Structure of the bovine ET_B endothelin receptor gene

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The structure of the gene encoding the bovine type B endothelin receptor (ET_B) has been established and compared with those of other heptahelical receptors. The gene is present as a single copy in the bovine genome, as demonstrated by Southern blot analysis, and spans at least 36 kb. The coding region is divided into 7 exons separated by 6 introns, one of which is more than 23 kb in length. The exons correspond well to the structural domains of the receptor: the first exon encodes the first and second transmembrane domains, and each of the following transmembrane domains is encoded by a separate exon. The portion of the ET_B protein sequence encoded by exon 3 is quite different from the corresponding ET_A sequence, suggesting that this region is responsible for the distinct ligand specificities of the two receptor subtypes. The second intron interrupts the canonical Asp-Arg-Tyr sequence, which is located at the end of the third transmembrane domain of the heptahelical receptors, as with the substance P, substance K, dopamine D₂ and dopamine D₃ receptor genes. To map the 5' region of the gene and determine the start of transcription, primer-extended cDNAs were cloned and sequenced: multiple start sites were deduced with no apparent TATA box in the expected upstream region. Similar results were obtained by ribonuclease protection analysis.

INTRODUCTION

The presence of an endothelial-cell-derived vasoconstrictor peptide was first demonstrated by Hickey *et al.* [1] and later confirmed by O'Brien *et al.* [2]. Soon after the isolation of the substance (endothelin) by Yanagisawa *et al.* [3], it became apparent that the endothelin family has three members: endothelin-1, endothelin-2 and endothelin-3. Two types of receptors that mediate the smooth-muscle-contracting activity of endothelins have been identified and their primary structures determined by cDNA cloning and sequencing [4,5]. The receptor subtypes, ET_A and ET_B, have characteristic ligand specificities and patterns of tissue expression. For example, the ET_A endothelin receptor which binds endothelin-1 and endothelin-2 much more strongly than endothelin-3, predominates in arterial smooth muscle cells [6] and testicular myoid cells [7], whereas the ET_B receptor, which recognizes the three ligands equally, appears to be the major form in endothelial cells [8,9] and in the ureter [10]. Elucidation of the structural basis for this differential expression requires a detailed analysis of their genes.

Our interest in the endothelin receptor genes also stems from an evolutionary point of view. Both ET_A and ET_B receptors belong to the G-protein-coupled receptor family and have seven membrane-spanning domains. The members of this heptahelical receptor family can be divided into two groups in terms of their gene structure: one has introns, whereas the other is intronless. To elucidate which type of gene encodes the endothelin receptor, we carried out a structural and comparative analysis of the bovine ET_B receptor gene.

MATERIALS AND METHODS

Materials

Fresh bovine lung and adrenal gland were obtained from the Shibaura Abattoir Sanitation Inspection Station, Tokyo, Japan.

Restriction enzymes and T4 polynucleotide kinase were from Toyobo, Osaka, Japan; ribonuclease A was from Boehringer Mannheim, Mannheim, Germany; DNA ligation kit and random primer DNA labelling kit were from Takara, Kyoto, Japan; You-Prime cDNA synthesis kit, mRNA purification kit and double-stranded Nested Deletion kit were from Pharmacia, Uppsala, Sweden; λ ZAP II, Gigapack Gold *in vitro* packaging kit, pBluescript II and RNA transcription kit were from Stratagene, San Diego, CA, U.S.A.; Sequenase version 2.0 sequencing kit was from United States Biochemical Corp., Cleveland, OH, U.S.A.; a bovine genomic DNA library was from Clontech, Palo Alto, CA, U.S.A.; nitrocellulose filters were from Schleicher & Schuell, Dassel, Germany; Gene Screen Plus was from Du Pont–New England Nuclear, Boston, MA, U.S.A.; GeneAmp DNA amplification reagent kit was from Perkin–Elmer Cetus, Norwalk, CT, U.S.A.; ribonuclease T1 and Superscript MMLV reverse transcriptase were from Life Technologies, Gaithersburg, MD, U.S.A.; ³²P-labelled nucleotides were from Amersham International, Amersham, Bucks., U.K. Oligonucleotides were synthesized with a Milligen/Biosearch Cyclone Plus DNA synthesizer. DNA sequences were analysed using the computer software GENETYX purchased from Software Development Co., Tokyo, Japan.

