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MOLECULAR CLONING OF A NON-ISOPEPTIDE-SELECTIVE HUMAN ENDOTHELIN RECEPTOR

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Summary: We isolated several complementary DNA (cDNA) clones encoding a non-isopeptide-selective human endothelin receptor (ET_BR) from a human placenta cDNA library. The clones, different in the length of their 3'-untranslated regions, encoded the same 442-amino acid protein with a transmembrane topology similar to that of other G protein-coupled receptors. The rank order of the binding of ET isopeptides (ET-1, ET-2 and ET-3) to the receptor expressed in COS-7 cells was ET-1 = ET-2 = ET-3. Northern blot analysis identified three mRNA species, 4.3 kb, 2.7 kb and 1.7 kb in size, probably generated by their use of alternative polyadenylation sites. These mRNAs were expressed in a wide variety of human tissues, at the highest level in the brain and at a significant level in cultured endothelial cells.

Endothelin (ET), a 21-amino acid endothelium-derived peptide first identified by Yanagisawa et al., induces a potent vasoconstriction in a wide variety of vascular beds (1) and serves as an important modulator of vascular tonus. Subsequently, analysis of the human ET gene has predicted the presence of a family of three distinct peptides (ET-1, ET-2 and ET-3) , which exhibit different pharmacological profiles *in vivo* and *in vitro* (2), suggesting the existence of multiple ET receptor (ETR) subtypes.

We have recently succeeded in isolating a complementary DNA (cDNA) encoding an ET-1-selective bovine ETR (ET_AR) using the *Xenopus* oocyte expression system coupled with the voltage clamp method (3) and Sakurai et al. have described a non-isopeptide-selective rat ETR (ET_BR) (4), both of which belong to a superfamily of G protein-coupled receptors. More recently, we have cloned a cDNA encoding human ET_AR (5). Here we have isolated several cDNA clones for a non-isopeptide-selective human ET_BR from a human placenta cDNA library under low-stringency conditions with a bovine ET_AR cDNA fragment as a probe.

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Abbreviations: ET; endothelin, ETR; endothelin receptor.

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MATERIALS AND METHODS

Molecular cloning: A human placenta cDNA library was constructed in bacteriophage λ ZAPII (Stratagene, La Jolla, CA) (6) and approximately 10^6 plaques were screened under low-stringency conditions with ^{32}P-labelled 0.96-kilobase (kb) NcoI-EcoRI restriction fragment of bovine ET_AR cDNA as a probe (3). Plaque hybridization was carried out for 18 hr at 65°C in a solution containing 1 % SDS, 1 M NaCl, 10 % dextran sulfate, 200 μg/ml yeast tRNA and 250 μg/ml denatured salmon sperm DNA. The membranes were then washed twice (30 min x 2) in 0.5 x SSC (1 x SSC is 0.15 M NaCl, 15 mM sodium citrate [pH 7.0]), 0.1 %SDS at 50°C. The hybridization-positive clones were purified and rescued as Bluescript plasmids utilizing the in vivo excision process of λ ZAPII. DNA sequencing was performed by the dideoxy nucleotide chain termination method (7) and was confirmed by reading both DNA strands.

Transfection of DNA to COS-7 cells: The 2.7-kb XbaI restriction fragment of pHETBR34 (Fig. 1) was subcloned into the expression vector CDM8 (8) (CDM8-pHETBR). COS-7 cells were transfected with 20 μg of DNA/100-mm plate, using the calcium phosphate method (9). One day after the transfection, cells were harvested from 100-mm plate and seeded at 5 x 10^4cells/24-well culture plate (Corning, Corning, NY) for binding assay.

Competitive binding assay: [^{125}I] ET-1 (2000 Cimmol^{-1}) was purchased from Amersham (Buckinghamshire, UK). Transfected cells were incubated for 60 min at 37°C in 0.5 ml of Hank's balanced salt solution containing 0.1 % bovine serum albumin with 50 pM of [^{125}I] ET-1 plus various concentrations of unlabelled ET-1, ET-2 and ET-3 (Peptide Institute Inc, Minoh, Japan). Following the incubation, cells were solubilized in 0.5 ml of 1 N NaOH and associated radioactivity was measured by an autogamma counter (Aloka, Tokyo, Japan). Background binding was determined in the presence of 10^{-6}M unlabelled ET-1.

