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# THE HUMAN BRADYKININ $B_2$ RECEPTOR GENE: FULL LENGTH cDNA, GENOMIC ORGANIZATION AND IDENTIFICATION OF THE REGULATORY REGION*

S. Kammerer[1,2], A. Braun[1]+, N. Arnold[2], and A.A. Roscher[1]

[1]Children's Hospital, Department of Clinical Chemistry and Biochemistry, University of Munich, Lindwurmstr. 4, D-80337 Munich, Germany

[2]Institute of Anthropology and Human Genetics, University of Munich, Richard-Wagner-Str. 10/I, D-80333 Munich, Germany

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**Summary** Hybridization of Northern blots with a probe corresponding to the coding region of the bradykinin $B_2$ receptor revealed a single transcript of approximately 4.0 kb. Using the same probe, we detected a 2.6 kb human cDNA clone that overlapped 103 bp with the 3' end of the known coding region and contained a classical polyadenylation site and a polyA tail. The gene for the human $B_2$ receptor was isolated from a human placenta genomic library. Analysis of several genomic $\lambda$ clones indicated that the $B_2$ receptor gene is organized in three exons. Genomic Southern analysis revealed the $B_2$ receptor to be encoded by a single copy gene. In situ hybridization showed that the gene is located on chromosome 14q32. By testing different putative regulatory fragments in a luciferase reporter assay, a gene activating function of the 5' upstream region of exon 1 was demonstrated.


Bradykinin (BK) is a vasoactive nonapeptide of the kinin family, released from kininogenes by proteolytic activity of kallikreins (1,2). It is involved in multiple physiological processes such as cardiovascular homeostasis, inflammation, pain, neurotransmission and cell proliferation (3,4). The majority of effects are mediated by the cell surface BK-receptors of the $B_2$-subtype which exhibits high affinity for kallidin (Lys-BK) and BK (2,5,6,8). The BK-receptor of the $B_1$-subtype is merely expressed under certain pathological conditions e.g. inflammation and trauma and shows high affinity for the kinin metabolites [desArg$^9$]BK and [desArg$^{10}$]kallidin (5-7).

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+Correspondence should be addressed to Andreas Braun, M.D., Ph.D.
Fax: +49-89-5160-4486.

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In 1992, the coding part of the cDNA of the human $B_2$ receptor has been described (9). The deduced amino acid sequence showed 81% homology with the rat $B_2$ receptor. Expression studies in COS cells confirmed that the cloned receptor shows the typical pharmacological properties expected for a $B_2$ receptor. Subsequently, the genomic structure of this gene was partially elucidated and the authors postulated that it is intronless (10). However, a discrepancy between the 5' end of the gene identified by Powell et al. (10) and the 5' end of the cDNA described by Hess et al. (9) was evident. Therefore, at least one additional exon in the 5'-region was postulated (11). Recently, Ma and co-workers (12) were able to demonstrate that there are two further non-coding exons. Up to now, however, the full length cDNA of the human $B_2$ receptor and the regulatory region of the gene including enhancer and regulatory elements are not characterized.

In this study, we elucidated the full length cDNA and the genomic structure of the gene, including sequences of the exon-intron boundaries and the 5' and 3' flanking regions. In particular, a fragment of the 5' upstream region showed a regulatory role by its activating effect on a reporter gene in transfection experiments.

## MATERIALS AND METHODS

**Genomic Southern and Northern analyses.** Southern blot was performed using 10μg of genomic DNA each, digested with different restriction enzymes, separated in a 0.8% agarose gel and transferred to a Hybond $N^+$ membrane (Amersham, Braunschweig, Germany). Northern blot was done with 15 μg of total RNA of human foreskin fibroblasts, prepared by the procedure of Chirgwin et al. (13), separated on a 2.2 M formaldehyde/1.2% agarose gel and transferred to a Hybond N membrane (Amersham). The probes were amplified using 10 ng of genomic DNA and two $B_2$ receptor specific primers and radiolabelled by PCR with [$\alpha$-$^{32}$P]dATP (Amersham). Hybridization was performed by a modified method of Church and Gilbert (14) as described elsewhere (15).

