier's protocols. After PCR cycling and addition of the stop solution to samples, the reaction mixture was subjected to electrophoresis in 6% acrylamide TBE gels containing 7 mol/L urea. After electrophoresis, gels were fixed in 5% acetic acid and 15% methanol for 30 minutes, vacuum-dried, and exposed to Kodak XAR 5 x-ray film at room temperature without intensifying screens. Nucleotide sequences were determined in both DNA strands. The DNA sequences of amplified PCR products were also confirmed by automated fluorescent DNA sequencing as described above.

The hB2BKR exon 1 sequence was analyzed with University of Wisconsin Genetic Computer Group Sequence Analysis Software Package by using the REPEAT program on SUN workstations.

Human subjects and tissues

After informed consent was obtained, blood was drawn from donors either from the General Clinical Research Center at The Scripps Research Institute or from the University of Southern California Medical Center (Los Angeles, Calif.) and processed for extraction of genomic DNA. Donors with hepatitis, human immunodeficiency virus, or cancer were excluded from the study populations. Patients with C1 inhibitor deficiency were diagnosed on the basis of a low C1 inhibitor antigenic or functional level and were categorized as having either hereditary angioedema (HAE) or acquired C1 inhibitor deficiency on the basis of standard criteria.²³ Patients with C1 inhibitor deficiency were further characterized according to whether they had ever had an episode of angioedema.

Nasal scrapings were performed by using a nasal mucosal curette (Rhino-Probe, Apotex Scientific, Arlington, Texas) in selected normal subjects after informed consent was obtained. The nasal scrapings were immediately placed into RNazol B and processed as described above. A sample of surgically removed human colon tissue was obtained from the Department of Pathology at Scripps Clinic with Institutional Review Board approval.

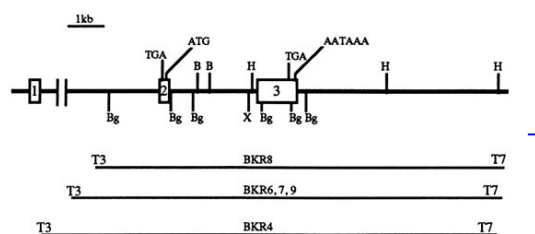
Statistical analysis

Comparisons among subject groups were performed by using the chi square test. A *p* value less than 0.05 was considered to be statistically significant. Degrees of freedom (df) are indicated. Statistical analysis was performed with Statview 4 (Abacus Concepts, Berkeley, Calif.).

Complementary DNA and genomic clones of hB2BKR gene

A 1.2 kb cDNA clone (phB2BKR) was obtained from RT-PCR of RNA derived from human T24 cells. Sequence analysis showed that the complete open reading frame sequence in phB2BKR was identical to the complete open reading frame sequence reported by Hess et al.¹⁴ This 1.2 kb cDNA probe was used to screen the genomic DNA library. Five phage clones designated as BKR 4, 6, 7, 8, and 9 were isolated and subcloned to determine the hB2BKR gene structure. Restriction mapping demonstrated that these five phage clones represented three independent overlapping clones (Fig. 2).

Fig. 2. Genomic DNA clones for hB2BKR gene. From a human IFIX II genomic DNA library, three independent overlapping phage clones (BKR 4, BKR 6, and BKR 8) were obtained containing exons 2 and 3 of hB2BKR gene. The upper part of the figure shows a restriction map and the relative positions of hB2BKR gene exons 1, 2, and 3 (*open boxes*). The double slash lines in intron 1 indicate that this intron (>20 kb) is not fully displayed. Restriction enzymes are abbreviated as follows: *B*, *Bam*HI; *Bg*, *Bgl* II; *H*, *Hind*III; *X*, *Xho* I. The translational start codons (ATG), stop codons (TGA), and polyadenylation signal codon (AATAAA) are indicated. The lower part indicates three independent clones. BKR 6, 7, and 9 are identical clones. T3 and T7 indicate the insert orientation in IFIX II clones.



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Compared with the previously reported cDNA sequence,¹⁴ these phage clones contained only exons 2 and 3 but not exon 1, which has been recently reported to be about 20 kb upstream of exon 2 (Fig. 2■).¹⁸ Re-screening the library with a 51 bp exon 1 oligonucleotide probe also failed to identify an exon 1 clone under stringent conditions of hybridization and washing.