Northern blot analysis: Human tissues obtained at autopsy or operation, were frozen in liquid nitrogen, and stored at -70°C until use. Informed concent was obtained from each patient's family. Human umbilical vein endothelial cells were purchased from Colonetics Corp.(San Diego, CA), and cultured as previously reported (10). RNA was extracted in 4 M guanidinium thiocyanate buffer (6). Using ^{32}P-labelled 1.2-kb EcoRI restriction fragment of pHETBR34 (Fig. 1) as a probe, Northern blot analysis was carried out as described (3,6).

RESULTS AND DISCUSSION

Isolation and sequence determination of cDNA clones

A total of 20 clones were obtained by plaque hybridization under low-stringency conditions with the above-mentioned probe, three of which gave positive signals even under high-stringency. These were idetified as human ET_AR cDNA clones and will be reported elsewhere (5). Of the isolated 17 clones, representative 4 clones, which covered the entire coding region, were characterized. Restriction endonuclease mapping revealed that all the clones fell into the same group, which were different in the length of their 3'-untranslated regions (Fig. 1). The nucleotide and deduced amino acid sequences of two overlapping clones (pHETBR31 and pHETBR34) are presented in Fig. 2.

The 4,286-basepair (bp) nucleotide sequence included an open reading frame of 1,329 bp, predicting a protein of 442-amino acid residues with a relative molecular mass of 49,629. The nucleotide sequence around the putative initiation codon fits the consensus sequence for eukaryotic translation initiation sites (11) In the 3'-untranslated region, there were 12 copies of