**Amplification of cDNA 5' fragments from a cDNA library.** 1 μg plasmid DNA prepared from a testis cDNA library in the CDM8 vector were submitted to a PCR amplification with a CDM8 specific forward primer (5'-GAGCTCTCTGGCTAACTAG AG-3') and the $B_2$ receptor specific primer P461 (sequence see Fig.2). The obtained fragments were subcloned in pUC19 and sequenced by the dideoxy chain termination method of Sanger et al. (16) using [$\alpha$-$^{32}$P]dATP (Amersham) and Sequenase sequencing kit (USBiochemical, Bad Homburg, Germany)

**Screening of genomic and cDNA libraries.** $10^6$ plaques of a human placenta genomic library in λFIXII (Stratagene, Heidelberg, Germany, 946205) were screened twice with different $B_2$ receptor cDNA probes amplified by PCR using the following primer combinations: P666/P1317 and P14/P132 (sequences see Fig.2). A human oligo(dT) primed fibroblast cDNA library in λgt11 (Clontech Laboratories, Heidelberg, Germany, HL1052b) was screened with the probe P666/P1317. Labelling of probes and hybridization were done as for genomic Southern blots with the exception that the probe P14/P132 was hybridized and washed at 55°C. The clones detected were purified by standard methods and inserts of prepared phage DNA were subcloned and sequenced as described above.

**Chromosomal localization.** Human metaphase chromosomes of a healthy male donor were prepared following standard methods. A mixture of plasmids containing inserts of the two genomic λ clones, 2 μg of each, were nick-translated with digoxigenin-11-dUTP. In situ hybridization followed the protocols described elsewhere (17). Digital images were obtained using a Zeiss Axioskop epifluorescence microscope coupled to a cooled CCD camera (Photometrics Kodak KAF1400) controlled by a Macintosh Quadra 950 computer. The images were then pseudocolored separately and merged using Gene Join MaxPix software, as described by Ried et al. (18).

**Analysis of gene regulatory activity in cell culture.** Two fragments of the putative human $B_2$ receptor regulatory region and one fragment of intron 2 were cloned to the 5' end of the firefly luciferase in pXP-1 (19). This vector containing no regulatory element (pXP-1), and the vector pRSV, bearing a strong long terminal repeat promoter (20), served as negative and positive controls, respectively. Triplicate cultures of the fetal kidney cell line 293 (ATCC, Rockville, USA, CRL1573) grown in six-well plates were transfected with 2 μg CsCl gradient purified DNA of the five different plasmid constructs using 10 μg lipofectin reagent (Gibco BRL, Eggenstein, Germany). According to the manufacturer's procedure for transient transfection of adherent cells, the transfection was performed for 6 hours, followed by 40 hours of incubation in 2 ml Dulbecco´s modification of Eagle´s medium supplemented with 10% fetal calf serum. Cell harvest and luciferin reaction were done using a luciferase assay system supplied by Promega (Heidelberg, Germany). Light emission was detected in a luminometer (Berthold, Lumat LB9501) and integrated over a 10-s interval.

## RESULTS AND DISCUSSION

### Full length cDNA of the $B_2$ receptor

By using a $B_2$ receptor specific probe, corresponding to the 3' part of the coding region, northern blot hybridization showed a unique signal of approximately 4.0 kb (Fig.1). This result is in accordance with previously published data on the length of the complete rat cDNA (21), with the transcript length determined for the mouse (22) and with the size range derived from sucrose gradient centrifugation studies followed by $B_2$ receptor specific expression experiments of human mRNA fractions in Xenopus oocytes (23). The 5' end of the largest PCR products available from a testis cDNA library (data not shown) coincided with the partial cDNA that determined previously by Hess et al. (9). However, the sequence differed in nucleotide positions 24 and 25 where we determined GG instead of CC and in the positions 71 and 87 where the T and the G, respectively, were missed. The accuracy of our sequence was confirmed by sequencing of genomic λ clones (see below) and several individual genomic amplicons. Subsequent screening of a human fibroblast cDNA library resulted in a 2.6 kb clone (λF2.6) that exceeded the 3' end of the sequence published by Hess et al. (9) about 2.5 kb and contained a classical polyadenylation site (AATAAA) and a polyA tail 16 bp downstream. These data clearly indicate, that there is no usage of more than one polyadenylation site. In particular, the usage of the polyadenylation site suggested by Powell et al. (10) and by Ma et al. (12) from studies at the genomic level must be dismissed.

