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CLONING AND CHROMOSOMAL LOCALIZATION OF A HUMAN ENDOTHELIN ETA RECEPTOR

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Summary A cDNA clone encoding a human endothelin receptor was isolated from a placenta cDNA library. The deduced amino acid sequence of the clone is 94% identical to the bovine endothelin ETA receptor and represents the human homologue. The human endothelin ETA receptor gene was localized to chromosome 4 by analysis of its segregation pattern in rodent-human hybrids.

Endothelin is a twenty-one amino acid peptide originally isolated from porcine aortic endothelial cells and is a potent endogenous vasoconstrictor (1). Cloning and sequencing analysis showed that there are at least three genes encoding the endothelin family (2). One of these genes codes for endothelin 1 (ET1) and the other two genes code for peptides of the same length designated as endothelin 2 and 3 (ET2 and ET3). This family of proteins is remarkably similar to the sarafotoxins, a group of snake venoms derived from the Israeli burrowing asp (*Atractaspis engaddensis*) (3). The sarafotoxins are one of the most potent venoms known.

Two ET receptor subtypes have recently been cloned and sequenced. The receptors are members of the rhodopsin-like superfamily of receptors that have seven putative transmembrane domains and are coupled with G-proteins for signal transduction. One ET receptor subtype was cloned from a rat lung cDNA library and human placenta library (4,5). Displacement experiments performed on the expressed endothelin receptor revealed similar affinities for ET1, 2 and 3. This receptor subtype has been

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Abbreviations used in this paper: Endothelin: ET; ETA: endothelin type ETA.

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designated ETB. The endothelin receptor subtype cloned by Arai et al. (6) from a bovine lung cDNA library utilizing a *Xenopus* oocyte expression system has the highest affinity for ET1 with a Kd of 0.18 nM. This ET receptor subtype has been named ETA (6). More recently, a rat ETA receptor has been cloned from a rat heart cell line and is over 90% similar to the bovine sequence (7). The rat and bovine sequences differ most widely at the amino terminus. A cloned human endothelin receptor could make screening for specific human endothelin antagonists much easier. In addition, a human clone can be used to probe the role of endothelin receptor in disease states. Here we describe the cloning, sequencing and chromosomal localization of a human ETA receptor isolated from a human placenta cDNA library.

Materials and Methods

Cloning technique: A probe for the human endothelin receptor was produced using polymerase chain reaction amplification. Two oligonucleotides (20 bases each) were produced from conserved regions corresponding to the bovine ETA sequence. The sense oligonucleotide was produced starting from base number 296 and the antisense was produced starting from base 830. The template DNA was cDNA produced from human placenta mRNA using a kit from Invitrogen. The resulting PCR product was 534 base pairs and was subcloned into pBluescript (Stratagene) (8). The insert (ET8) was sequenced for verification and removed from the plasmid using restriction enzymes BamHI and EcoRI. ET8 was labelled with a-32P-CTP by random priming and used to screen human brain and placenta libraries. Plaque hybridization was carried out at 55°C overnight in 6xSSPE (1xSSPE is 0.15M NaCl, 0.1M NaH_2PO_4, 0.001M EDTA, pH 7.4) and blotto (0.2% nonfat dried milk) and the membranes washed once at room temperature with 6xSSC, 0.1% SDS and twice at 65°C with 0.1xSSC and 0.1% SDS (8). Positive clones were purified and inserts removed using EcoRI, subcloned into pBluescript and subjected to didioxy double stranded sequencing from both ends (9).

Cells: Isolation, propagation and characterization of most somatic cell hybrids used in this study have been described elsewhere (10). Many of the hybrids were obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, N.J.). Chromosome regions retained by each hybrid are diagrammed in Fig. 3.

Southern Blot Analysis: Hybrid and control DNAs were prepared by cell lysis, proteinase K digestion, phenol extraction, and ethanol precipitation. Cellular DNAs were digested with restriction enzyme EcoRI, fractionated on 0.8% agarose gels, transferred to nylon membrane and hybridized at 42°C for 16 hours in 50% formamide, 5x SSPE, 5X Denhardts solution, 0.1% SDS and 100 ug/ml salmon sperm DNA. ET8 probes (see above) for filter hybridizations were radiolabelled by nick translation with a-32P-CTP to a specific activity of 10^8 cpm/0.1 ug and 10^8 cpm were used for each filter hybridization.

Results and Discussion

10^6 clones from each of three libraries (human brain stem, caudate, and placenta) were screened using a humanized PCR probe specific for the endothelin ETA receptor (see methods). Positive clones were obtained from human brain stem, caudate, and placenta libraries. The positive clones from brain stem and caudate were not full length and repeated screenings did not produce a full length clone from these libraries (data not shown). All three clones had an internal EcoRI site and were subcloned into pBluescript for sequencing. The nucleotide sequence of the full length placenta clone is shown in figure 1. The cDNA consists of 1661 nucleotides with a coding region of 1263 nucleotides. The sequences of the partial clones were identical to the full length clone obtained from the placenta library. However, the 5' end of the caudate clone started at base 380 of the placental clone and the 5' end of the brain stem clone started at nucleotide 504 of the placental clone.