RACE analysis of the 5' sequence for hB2BKR mRNA

To determine the 5' mRNA sequence, 5' RACE was performed with RNA samples from human placenta and colon tissue. A 229 bp PCR product was expected after the second PCR on the basis of the longest published cDNA sequence (Fig. 1, **A**).¹⁴ Three bands were observed after the second PCR (lane 2, Fig. 1, **B**). The longest PCR product was estimated to be approximately 280 bp compared with DNA size standards. These amplified PCR products were further confirmed by Southern blot analysis with the 1.2 kb cDNA probe containing hB2BKR exons 2 and 3 (lane 2, Fig. 1, **C**).

The amplified PCR products were cloned into the pCR II vector, and 39 clones were isolated and analyzed by restriction mapping. Nine independent clones were further sequenced and found to represent five overlapping groups compared with both reported genomic DNA and cDNA sequences (Fig. 3, **A**).

Fig. 3. Sequence comparison among reported genomic DNA, cDNA, and 5' RACE clones. **A** and **B** show sequences of the hB2BKR gene exon 1 and the 5' end of exon 2, respectively. The gaps in **A** show the differences between the reported sequences and 5' RACE clones, indicating that those nucleotides are not detected in any of the sequenced 5' RACE clones ($n = 9$). In both panels: *line 1*, genomic DNA sequence determined by Ma et al.¹⁸; *line 2*, cDNA sequence described by Hess et al.¹⁴; *line 3*, longest 5' RACE clones ($n = 2$) obtained from human placenta tissue; *line 4*, 5' RACE clone obtained from human placenta tissue with the 5'-cap-guanosine nucleotide indicating the transcriptional start site; *line 5*, 5' RACE clones obtained from human placenta tissue ($n = 4$) with alternative usage of 3' acceptor splicing site; *line 6*, 5' RACE clone obtained from human colon tissue with alternative usage of 3' acceptor splicing site; *line 7*, 5' RACE clone obtained from human placenta tissue. An arrow indicates the probable primary transcriptional start site. Both 5' donor splicing and 3' acceptor splicing sites are underlined. Double-underlined nucleotides indicate the first in-frame translational start site. **C**, Schematic map denoting alternative usage of 3' acceptor splicing sites. Arrows indicate positions of both 5' donor splicing and 3' acceptor splicing sites. Exonic sequences are shown in capital letters, and intronic sequences are shown in lowercase letters.

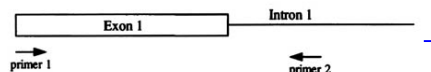
compared with the published sequence.¹⁴ Each of the sequenced 5' RACE clones contained a 9 bp and two single base deletions in the hB2BKR exon 1 (Fig. 3, A ■). The position of the 9 bp deletion corresponded to the middle of the 51 bp oligonucleotide probe used to screen the genomic library and therefore might explain the failure to obtain genomic DNA clones containing exon 1 in the second genomic DNA library screening under stringent conditions of hybridization and washing.

hB2BKR exon 1 PCR and sequence from genomic DNA

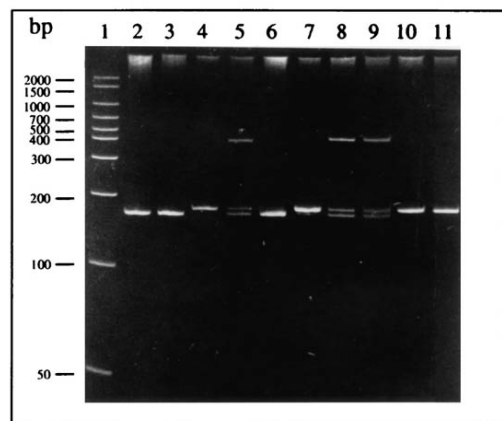
Fig. 4. Genomic DNA PCR of exon 1 of the hB2BKR gene. **A**, Schematic map of relative locations of forward primer 1 and reverse primer 2 for genomic DNA PCR. **B**, Representative acrylamide TBE gel shows results of three different patterns of genomic DNA PCR after ethidium bromide staining. *Lane 1*: Bio-Rad DNA size standards; *lanes 2, 3, and 6*: homozygotes with low band pattern (167 bp); *lanes 4, 7, 10, and 11*: homozygotes with high band pattern (176 bp); *lanes 5, 8, and 9*: heterozygotes with the pattern containing both low and high bands plus an extra band (approximately 400 bp). **C**, Acrylamide TBE gel shows that the 400 bp band represents heteroduplex DNA. *Lane 1*, Exon 1 genomic PCR with DNA template from a homozygote with the

high band pattern; *lane 2*, exon 1 genomic PCR with DNA template from a homozygote with the low band pattern; *lane 3*, exon 1 genomic PCR with a 1:1 mixture of DNA template from homozygotes in lanes 1 and 2; *lanes 4 and 5*, DNA templates from two heterozygous individuals.