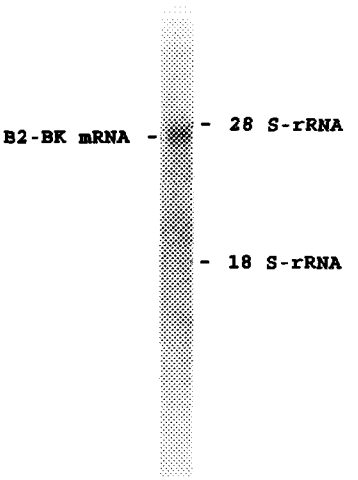



Fig.1. **Northern blot analysis.** For detection of the $B_2$ receptor transcript of about 4.0 kb we used the PCR radiolabelled cDNA probe P666/P1317 located in the 3' part of the coding region (position of exon primers and sequences see Fig.2). 15 µg of total mRNA from human foreskin fibroblasts was used. 28S and 18S rRNA are indicated at the right margin.

**Genomic organization and chromosomal localization of the $B_2$ receptor gene**

A human genomic DNA library was screened successively with PCR derived cDNA probes corresponding to a part of the coding region of the $B_2$ receptor cDNA (P666/P1317) and the 5' noncoding region (P14/P132), respectively (primer sequences see Fig.2). Exact examination of two clones detected (λBK-3 and λBK-7) revealed the complete gene structure except an uncloned gap in intron 1. The gene consists of three exons (Fig.2). The first exon is not-translated whereas the second and third one comprise the entire open reading frame and a large 3' not-translated region. Data from N-terminal sequencing of the immunoprecipitated human $B_2$ receptor appear to indicate that it is the first ATG in frame and not the third one that is used as initiation codon (AbdAlla et al., personal communication). This finding is different to artificial expression experiments in Xenopus oocytes that resulted in a pharmacological active $B_2$ receptor protein starting from the third in frame ATG (10,11). N-terminal sequencing, however, points to the start codon that is used in vivo. This codon is located in the middle of exon 2 at nucleotide position 142 referring to the A of the published cDNA sequence (9). The recent elucidation of the genomic structure of the rat $B_2$ receptor gene revealed the existence of four exons, the third one being alternatively spliced (24). In the human cDNA sequence, however, we did not find any evidence for alternative splicing and/or a fourth exon as thoroughly examined by RT-PCR procedures (data not shown).

Although we did not obtain an overlapping clone spanning entire intron 1, we were able to estimate that intron 1 is larger than 17 kb. Therefore, the entire $B_2$

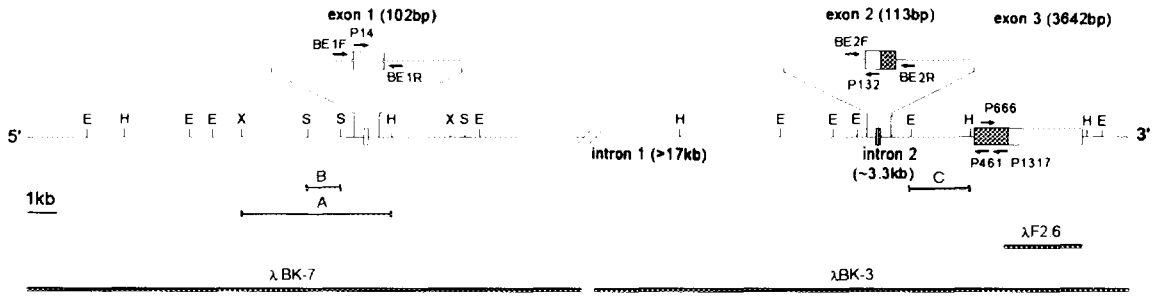



Fig.2. **Structural organization of the human $B_2$ receptor gene.** The upper part of the figure represents the gene structure with the location of exons and introns and their respective length. Exons are indicated by closed boxes (exons 1 and 2 are enlarged above), the coding region is marked by dotted boxes. The introns and the 5' and 3' flanking regions are shown as fine lines. The double dash indicates the uncloned gap in intron 1. The gene organization was determined based on the analysis of the genomic clones shown as bold lines in the lower part of the figure. A partial restriction map was determined digesting both genomic λ clones with *Eco*RI (E) and *Hin*dIII (H). Clone λBK-7 was additionally digested with *Xba*I (X) and *Sac*I (S). The localization of the 2.6 kb fibroblast cDNA clone λF2.6 is shown as bold line as well. The fragments A, B and C, subcloned in the luciferase expression vector pXP-1, are denoted as lines below the gene structure. Primers used for amplification of the different probes are marked by arrows. Numbers of the exon primers (P) indicate the 5' site according to the cDNA sequence published by Hess et al. (9): BE1F, 5'-GCCCTTGAAAGATGAGCTG-3'; BE1R, 5'-AACTCCCCA CGACCACAG-3'; BE2F, 5'-CCTGCTGGAGAATGCGTG-3'; BE2R, 5'-CCAC CTCCCCTAAGTAGG-3'; P14, 5'-TGGGGACGGTCCTGACGGTG-3'; P132, 5'-GAGTGGGATGTGAGTGAGGC-3'; P461, 5'-GCCAGGATCAGGTCTCTG-3'; P666, 5'-GCTCTACAGCTTGGTGATC-3'; P1317, 5'-TCACTGTCTGCTC CCTGCCCAG-3'.