The placenta cDNA sequence includes an open reading frame of 1263 bases which would encode a protein of 421 amino acids with a relative

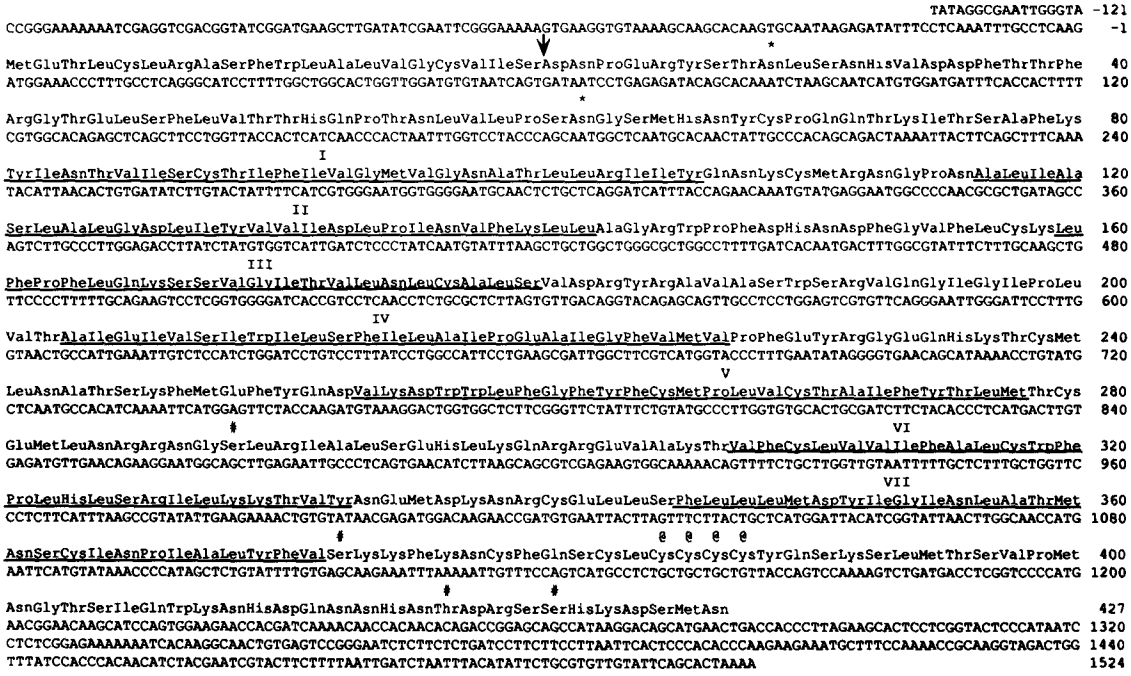

Fig. 1. The nucleotide sequence and deduced peptide sequence of the human endothelin ETA receptor. The arrow denotes a potential cleavage site of a hydrophobic signal sequence. Predicted hydrophobic transmembrane regions are underlined. Potential N-linked glycosylation sites are denoted by * and predicted protein kinase C phosphorylation sites by #. Possible palmitoylation sites are marked by @.

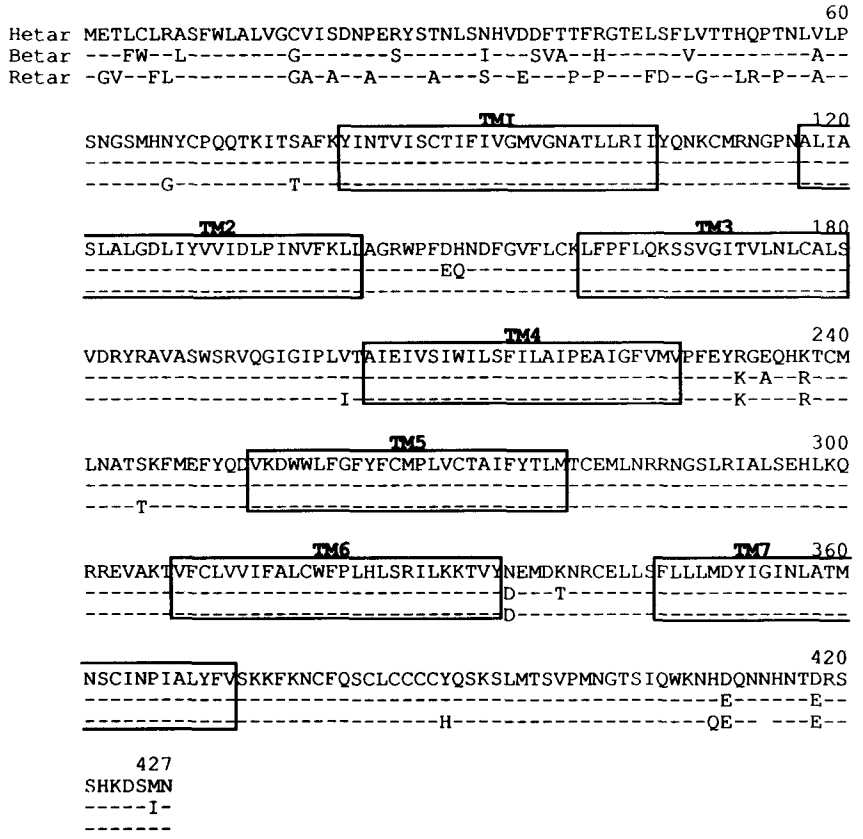

Fig. 2. Alignment of deduced human, bovine and rat ETA receptor protein sequences. The seven transmembrane regions are denoted by boxes. Amino acids identical to the human sequence are denoted by a dashed line (-). The sequences of bovine and rat ETA receptor sequences are from ref 6 and 7, respectively.