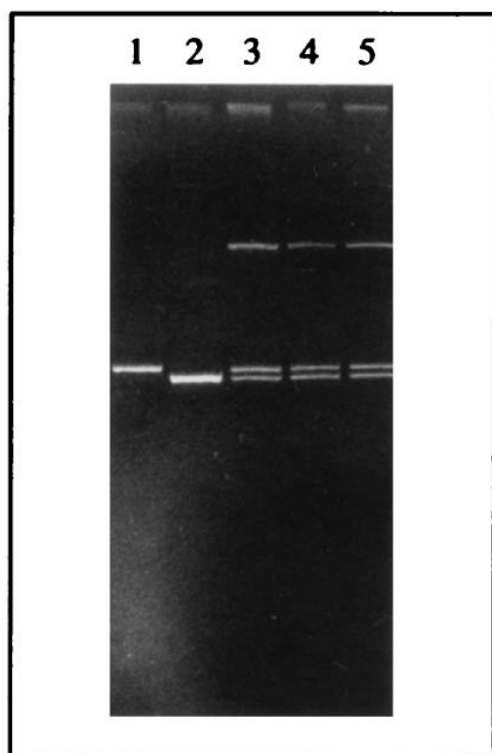
A.



B.



C.



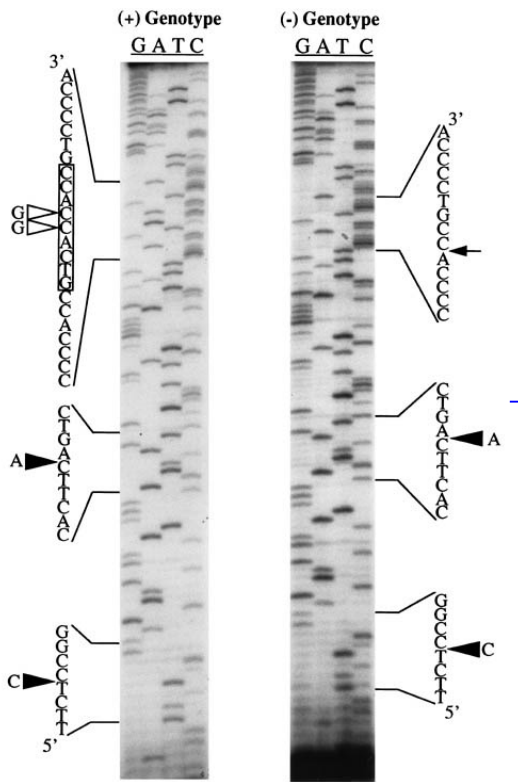
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Amplified PCR products were resolved in acrylamide TBE gels and revealed three specific patterns

of bands. Some subjects showed a product at 176 bp, some subjects showed a product at 167 bp, and others showed both bands (Fig. 4, B ■). A band migrating at 400 bp was also observed in the PCR products from subjects showing both the 167 bp and 176 bp bands. This 400 bp product was shown to be heteroduplex DNA because it was not seen in either 167 bp band or 176 bp band homozygous subjects alone but could be artificially generated by performing PCR with a 1:1 mixture of genomic DNA from a 167 bp band homozygous subject and a 176 bp band homozygous subject (Fig. 4, C ■). Similar DNA heteroduplex results have also been observed from two alleles of human corticosteroid-binding globulin gene²⁴ and mouse testis-determining Y gene.²⁵

Direct manual sequencing of the two specific PCR products amplified from hB2BKR exon 1 genomic DNA showed that the difference between the 167 bp and 176 bp bands was accounted for by the presence or absence of the nonanucleotide sequence, 5'-GGTGGTGAC-3', corresponding to the 9 bp deletion found in the 5' RACE (Fig. 5).