Isolation of bovine ET_B receptor cDNA clones

To obtain cDNA clones of the bovine ET_B receptor covering the 5' end of the mRNA, a cDNA library was constructed with a specific primer. Poly(A)⁺ RNA was prepared from bovine lung as described previously [11] and reverse-transcribed with the synthetic oligonucleotide 5'-GTCCCCGGAAGTGGCTGAGT-3', corresponding to the 5' region of the partial cDNA clone bETR5 [11], using a Pharmacia You-Prime cDNA synthesis kit according to the directions provided. The double-stranded, blunt-ended and adaptor-ligated cDNA obtained was cloned into λ ZAP II. Approx. 6 × 10⁶ phage clones were screened with

Abbreviations used: ET_A, type A endothelin receptor; ET_B, isopeptide-nonspecific type B endothelin receptor.

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a random-primed labelled 127 bp *Bam*HI/*Xba*I restriction fragment of the previously cloned bETR5 [11] by the plaque hybridization method [12]. Positive clones were characterized and sequenced [12].

Isolation and characterization of bovine ET_B receptor gene

The bovine ET_B receptor gene was isolated from a bovine genomic library constructed in EMBL-3. Approx. 3×10^6 phage clones were screened with various random-primed labelled restriction fragments of bovine ET_B receptor cDNA (bETR5) [11]. Positive clones were purified, characterized by restriction mapping, subcloned into the pBluescript II vector and sequenced by the dideoxy chain-termination method [12] using Sequenase version 2.0. All exons and intron/exon junctions with adjacent intronic sequences were subjected to sequence analyses. The nucleotide sequence of 1000 bp of 5'-flanking DNA was also determined.

Genomic Southern blot analysis

Bovine genomic DNA (20 µg) prepared from cultured endothelial cells was digested with 200 units of *Eco*RI and *Hind*III, electrophoresed and blotted on to Gene Screen Plus by the standard method [12]. Hybridization was performed with a random-primed labelled 500 bp *Eco*RI/*Eco*RI fragment of λBerG1 as described previously [13].

Primer extension

A synthetic oligonucleotide corresponding to nucleotides -210 to -191 and -128 to -112 in the 5'-flanking region of the gene was labelled with [γ -³²P]ATP, and 2×10^5 c.p.m. was annealed to 2 µg of bovine adrenal poly(A)⁺ RNA for 16 h at 30 °C in 30 µl of hybridization buffer containing 40 mM-NaCl, 40 mM-Pipes, pH 6.4, 1 mM-EDTA and 80 % (v/v) formamide. The annealed RNA/primer mixture was precipitated and resuspended in 20 µl of 50 mM-Tris/HCl, pH 8.3, 75 mM-KCl, 3 mM-MgCl₂, 10 mM-dithiothreitol, 50 µg of actinomycin D/ml and 0.5 mM-deoxyribonucleotide triphosphate, and extended with 100 units of Superscript MMLV reverse transcriptase at 37 °C for 60 min. Remaining RNA was digested with DNAase-free ribonuclease A, extracted with phenol/chloroform and ethanol-precipitated. The precipitated extension products were resuspended in sample buffer and electrophoresed through a 5 % (w/v) denaturing acrylamide sequencing gel. The size of the extended product was determined by reference to sequence reactions run on the same gel.

RNAase protection analysis

A 315 bp *Hind*III/*Xba*I genomic fragment containing the 5'-flanking region of the gene was subcloned into pBluescript II. Antisense riboprobes were generated with T3 RNA polymerase in the presence of [α -³²P]UTP according to the specifications of the manufacturer (Stratagene). Bovine lung total RNA and the ³²P-labelled cRNA was hybridized in 30 µl of 40 mM-Pipes containing 80 % (v/v) formamide, 40 mM-NaCl and 1 mM-EDTA at 45 °C or 55 °C for 16 h. Non-annealed RNA was digested for 1 h at 30 °C with 20 µg of ribonuclease A/ml and 2 µg of ribonuclease T1/ml in 300 µl of digestion buffer containing 10 mM-Tris/HCl, pH 7.4, 300 mM-NaCl and 5 mM-EDTA, followed by digestion with proteinase K and ethanol precipitation. Protected fragments were analysed by electrophoresis.

RESULTS AND DISCUSSION

Isolation and mapping of genomic clones

A bovine genomic library in EMBL-3 was screened with bovine ET_B endothelin receptor cDNA and numerous positive

phage clones were obtained. Four overlapping clones, λBerG1, λBerG2, λBerG3 and λBerG4, were selected for mapping (Fig. 1) and analysis of intron/exon organization (Fig. 1c).