molecular mass of 48,689 (Fig. 1). This protein appears to have seven transmembrane regions predicted by hydrophobicity analysis (11) and has a number of motifs found in the bovine and rat ETA receptors (6,7). There is a long N-terminal domain that includes a potential signal sequence and two potential N-linked-glycosylation sites (11). A signal sequence has been detected in very few G-protein coupled receptors (14). The C-terminal domain has a group of four cysteine residues at least one of which may be palmitoylated in a manner similar to rhodopsin and beta-adrenergic receptor (12,13). There are three predicted protein kinase C phosphlorylation sites in the C-terminus and one in the third cytoplasmic domain between transmembrane regions five and six (11).

A comparison of the human, bovine and rat ETA receptor protein sequences is shown in Fig. 2. The human protein sequence is 94.6% identical to the bovine sequence and 92.7% identical to the rat sequence. The N-terminal portion of the receptors represents the area of least

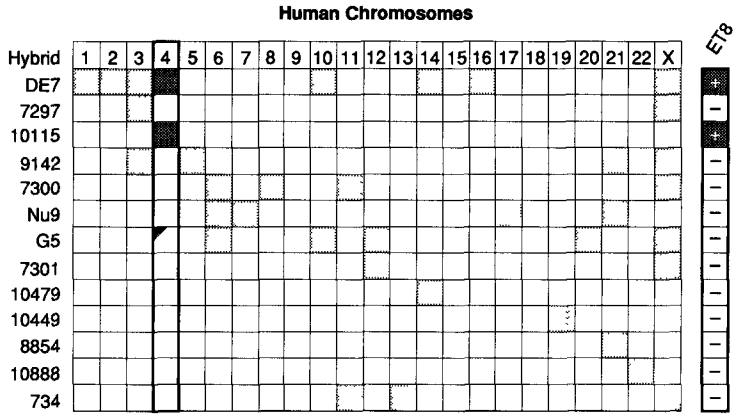

Fig. 3. Presence of the ET8 gene in 13 rodent-human hybrids. A completely stippled box indicates that the hybrid named in the left column contains the chromosome indicated in the upper row; lower-right stippling indicates presence of the long arm (or part of the long arm, indicated by a smaller fraction of stippling) of the chromosome shown above the column; upper-left stippling indicates the presence of the short arm (or partial short arm) of the chromosome listed above the column; no stippling indicates the absence of the chromosome listed above the column. The column for chromosome 4 is boldly outlined and stippled to highlight correlation of presence of this chromosome (or region of chromosome) with presence of the probe. The pattern of retention of the ET8 gene in the panel is shown in the column to the right of the figure where presence of the probe in the hybrid is indicated by a stippled box with a plus sign and absence of the probe is indicated by an open box enclosing a minus sign.

homology between the different species, suggesting that it may not be involved in ligand binding. The human and bovine sequences have the same number of amino acids while the rat sequence has one residue deleted near the C-terminus relative to the human and bovine sequences.

More than 20 rodent-human hybrids were tested for the presence of the ET8 locus by hybridization of a radiolabelled human ET8 probe to EcoRI cleaved hybrid and control DNAs immobilized on nylon filters. Results of testing of the entire panel demonstrated that the endothelin receptor locus was present only in hybrids retaining chromosome 4 and was absent in all hybrids which did not contain chromosome 4, as summarized in Fig. 3.

The significance of the linkage of the endothelin A receptor to chromosome 4 is difficult to gauge without a narrower regional localization. There are several chromosome 4-linked disease loci such as Huntington's at 4p16, Dentiogenesis imperfecta at 4q12-23, Williams syndrome at 4q33-qter (15). Since one of our hybrids which was negative for the ETA receptor locus was positive for the craf-2 probe at 4p16 (K. Huebner, unpublished), it is unlikely that the ETA receptor locus is near the Huntington's locus. Several other receptor genes (KIT and PDGFRA on 4q) and growth factor genes (FGF5, EGF, FGFB, IL2) have been regionally

localized on 4q (15) and it will be of interest to determine if the endothelin A receptor is near any of these loci.

In conclusion, a human endothelin ETA receptor has been cloned and mapped to human chromosome 4. The sequences of the partial clones obtained from human brain libraries suggest that the sequence of the ETA receptor expressed in the brain is identical to the ETA receptor expressed in the placenta. The human ETA receptor can be used to analyze the role of endothelin receptors in disease states and for the analysis of antagonists specific for the human endothelin ETA receptor.

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