Fig. 5. Autoradiogram of a 6% acrylamide/7 mol/L urea gel showing the noncoding strand sequence ladder of hB2BKR exon 1 genomic DNA PCR products from one homozygous individual with the (+)²¹⁻²⁹ genotype [indicated by (+)] and one homozygous individual with the (-)²¹⁻²⁹ genotype [indicated by (-)]. *The arrow* in the (-)²¹⁻²⁹ genotype side indicates the sequence difference, 5'-CCGTCACCA-3', between the (+)²¹⁻²⁹ genotype and the (-)²¹⁻²⁹ genotype. *Open arrowheads* in both (+)²¹⁻²⁹ and (-)²¹⁻²⁹ genotype panels indicate the difference between the published genomic sequence data and the current sequence data. *Filled arrowheads* in both (+)²¹⁻²⁹ and (-)²¹⁻²⁹ genotype panels indicate positions of extra single nucleotides shown only in the published genomic sequence data.



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The hB2BKR allele containing this nonanucleotide sequence was referred to as the (+)²¹⁻²⁹ allele, whereas the allele without the nonanucleotide sequence was referred as the (-)²¹⁻²⁹ allele. Compared with the previously reported DNA sequence, there were also two separate single nucleotide deletions, one A and one C, further upstream of the nonanucleotide sequence in both (+)²¹⁻²⁹ and (-)²¹⁻²⁹ genotypes (Fig. 5 ■). As shown in Fig. 3 ■, these were also absent in all 5' RACE clones. DNA sequencing analysis of amplified exon 1 PCR products from 16 homozygotes [8 with the (+)²¹⁻²⁹ genotype and 8 with the (-)²¹⁻²⁹ genotype] showed that all (+)²¹⁻²⁹ samples had the 21-29 nonanucleotide, whereas all (-)²¹⁻²⁹ samples lacked the 21-29 nonanucleotide. All 16 sequences also contained the two single nucleotide deletions. Moreover, the GG dinucleotide in the nonanucleotide, 5'-GGTGGTGAC-3', were different from the corresponding CC dinucleotide in the 5'-GGTCCTGAC-3' sequence previously reported.¹⁸ The genomic DNA sequence results further substantiated the exon 1 sequences obtained from 5' RACE (Fig. 3, A ■) and demonstrated the existence of an exon 1 polymorphism. This polymorphism was also detected in several human cell lines (Table I).

Table I. Genotypes and transcripts of human cell lines

Cell line	Origin	hB2BKR genotype	hB2BKR RT-PCR
A431	Epidermis	(-) ²¹⁻²⁹ *	ND
T24	Bladder	(-) ²¹⁻²⁹ †	(-) ²¹⁻²⁹
MCF-7/6	Breast	(+) ²¹⁻²⁹ /(-) ²¹⁻²⁹ *	(+) ²¹⁻²⁹ /(-) ²¹⁻²⁹
T47D	Breast	(+) ²¹⁻²⁹ /(-) ²¹⁻²⁹ *	ND

HeLa S3	Cervix	(+) ²¹⁻²⁹ /(-) ²¹⁻²⁹ *	(+) ²¹⁻²⁹ /(-) ²¹⁻²⁹
WI38	Lung	(+) ²¹⁻²⁹ †	ND

ND, Not determined.

*Determined only by genomic DNA PCR.

†Determined by both genomic DNA PCR and sequencing analysis.

Distribution of hB2BKR gene exon 1 alleles in different ethnic groups

To establish the frequency of the (+)²¹⁻²⁹ and (-)²¹⁻²⁹ alleles, we genotyped 102 healthy donors (39 Caucasians, 31 African Americans, and 32 Asians). Table II shows the distribution frequency of the genotypes in these groups.

Table II. Distribution of hB2BKR genotypes in three ethnic groups

hB2BKR Genotype	Ethnic group		
	Caucasian	African-American	Asian*
(+) ²¹⁻²⁹ /(+) ²¹⁻²⁹	12 (30.8%)	11 (35.4%)	0 (0%)
(+) ²¹⁻²⁹ /(-) ²¹⁻²⁹	17 (43.6%)	14 (45.2%)	0 (0%)
(-) ²¹⁻²⁹ /(-) ²¹⁻²⁹	10 (25.6%)	6 (19.4%)	32 (100%)
Total	39	31	32

*The Asian group consisted entirely of subjects who came from Japan, China, or Taiwan.