Intron/exon structure

The location of 7 exons of the ET_B receptor gene was determined by Southern hybridization analysis and DNA sequencing; the positions of splice junctions were deduced by comparing the nucleotide sequence of the gene with that of the receptor cDNA (Fig. 2). The gene, spanning more than 36 kb, is relatively large and composed of 7 exons and 6 introns, one of which is more than 23 kb. The other introns ranged from 130 bp to 4.2 kb. The exons varied in size from 109 to 2000 bp. The

Fig. 1. Restriction enzyme map and organization of the bovine ET_B receptor gene

(a) The four lines represent cDNA clones used to determine the 5'-flanking region. All clones were obtained from a specific-primed cDNA library using a synthetic oligonucleotide corresponding to bases 107-126 (arrow); however, two clones, bETR13 and bETR15, turned out to be primed non-specifically, a phenomenon often observed. (b) Schematic representation of the cDNA structure of the bovine ET_B receptor. Non-coding regions are shown by hatched boxes. Putative membrane-spanning domains are shown by closed boxes and are numbered I-VII. (c) The structure of the bovine ET_B gene. The exons are indicated by boxes and are numbered 1-7. Beneath the genomic map is shown the restriction enzyme map; *Eco*RI and *Hind*III sites are indicated by vertical lines. (d) The four lines labelled λBerG1, λBerG2, λBerG3 and λBerG4 represent the overlapping phage clones used to determine the gene structure.

Exon	Intron 3' junction	Exon size (bp)	MSD (%)	Intron 5' junction	Intron size (kb)	Amino acids interrupted
1	TCTA	712	I,II (70.0)	CAAG gtaagg	>23.1	Lys ¹ Leu -161
2	ctcaatgcag CTGC	113	III (78.9)	ACAG gtaaga	0.13	Ar g -199
3	tgttcttcag ATAT	205	IV (52.9)	GCAG gtaaat	1.7	Gln Phe -267
4	tcccccatag TTTT	150	V (68.0)	ACAG gtaagg	4.2	Gln Arg -317
5	ttatttcag AGAC	134	VI (77.8)	TAAG gtaagt	0.16	Se r -361
6	atttttcag CTTT	109	VII (77.8)	TAAG gtaaga	2.0	Lys Ser -398
7	ctttgttcag TCAT	>2000	(36.4)	TTTG		

Fig. 2. Intron/exon splice sites of the bovine ET_B receptor gene

The sizes of the introns and exons in bp and the nucleotide sequences around the splice sites are indicated. The putative membrane-spanning domains (MSD) in each exon are shown, and the percentages of identical amino acids in ET_A and ET_B are also indicated in parentheses, although the non-homologous N-terminal region in the first exon is omitted. We have demonstrated that this N-terminal region is not responsible for ligand specificity [28]. The sequences at the beginning of the first exon correspond to the major transcription start site determined by RNAase protection analysis.

intron/exon boundaries conform to the consensus splice sequences [14]. Two of the junctions split codons (type 2 introns [15]), whereas codons are preserved in the four remainders (type 0 introns).

Comparison of genomic and protein organization has revealed that there is considerable correspondence between individual exons and the functional domains of the protein. The exon structure determined here, therefore, may provide useful information for determining the ligand-specificity-conferring region by, for example, making chimaeric receptors of ET_A and ET_B; i.e. the exons or the locations of introns can effectively be used to determine the portions to be exchanged between the two receptor subtypes. We have thus included in Fig. 2 the amino acid sequence similarities of the portions encoded by the individual exons of ET_B compared with the corresponding regions of ET_A. The region corresponding to exon 3 showed the lowest similarity (52.9%), and therefore is the region most likely to be responsible for the ligand specificity. This point will be resolved by future studies using chimaeric constructions.