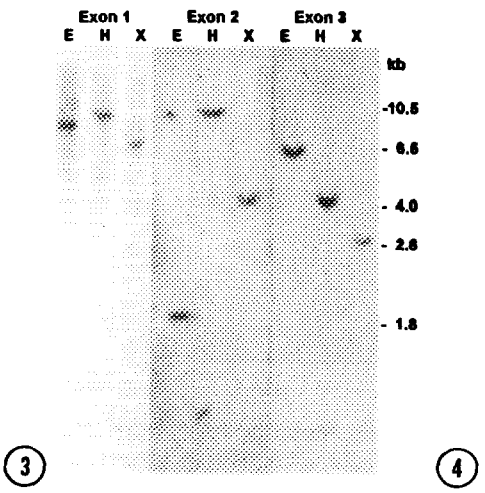
receptor gene must be larger than 25 kb. A similar large size of the first intron is also reported for the rat gene (24).

Genomic Southern hybridizations with probes corresponding to exons 1, 2, and 3 (Fig.3) showed restriction fragment lengths as expected from the restriction maps of the different λ phages containing fragments of the $B_2$ receptor gene (Fig.2). While these results are in agreement with the sequence data published by Ma et al. (12), the genomic Southern blot depicted there clearly differs from ours. The authors did not discuss the discrepancies between their Southern blot analysis and DNA sequence data.

The in situ hybridization experiments localized the receptor gene to chromosome 14q32 (Fig.4) which is in accordance with this report (12).

**The regulatory region of the $B_2$ receptor gene**

Several DNA fragments of the genomic λ clones containing the $B_2$ receptor gene were subcloned in a luciferase reporter vector. Locations of the DNA fragments are shown in Fig.2. Clone A contains a 5.0 kb *Xba*I/*Hin*dIII fragment including exon 1 and



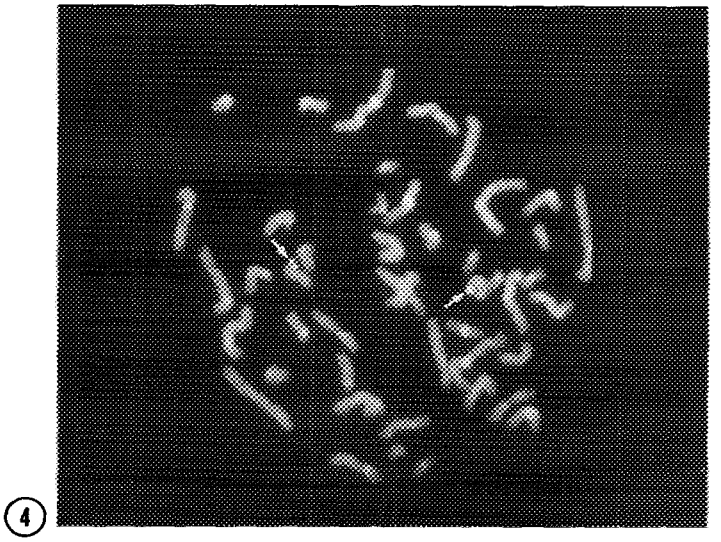




Fig.3. **Genomic Southern blot analysis.** A Southern blot with three lanes of human genomic DNA, digested by the restriction enzymes indicated, was hybridized successively to cDNA probes representing the three exons of the $B_2$ receptor gene amplified by PCR using the following primers (see Fig.2): BE1F/BE1R (exon 1), BE2F/BE2R (exon 2) and P666/P1317 (exon 3). E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. Size markers are indicated on the right side of the blot.