The overall frequencies of the (+)²¹⁻²⁹ and (-)²¹⁻²⁹ alleles were calculated as follows: Caucasians, (+)²¹⁻²⁹ = 0.53, (-)²¹⁻²⁹ = 0.47; African Americans, (+)²¹⁻²⁹ = 0.58, (-)²¹⁻²⁹ = 0.42; and Asians, (+)²¹⁻²⁹ = 0.00, (-)²¹⁻²⁹ = 1.00. The genotypic distribution of hB2BKR exon 1 alleles was at Hardy-Weinberg equilibrium in all three groups. Chi square analysis showed a highly significant difference in the frequency of the (+)²¹⁻²⁹ and (-)²¹⁻²⁹ alleles between the ethnic groups (chi square = 54.1, *p* < 0.0001, *df* = 2). Analysis of the post hoc cell contributions revealed that the Asian group was significantly underrepresented for the (+)²¹⁻²⁹ allele and overrepresented for the (-)²¹⁻²⁹ allele. There was no significant difference in the distribution of alleles between Caucasian and African-American subjects (chi square = 0.42, *p* = 0.52, *df* = 1).

Functional differences between the hB2BKR gene exon 1 alleles

To determine whether the exon 1 polymorphism was associated with any difference in the expression of the hB2BKR gene, we measured the relative steady-state levels of (+)²¹⁻²⁹ and (-)²¹⁻²⁹ mRNA by RT-PCR. Because hB2BKR expression in peripheral blood cells is minimal,¹⁸ we used nasal scraping tissue for this analysis. Nasal scrapings were obtained from five normal subjects

who were previously found to be (+)²¹-29/(-)²¹-29 heterozygous. After reverse transcription, the cDNA was amplified by using a forward primer in exon 1 and a reverse primer in exon 2. Each sample produced two hB2BKR exon 1 bands, corresponding to the (+)²¹-29 and (-)²¹-29 transcripts. The PCR products were separated on 10% acrylamide TBE gels and stained with ethidium bromide, and the band intensities were measured by densitometry. All five heterozygote subjects showed more intense (-)²¹-29 product bands than (+)²¹-29 product bands, with the ratio ranging from 1.30 to 3.22 (mean $\pm$ SD = 1.86 $\pm$ 0.78).

We next genotyped 21 patients with angioedema caused by C1 inhibitor deficiency. This group consisted of 17 patients with HAE and four patients with acquired C1 inhibitor deficiency in association with anti-C1 inhibitor autoantibodies (Table III).

Table III. Distribution of hB2BKR genotypes in patients with C1 inhibitor deficiency

Subject	Diagnosis	Kindred	Sex	Symptomatic	Exon 1 genotype*
CW	Type I HAE	1	F	Yes	+/-
SW	Type I HAE	2	M	Yes	+/-
MB	Type I HAE	3	F	Yes	-/-
BM	Type I HAE	4	M	Yes	+/-
BG	Type I HAE	5	F	Yes	+/-
CM	Type I HAE	5	F	Yes	-/-
DF	Type I HAE	5	F	Yes	+/-
PM	Type I HAE	6	F	Yes	+/-
EM	Type I HAE	7	M	Yes	-/-
DH	Type I HAE	8	M	Yes	+/-
CH	Type I HAE	8	M	Yes	+/-
PH	Type I HAE	8	M	Yes	+/-
VC	Type I HAE	9	F	Yes	+/-
LS	Type I HAE	10	F	Yes	+/-
EC	Type I HAE	11	F	Yes	-/-
MT	Type I HAE	12	F	Yes	+/-
LF	Type II HAE	13	F	Yes	+/-
RL	Acquired	14	M	Yes	-/-
JS	Acquired	15	F	Yes	-/-
LS	Acquired	16	M	Yes	+/-
RW	Acquired	17	F	Yes	+/-
MW	HAE	2	M	No	+/+

AM	HAE	4	M	No	+/+
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*Genotypes are abbreviated as follows: $+/+ = (+)^{21-29}/(+)^{21-29}$; $+/- = (+)^{21-29}/(-)^{21-29}$; and $-/- = (-)^{21-29}/(-)^{21-29}$

None of the patients with symptomatic C1 inhibitor deficiency were $(+)^{21-29}$ homozygous. The exon 1 genotypes in the patients with symptomatic C1 inhibitor deficiency (none of whom were Asian) were significantly different from those of the non-Asian normal subjects described above (chi square = 9.47, $p = 0.0088$, $df = 2$). We also identified two patients (a 9-year-old boy, MW, and an 18-year-old man, AM) who had immunochemical evidence of HAE but had never experienced an episode of angioedema. Both patients had a family member with symptomatic HAE who was $(+)^{21-29}/(-)^{21-29}$ heterozygous, but both of these patients without symptoms were found to be $(+)^{21-29}$ homozygous.