Nucleotide sequence of 5'-untranslated region and 5'-genomic flanking sequence

The nucleotide sequence upstream of the translation initiation site was determined from a *Bam*HI/*Xba*I subclone of the λ BerG3 genomic clone. Approx. 1000 bases upstream were sequenced (Fig. 3). The sequence of the 5'-untranslated region was determined exclusively from the genomic clones for bases -1019 to -148, and from the genomic and cDNA clones for bases -147 to -1. When the 5'-untranslated region of the genomic clone λ BerG3 was sequenced, it turned out to be completely different from the previously reported cDNA sequence [11] at nucleotide -148 and further upstream. The sequence surrounding nucleotide -148 did not fit the consensus of the splice site. We suspected that the difference might result from an artifact arising by rearrangement of DNA sequences during construction of the cDNA library, and sought additional cDNA clones. New clones containing a longer 5'-region of the cDNA sequence were obtained by screening a newly prepared specific-primer-extended bovine lung cDNA library. Four independent clones were sequenced; the longest clone, bETR15, extended 269 additional bases in the 5' direction from position -148. The nucleotide sequence of the extended region was consistent with that of the genomic clone. The previously published cDNA sequence was therefore revised in its 5'-untranslated region, deleting the sequence corresponding to nucleotides 1-924 of the previous clone bETR5 [11].

The above specific-primer-extended cDNAs may also help to determine the start site of transcription. Three out of the four cDNAs had similar 5'-termini (Fig. 1a) which correspond to bases -239, -232 and -226, suggesting that transcriptional initiation begins close to these sites. To confirm this, RNAase protection analysis was performed. Bovine lung total RNA was hybridized to a radiolabelled 352 bp antisense cRNA probe corresponding to exon 1 and sequence upstream from this exon, and digested with ribonuclease A and T1. In complete agreement with the result of the primer-extended cDNA analysis, multiple protected fragments were recovered (Fig. 4), which located the transcription initiation site at positions -237 to -229. The hybridization conditions we used were stringent [55 °C in 80% (v/v) formamide] and this pattern was reproducible with two different preparations of bovine lung total RNA. No apparent TATA box was found in the promoter region (-350 to -260; Fig. 3).

In addition to these major sites there must be an additional upstream transcription start site(s), since we obtained, although as a minor species, a longer cDNA (bETR15, Fig. 1a) and the

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GGGATCCCGAGCAAACTGCGAGGCCACACTGTGAGGCATTCCTCGGCGTTTCGTGAGA -956
GCCAGACCTCCCGCTGCTGAAGGAGGCGCGCTGCTCCCGGCTGCCGGGAACCCAG -896
CCGCGCGCCGCGGGAAGGAGGTCCTGCTGCTGCTGCTGCGGACCAAGAGGTAACCGTTCTG -836
CTTGCTGTTAAATTCAAATCTGTGATTGAACCTTATCTGGGCTTCAGTTGGATTACT -776
TCTTAGGTTTGTGTTTGTGTTTGTGTTTGTGTTTAACTGTAGCTACAGCAGATTAAAA -716
TGGGATTGGAGTGAAGGAGAGGCTTGAGTGTCTGAAGTCACTCGAAGGGGATAGAACT -656
TCCGAGTTAATCCAGATAGAGACTCTTCCAATAGACCAAGTGGAGATGCTCCGGGAGATT -596
      MyoD
TCCGAGTCAGCCGGCTCAGTCTCGGGTGTGTATGGGGGTGGGGCGGTCGCCAAG -536
      Sp-1
AGCGGAAGGAAGGGGTCTGAAAGTCCAGACATCGGCATCCGGGTTGCTGTGGAGTCGA -476
AAGCAGAGACTGGCGATGCCCTTGATCTCTAACCTGCCTTGATTGCCCGTCCCGCGC -416
GCCCTTGGCCAGAACAACTAGTCTCCTCTGCGCACACAGGAGCTCAAAGCTTTCCT -356
TGGACACCGTCTCTCCTCCCGTCACACCCCTTCAGGATACCCGCGCCACTGCGAG -296
      Sp-1
TCATTACCCCTCAGGGGACGCGGGGAGGAAAAACAGCGAAAGCTGGCTCTGGAGGA -236
      ↓
CGGTCATGGGCGCAAGCGAGTCCCACGGCCAGAGTTCCCACTCCCGGCCAGCTCGA -176
      ↓
CTTATTAATACTTTTGGCAGTGAAGTGGGGGCTGCTGCGCTTGAGGATAGCGGCTT -116
GCAGGGGCGTGGCTGGGACGCTGACCCGGGTGCTGTCTTCTTCCCTTGTGCTGCTC -56
      +1
TAGACTCGGAAAGCGGAGGAGCGGCCACCCGACTCCCTCTGGAGCAGGACAGCCTG +3

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Fig. 3. Nucleotide sequence of the 5'-region of the bovine ET_B receptor gene

Numbers refer to nucleotide position relative to the initiation ATG codon. Arrows represent 5'-termini of cDNA clones obtained from a specific-primed cDNA library. The closest AT-rich sequence to these 5'-termini and possible CAATT sequence are boxed. The other AT-rich sequences are underlined, and Sp-1 and MyoD sequences are underlined and indicated. The ATG start codon is shaded.

corresponding cRNA-mRNA hybrid was protected in the RNAase protection analysis (Fig. 4). However, it is not clear whether the upstream start site is located close to the 5' end of the longest cDNA clone bETR15 or further upstream because, in primer extension analysis using the primers corresponding to bases -210 to -191 and -128 to -112 (Fig. 3), the appearance of specific transcripts was influenced by the locations of the primer binding region, probably due to a stem-and-loop structure in the bovine ET_B mRNA.