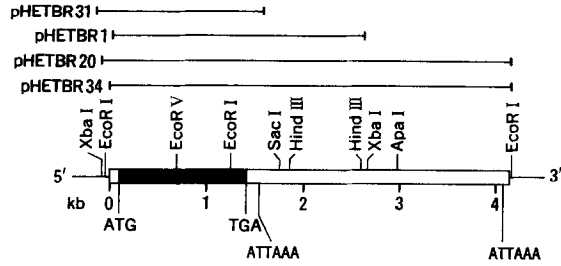


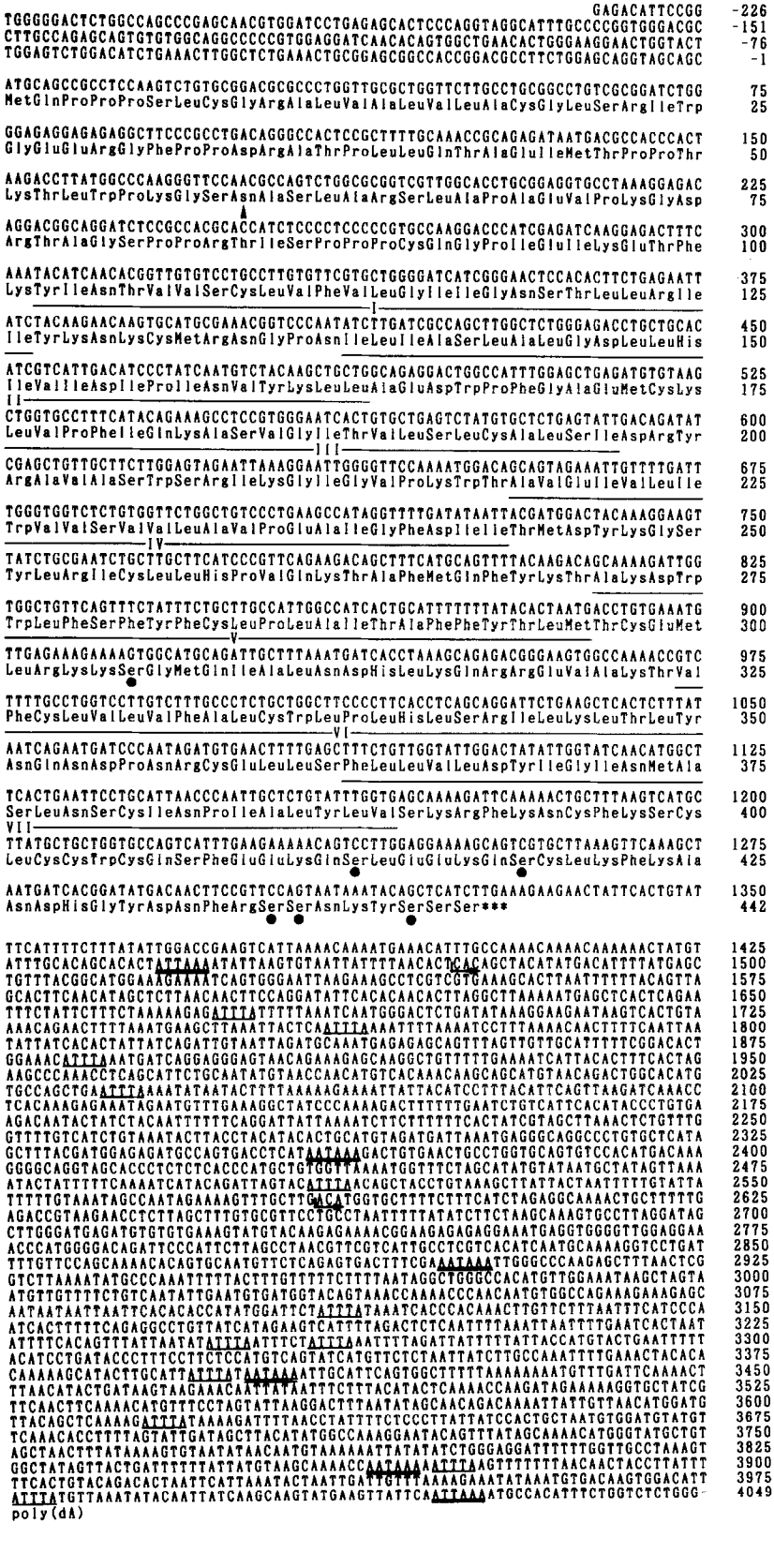
Fig. 1. Restriction endonuclease mapping of human ET_BRcDNAs. The four overlapping clones (pHETBR1, pHETBR20, pHETBR31 and pHETBR34) are indicated by solid bars above the restriction mapping of pHETBR34. The box denotes the insert of pHETBR34 and the flanking vector regions (Bluescript) are also depicted by solid horizontal bars. The coding region for human ET_BR is indicated by the closed box. The location of the initiation codon ATG, the termination codon TGA and the putative polyadenylation signal ATTAAA are also marked.

ATTTA motif implicated in mRNA instability (12), all of which occurred downstream from the polyadenylation site of the shortest clone (pHETBR31). The 3'-untranslated region contained the typical polyadenylation sequence, AATAAA, and its variants (13)(Fig. 2). Two clones (pHETBR20 and pHETBR34) with approximately 4.1-kb inserts and one clone (pHETBR31) with an approximately 1.6-kb insert contained ATTAAA sequence at 22 bp and 26 bp upstream from each polyadenylation site, respectively. One clone (pHETBR1) with an approximately 2.6-kb insert was isolated, which had no putative polyadenylation signal.

Hydropathicity analysis of the protein predicted the presence of seven stretches of 22-27 hydrophobic amino acid residues, suggesting that it has seven membrane-spanning domain, an extracellular N-terminus and a cytoplasmic C-terminus. Thus, the protein belongs to a superfamily of G protein-coupled receptors. The extracellular N-terminal region contained one potential N-glycosylation site. The third cytoplasmic loop and intracellular C-terminal region had serine residues which may be phosphorylated by serine/threonine kinase (14)(Fig. 2).