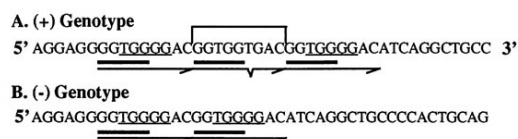
Discussion

TOP

Bradykinin is thought to be important in the initiation and propagation of diverse forms of inflammation.^{1 3 4} To study the regulation of hB2BKR expression, we investigated its gene structure. This study confirmed the three-exon structure of the hB2BKR. Compared with the published hB2BKR cDNA and genomic sequences,^{14 18} we found three deletions in exon 1, including a single nonanucleotide deletion (nucleotides 21-29) and two single nucleotide deletions at nucleotide 71 and nucleotide 97 of the cDNA sequence. There was also evidence for a possible alternative transcription start site, as well as an alternative usage of the 3' acceptor splicing site in the beginning of exon 2. Potentially significant functional and clinical consequences of the identified exon 1 polymorphism were also identified.

Using genomic PCR to amplify the hB2BKR exon 1 from peripheral blood leukocytes, we demonstrated that normal subjects were polymorphic for the presence or absence of the nonanucleotide sequence 5'-GGTGGTGAC-3', corresponding to the 9 bp deletion found by 5' RACE. This $(+)^{21-29}/(-)^{21-29}$ polymorphism is identical to the 2G/3T hB2BKR exon 1 polymorphism recently described by Braun et al.¹⁹ They also described a third allele, 3G, consisting of the nonanucleotide sequence, 5'-GGTGGGGAC-3' in place of 5'-GGTGGTGAC-3'. We did not detect a G/T transversion in the eight $(+)^{21-29}$ alleles we sequenced; however, the current genomic PCR screening assay would not be expected to specifically detect this transversion. Analysis of nucleotide sequences in the vicinity of the deletion site reveals the presence of the short direct repeat, 5'-GGTGGGGAC-3' (Fig. 6).

Fig. 6. Short direct repeat sequences in hB2BKR gene exon 1. Positions of short direct repeats in (+)²¹⁻²⁹ genotype and (-)²¹⁻²⁹ genotype are shown in **A** and **B**, respectively. Short direct repeats are either underlined by fine lines, bold lines, or arrows. *The bent arrow* indicates the single nucleotide difference. *Bracketed sequence* in **A** indicates one of the nonanucleotides not present in the (-)²¹⁻²⁹ genotype.



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This sequence is concordant with the deletion “hot spots” consensus sequence, TG(G/A)(A/G)(T/G)(C/A),²⁶ and also contains the immunoglobulin gene switch regions (Sm) core motif, 5'-TGGGG-3'.²⁷ The mechanisms for deletion are unknown at present. The presence of direct repeats could contribute to the deletion by slipped mispairing during DNA replication or recombination.²⁸

The distribution frequency of the (+)²¹⁻²⁹ and (-)²¹⁻²⁹ alleles in 102 normal Caucasian, African-American, and Asian subjects revealed a highly significant underrepresentation of the (+)²¹⁻²⁹ allele in the Asian group (chi square = 54.1, $p < 0.0001$). In contrast, the allelic frequency of the (-)²¹⁻²⁹ allele in Caucasian and African-American normal subjects was 0.47 and 0.42, respectively, which is similar to the allelic frequency of 0.53 for the 2G allele found in the southern German population described by Braun et al.¹⁹

The effect of the exon 1 polymorphism on hB2BKR expression was assessed by comparing the relative levels of (+)²¹⁻²⁹ and (-)²¹⁻²⁹ mRNA transcripts in heterozygous subjects. With total RNA extracted from nasal scrapings, RT-PCR was performed to amplify a 139 nucleotide product from the (+)²¹⁻²⁹ allele and a 130 nucleotide product from the (-)²¹⁻²⁹ allele. Because both products were amplified in a single reaction with the same primers, we measured the relative intensity of both bands within a single lane to obtain a ratio of the (-)²¹⁻²⁹ to (+)²¹⁻²⁹ PCR products. To minimize the influence of the exon 1 polymorphism on amplification efficiency, we designed the primers so that the 3' end of the forward and reverse primers annealed at least 15 bp upstream and downstream, respectively, from the polymorphic site. The primers were also designed to cross the exon 1–exon 2 boundary, so that genomic DNA would not be amplified. In each of the five subjects, the predominant form of hB2BKR mRNA was found to be the (-)²¹⁻²⁹ allele. The mean (-)²¹⁻²⁹/(+)²¹⁻²⁹ ratio in all five subjects was 1.86. Our studies did not address whether the difference in mRNA steady-state levels reflected transcriptional or posttranscriptional effects. Braun et al.,²⁹ however, recently coupled the putative hB2BKR regulatory region (including the exon 1 polymorphic site) to a luciferase reporter gene and demonstrated a decrease in the transcriptional activity when the