Fig.4. **Chromosomal localization of the human $B_2$ receptor gene.** The two genomic λ clones subcloned in pUC19 were labelled with digoxigenin and used as probe for in situ hybridization of a metaphase spread. Twin-spot signals on both chromosomes 14q32 are marked by arrows.

800 bp of intron 1 of the $B_2$ receptor gene. Clone B bears a 1.1 kb *Sac*I fragment located about 100 bp upstream of exon 1. The 1.9 kb *Eco*RI/*Hin*dIII fragment subcloned in clone C is located upstream of exon 3 and includes the putative $B_2$ receptor specific 5' regulatory region as proposed by Powell et al. (10).

In transient transfection experiments a gene regulatory function of clone B could clearly be shown (Fig.5). Clone A, although including clone B (Fig.2) showed an approximately ten-fold lower enhancement activity as compared with clone B alone. This decrease is likely to be caused by the presence of the exon 1 and parts of the intron 1 that might disturb the correct transcription start of the luciferase gene. Alternatively, an additional negative regulatory element in this fragment might exist. Determination of specific $B_2$ receptor binding at the surface of the cell line 293 showed only a low level expression of this protein under basal conditions. Therefore, it will be necessary to analyse if there is a tissue specific regulatory motif in the 3.9 kb *Xba*I/*Sac*I fragment that is absent in clone B. As expected, the strong RSV promoter of the positive control vector generated a luciferase activity that exceeded that of clone B about 80-fold (data not shown). The light intensity produced by clone C was not significantly higher than that produced by the negative control vector.

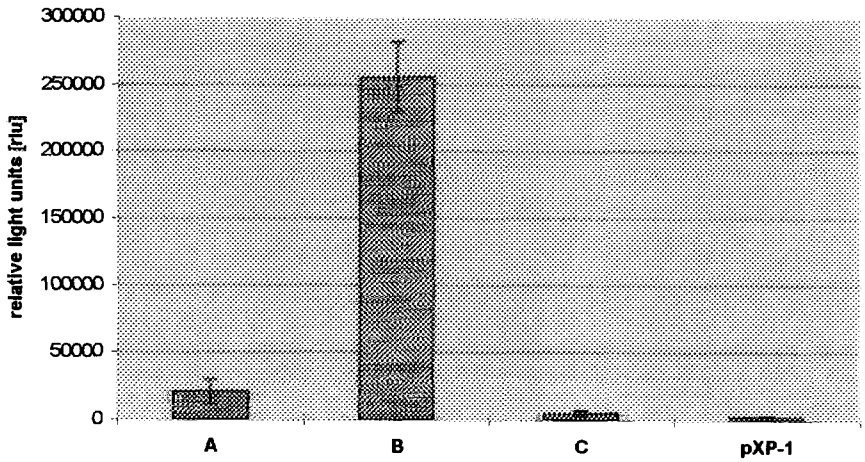



Fig.5. **Luciferase reporter gene assay for the human $B_2$ receptor regulatory region in the fetal kidney cell line 293.** The relative light units (rlu) represent a scale for the luciferase expression under the control of the different putative regulatory elements of the human $B_2$ receptor (fragments A, B and C, Fig.2). The vector containing no insert (pXP-1) indicates the background of the assay. The positive control construct pRSV, possessing a strong RSV promoter, generates a light emission of about 20 million relative light units (data not shown). The mean ± 1SD of three different experiments is shown.

Thus we prove that this fragment does not represent the 5' upstream promoter region as initially proposed by Powell et al. (10).

In accordance to data on the rat $B_2$ receptor gene (24), there are no typical TATA or CCAAT boxes in the putative promoter region of the human $B_2$ receptor gene. All assumptions on further transcription factor binding sites would be purely speculative. However, a comparison of the 5' upstream region in these two species revealed a homology of more than 80% in the first 85 bp upstream of the transcription start site determined for the rat $B_2$ receptor gene. Therefore, it is likely that this region represents the core promotor. The regulatory element in clone B is located immediately upstream of this region and shows a homology of approximately 50% with the equivalent region of the rat gene. Thus, this fragment could represent a promoter or enhancer element cooperating with the putative 85 bp core promoter.

Up to now very little is known about regulation of $B_2$ receptor expression. Indirect evidence appears to suggest that activation of adenylate cyclase by forskolin (25) and treatment with interleukin 1 (26) may enhance receptor expression. The identification of the regulatory region reported here will greatly facilitate a more detailed study of these and other influences on the transcriptional rate of the human $B_2$ receptor gene.

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