The deduced amino acid sequence of the protein was 88 % and 55 % identical to those of rat ET_BR (4) and human ET_AR (5), respectively, suggesting that the isolated cDNA clones encode a non-isopeptide-selective human ET_BR (Fig. 3). The length of the putative extracellular N-terminal regions was different between human ET_BR and human ET_AR (101 residues versus 80 residues). Between human ET_BR and rat ET_BR, there was a high degree of sequence homology within

Fig. 2. The nucleotide and deduced amino acid sequences of two overlapping clones (pHETBR31 and pHETBR34). The putative transmembrane domains I-VII are underlined. One potential N-glycosylation site and serine residues potentially phosphrylated are indicated by an arrowhead and circles, respectively. In the 3'-untranslated region, the ATTTA sequences implicated in mRNA instability are underlined and the putative polyadenylation signals, ATTAAA and AATAAA, are doubly underlined. Polyadenylation sites of the two shorter clones (pHETBR31 and pHETBR1) are indicated by arrows.



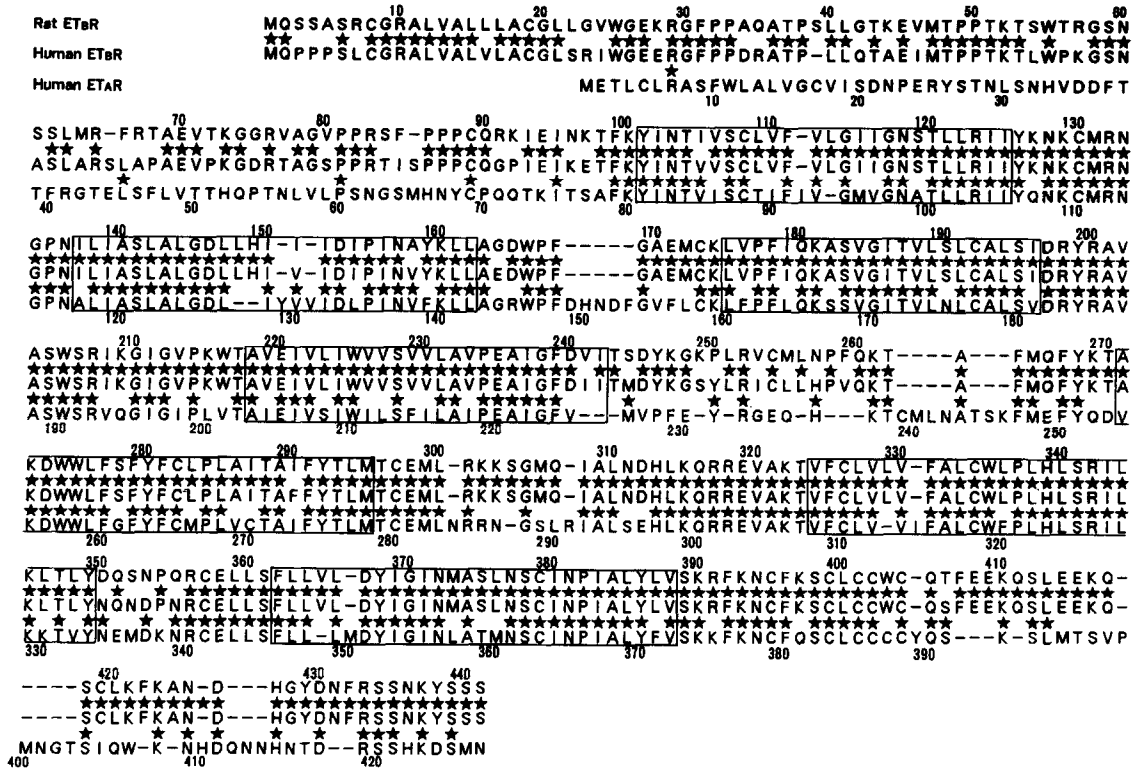


Fig. 3. Alignment of the deduced amino acid sequences of rat ET_BR, human ET_BR and humanET_AR. Identical amino acid residues are marked by asterisks. Dashes are inserted to maximize homology. Sequences of rat ET_BR and human ET_AR are from refs. 4 and 5, respectively.