construct contained the (+)²¹⁻²⁹ sequence compared with the (-)²¹⁻²⁹ sequence. Taken together, these results suggest that the exon 1 genotype may be capable of affecting hB2BKR expression.

We demonstrated a potentially important functional consequence of the hB2BKR exon 1 polymorphism in patients with C1 inhibitor deficiency. These patients were chosen because there is strong evidence that the angioedema in C1 inhibitor deficiency is a consequence of bradykinin generation.³⁰⁻³⁵ Twenty-one patients with angioedema caused by C1 inhibitor deficiency were genotyped, and none were (+)²¹⁻²⁹ homozygous. The distribution of exon 1 genotypes in the patients with C1 inhibitor deficiency was significantly different from that found in the normal control subjects (chi square = 9.47, $p = 0.0088$ compared with non-Asian normal subjects and chi square = 13.93, $p = 0.0009$ compared with all normal subjects). Because the C1 inhibitor gene is on chromosome 11, whereas the hB2BKR gene is on chromosome 14, this association cannot represent a linkage disequilibrium. An unusual syndrome of HAE without clinical expression of angioedema has been described.³⁶ Because these patients do not experience swelling, they are less likely to be diagnosed than other patients with C1 inhibitor deficiency. During our screening of unaffected family members in HAE kindreds, we identified two additional patients who had immunochemical evidence of HAE but no history of angioedema. Both of these patients were found to be (+)²¹⁻²⁹ homozygous. Taken together with the statistically significant absence of this genotype in patients with symptomatic HAE, these results led us to hypothesize that the ability of bradykinin to induce angioedema may, in part, be determined by the hB2BKR exon 1 genotype.

Although this polymorphism is located in the 5' untranslated region of the hB2BKR gene, the (-)²¹⁻²⁹ and (+)²¹⁻²⁹ alleles have measurably distinct effects on hB2BKR promoter activity and steady-state mRNA levels. Changes in steady-state hB2BKR mRNA levels lead to corresponding changes in cell surface expression of functional B2BKR, as well as bradykinin-stimulated intracellular calcium mobilization (Lung et al. Unpublished data). One possible explanation, therefore, for the absence of (+)²¹⁻²⁹ homozygous patients with symptomatic HAE in our sample group is that this genotype results in decreased B2BKR expression, which causes a clinically significant decrease in responsiveness to bradykinin. We cannot exclude the possibility that this represents a sampling error caused by the relatively small HAE group size, or alternatively, that the exon 1 polymorphism may be linked to another hB2BKR polymorphism. Additional studies are needed to examine these possibilities.

In conclusion, we cloned and analyzed exon 1 of the hB2BKR gene. Evidence for an alternative transcription start site and an alternative 3' acceptor splicing site in exon 2 were found. The functional significance of these observations await further studies. A polymorphism in exon 1 was shown to result from the presence or absence of the nonanucleotide sequence, 5'-GGTGGTGAC-3' belonging to a short direct repeat consensus sequence. A highly significant difference in the genomic frequency of the exon 1 alleles was found between Asian subjects and Caucasian or African-American subjects. Furthermore, the exon 1 polymorphism was shown to affect steady-state hB2BKR mRNA levels and possibly to determine the sensitivity of patients with C1 inhibitor deficiency to bradykinin-induced angioedema. Further studies are necessary to assess the relative contributions of the alternative transcription start site and 3' acceptor splice site on hB2BKR function and to define the importance of this exon 1 polymorphism. The results of this study suggest that differences in the structure of the hB2BKR gene may influence the severity of bradykinin-associated diseases, potentially including asthma, rheumatoid arthritis, hypertension, and C1 inhibitor deficiency.

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