Southern blot analysis of total bovine genomic DNA

To estimate the copy number of the bovine ET_B receptor gene, high-molecular-mass DNA extracted from bovine cultured endothelial cells was digested separately with two restriction enzymes, electrophoresed on a 1% (w/v) agarose gel, transferred to a nylon membrane and probed under stringent conditions with the radiolabelled *Eco*RI/*Eco*RI fragment derived from exon 7. As shown in Fig. 5, the probe hybridized to single genomic restriction fragments whose sizes agree with those predicted from the restriction map of the genomic clone λ BerG1 (Figs. 1 and 5), suggesting that the ET_B receptor gene is present as a single copy in the bovine genome.

Fig. 4. RNAase protection analysis of bovine lung RNA to determine the cap site

The cRNA riboprobe was designed as depicted in (b). The probe contained 315 bases of 5'-flanking region and exon 1 (open bar), and vector sequence (closed bar; V). Thick black lines represent protected fragments. cRNA was hybridized to total bovine lung RNA (two different preparations) at the indicated temperature, digested with ribonuclease A and ribonuclease T1, and protected fragments were separated on a denaturing acrylamide urea gel (a). Major and minor protected fragments are denoted by arrowheads. Nucleotide positions were determined by standard sequence ladder.

Comparison of the ET_B gene with other intron-containing heptahelical receptor genes

Many G-protein-coupled receptor genes lack introns; in fact, until 1990, only two exceptions were known: the genes for opsin [16] and the dopamine D₂ receptor [17,18]. However, a number of precedents now exist for the presence of introns within the coding region of such receptor genes [19]. Fig. 6 compares the splice sites of the heptahelical receptors encoded by the intron-containing genes. The splice sites occur at or near the borders of putative membrane-spanning domains. Similar cases have been reported for other membrane proteins such as the atrial natriuretic peptide receptors [13], the Cl⁻/HCO₃⁻ exchanger [20], the nicotinic acetylcholine receptor [21] and glucose transporters [22]. It is noteworthy that the second intron interrupts the canonical Asp-Arg-Tyr sequence at the end of the third transmembrane domain, since similar interruptions have been demonstrated in the case of substance P [23], substance K [24], dopamine D₂ [25,26] and dopamine D₃ [27] receptor genes. The location of the sixth intron of the bovine ET_B receptor gene is unique in that it interrupts the coding sequence at the external

Fig. 5. Southern blot analysis of the bovine ET_B receptor gene

Bovine genomic DNA (20 μg) prepared from cultured endothelial cells was digested with *Eco*RI (E) or *Hind*III (H) and hybridized with a restriction fragment containing an open reading frame in the seventh exon, as indicated at the bottom of the figure. The open box and closed box represent the C-terminal intracellular region and the 3'-non-coding region in the seventh exon respectively. Size standards are indicated on the left.

Fig. 6. Comparison of splice sites of heptahelical receptors

The locations of intron/exon splice sites of the bovine ET_B receptor (ET_BR) gene are compared with other members of the heptahelical receptor family including substance P receptor (SPR) [23], substance K receptor (SKR) [24], dopamine D₂ receptor (D₂R) [25,26], dopamine D₃ receptor (D₃R) [27], bovine opsin [16] and luteinizing hormone receptor (LHR) [29,30]. Arrows indicate the location of introns. The upper portion of the figure is a schematic representation of the coding region with the membrane-spanning domains, which are shown as hatched boxes and numbered I–VII.

border of the seventh transmembrane domain, where no introns occur in the other members of the family so far analysed.

Accumulation of information on the gene structure of the heptahelical receptors will help us to understand how this family of genes evolved through duplication, recombination, loss or insertion of introns and nucleotide substitution. The present study will also provide a starting point for the analysis of the mechanism of regulation of ET_B gene expression.

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