transmembrane domains (97 %) and three intracellular cytoplasmic loops (112 residues identical out of 113 residues). In the extracellular N-terminal region of human ET_BR, there was one potential N-glycosylation site (Fig. 2), while two were predicted in rat ET_BR (4). However, all the serine residues potentially phosphorylated were conserved between these two species (see Fig. 2 and Ref. 4).

Expression of the receptor in mammalian cells

To further characterize the ligand specificity of the receptor, human ET_BR in mammalian expression vector (CDM8-pHETBR) and control plasmid (pCDM8) were transiently expressed in COS-7 cells, which were then assayed for ET isopeptide binding. Fig. 4 presents the result of competitive binding assays. Cells transfected with CDM8-pHETBR specifically bound [^{125}I]ET-1 and unlabelled ET-1, ET-2 and ET-3 equally competed for its binding (IC_{50} was approximately 1.0 X 10^{-9}M), which is consistent with the ligand specificity of rat ET_BR (4). No specific binding was observed in cells transfected with pCDM8 (data not shown). Together with high sequence homology between the receptor and rat ET_BR, this result indicates that the receptor cloned in the present study is human ET_BR.

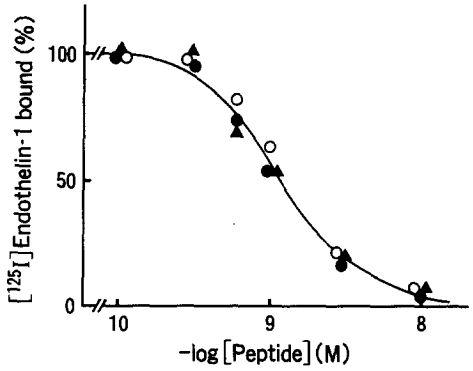


Fig. 4 Competitive binding of [^{125}I]ET-1 to COS-7 cells transfected with CDM8-pHETBR. [^{125}I]ET-1 (50 pM) was incubated with transfected cells in the absence or presence of various concentrations (10^{-10}-10^{-8}M) of ET-1(●), ET-2 (○) and ET-3(▲). Results are expressed as the percentage of specific [^{125}I]ET-1 binding and values are the means of triplicate determinations.

Tissue distribution of the receptor mRNA

To determine the expression of human ET_BR mRNA in different human tissues, we performed Northern blot analysis (Fig. 5). We detected two mRNA species with sizes of approximately 4.3 kb and 1.7 kb in total RNA from various human tissues. Using poly(A)$^+$RNA from the human placenta, we could detect a third mRNA band of approximately 2.7 kb in size. The sizes of ET_BR transcripts estimated from the Northern blot analysis roughly corresponded to those of the isolated cDNA clones (Fig. 1), suggesting that these mRNA species are generated by their use of alternative polyadenylation sites. Since the number of ATTTA motif in the 3'-untranslated region varies among the mRNAs, they could have different half-lives.

The two major forms of mRNA (4.3 kb and 1.7 kb) were expressed in a wide variety of human tissues, at the highest levels in the human cerebral cortex and cerebellum and at moderate levels in the placenta, lung, kidney, adrenal, colon and duodenum. Thus, the distribution of human ET_BR mRNA is very similar to that of rat ET_BR mRNA (4), and overlaps with that of human ET_AR mRNA (5). The rank order of the mRNA level, however, is different between ET_BR and ET_AR in human tissues. We could also detect significant amounts of human ET_BR mRNA in the aorta and cultured endothelial cells from the human umbilical vein. By contrast, in analysis on ET_AR mRNA in human tissues (5), the highest level of mRNA was detected in the aorta, while no appreciable amount was present in cultured endothelial cells. These results indicate that human ET_BR is expressed in vascular endothelial cells, possibly mediating the release of prostacyclin and endothelium-derived relaxing factor (15). Cloning of two human ETR subtypes (ET_AR and ET_BR) will facilitate our better understanding of clinical implication of the endothelin system in humans.

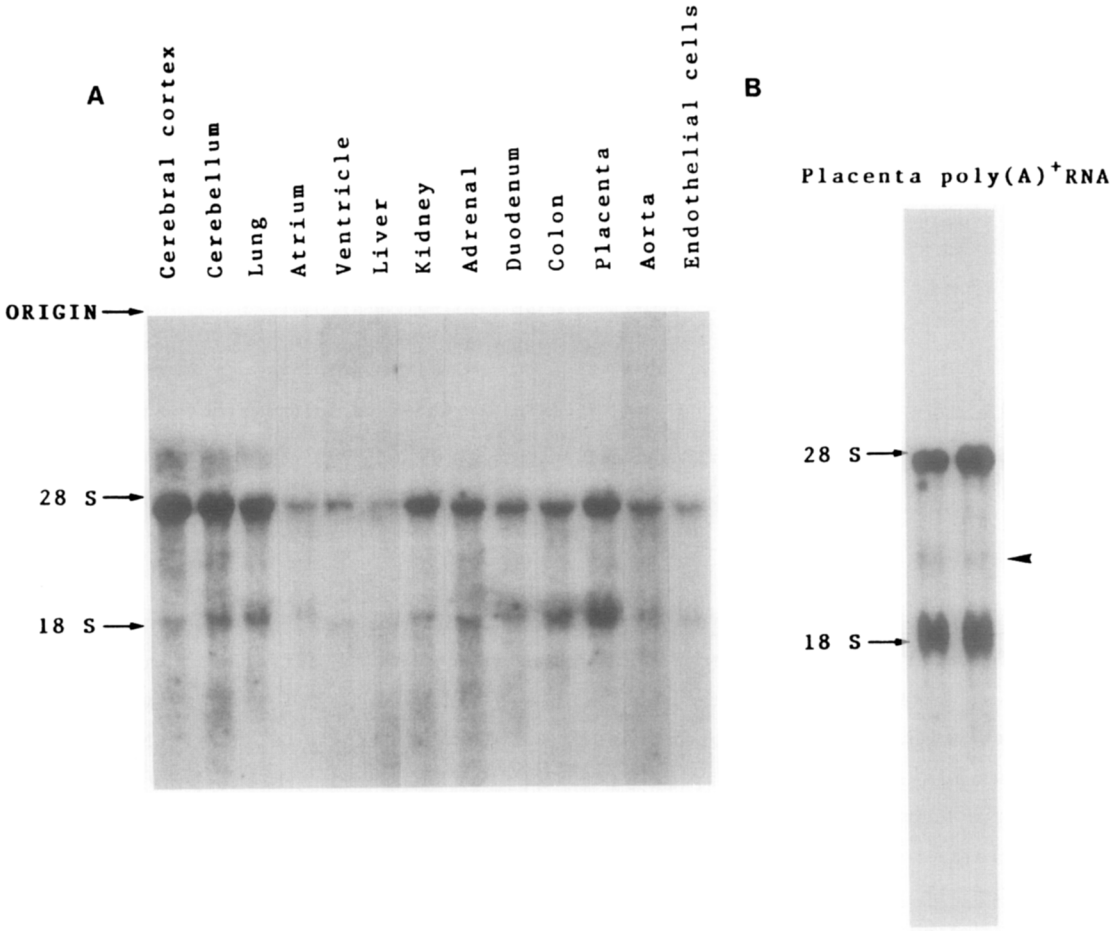


Fig. 5. Northern blot analysis of human ET_BR mRNA. Total RNA (20 μg) from various human tissues (A) and poly(A)$^+$RNA (10^2 μg) from two separate samples of the human placenta (B) are analyzed. A third mRNA of approximately 2.7kb in size is indicated by an arrowhead (B).

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