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Cloning and expression of human endothelin-1 receptor cDNA

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We isolated a human endothelin-1 (ET-1) receptor cDNA from a human placenta cDNA library. The cDNA encodes a 427-amino acid protein with seven putative transmembrane domains. The rank order of the binding to the receptor expressed in COS-7 cells was: ET-1 ≥ ET-2 ≫ ET-3. The receptor expressed in *Xenopus* oocytes showed a potent electrophysiological response to 1×10^{-7} M ET-1 under voltage clamp at −60 mV, while a much weaker response was produced by 1×10^{-7} M ET-3. Northern blot analysis with RNA from human tissues revealed a single band with a size of 4.3 kb in a wide variety of human tissues, especially highly in the blood vessel.

Endothelin (ET); Receptor; cDNA cloning; G-protein; *Xenopus oocyte*; Northern blot hybridization analysis

1. INTRODUCTION

Endothelin-1 (ET-1) was initially identified as a potent vasoconstrictor peptide with 21-amino-acid residues produced by porcine vascular endothelial cells [1]. Cloning and sequence analyses of the endothelin (ET) genes revealed that ETs comprise a peptide family consisting of three isopeptides, ET-1, ET-2 and ET-3, which have subsequently been found to distribute in a wide variety of vascular and non-vascular tissues [2–4].

The discovery of three isopeptides and their different biological activities raised the possibility of existence of multiple ET receptor subtypes. It has previously been shown in vivo that ET-1 and ET-2 are much more potent vasoconstrictors than ET-3, whereas the three isopeptides are roughly equipotent in producing the transient vasodilation, suggesting that the receptors mediating these opposing responses are different [2,5]. These pharmacological findings were further supported by detailed radioligand binding studies indicating the presence of, at least, two distinct subpopulations of ET binding sites; one has higher affinity for ET-1 or ET-2 than ET-3; the other has higher affinity for ET-3 than ET-1 or ET-2 [6,7].

Recently, we have succeeded in expression cloning of the bovine ET-1 receptor cDNA using the *Xenopus* oocyte expression system coupled with the voltage clamp method [8]. Here we report the cDNA cloning of human ET-1 receptor from a human placenta cDNA library and gene expression of this receptor in a wide variety of human tissues including the blood vessel.

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Abbreviations: ET, endothelin; IC_{50}, 50% inhibitory concentration

2. MATERIALS AND METHODS

2.1. *Isolation and sequencing of human ET-1 receptor cDNA*

Approximately 1×10^6 plaques of human placenta cDNA library in λZAPII were screened using the ^{32}P-labeled *Nco*I-*Eco*RI fragment of bovine ET-1 receptor cDNA as a probe [8,9]. A single clone was positively hybridized (phET1R), and restriction fragments of the cDNA insert were subcloned into Bluescript (Stratagene, La Jolla, CA). Both strands of the cDNA insert were sequenced by the dideoxy chain termination method [10].

2.2. *Expression of human ET-1 receptor in COS-7 cells*

The *Xho*I-*Not*I fragment of phET1R was subcloned into CDM8 (CDM8-phET1R) [11]. COS-7 cells were transfected with CDM8 or CDM8-phET1R (20 μg of DNA per 100 mm plate) by the calcium phosphate method [12]. Twenty-eight hours after transfection, cells were harvested and 5×10^4 cells/well were seeded into a 24-well culture plate.

2.3. *Competitive binding to human ET-1 receptor expressed in COS-7 cells*

Competitive binding assays of ^{125}I-ET-1 with unlabeled ET-1, ET-2 or ET-3, were performed 52 h after transfection as previously reported [13]. All determinants were in duplicate. Background binding in the presence of 4×10^{-7} M ET-1 was 150 cpm.

2.4. *Electrophysiological measurements of human ET-1 receptor expressed in* Xenopus *oocytes*

The mRNA (approximately 10 ng) synthesized in vitro from phET1R by T7 RNA polymerase was injected into *Xenopus* oocytes [8,14]. Whole-cell currents in response to the application of 1×10^{-7} M ET-1, ET-2 or ET-3 under voltage clamp at −60 mV were recorded [8,14].

2.5. *Northern blot hybridization analyses of human ET-1 receptor mRNA*

Total RNA was extracted from human tissues obtained at autopsy or operation, and human umbilical vein endothelial cells purchased from Colonetics Corp. (San Diego, CA) [9]. Northern blots of RNA on nylon membrane were hybridized with ^{32}P-labeled *Eco*RV-*Eco*RI fragment of the insert of phET1R. This study was approved by the ethical committee on human research of Kyoto University (No. 61-98).

3. RESULTS AND DISCUSSION

3.1. *Nucleotide and deduced amino acid sequences of human ET-1 receptor cDNA*

Fig. 1 shows the 4105-nucleotide sequence of the cloned cDNA containing a 1284-nucleotide open reading frame encoding a 427-amino acid protein with a relative molecular mass of 48726. The nucleotide sequence of the open reading frame was 91.2% identical to that of the bovine ET-1 receptor cDNA and the deduced amino acid sequence was 94.6% identical to that of the bovine ET-1 receptor. The nucleotide sequence surrounding the initiation codon agrees well with the consensus sequence [15]. The 3′-noncoding region contains ATTTA sequences implicated in mRNA instability [16]. There is a potential polyadenylation signal 22 nucleotides upstream of the poly(A)$^+$ tail [17]. Based on the consensus site for signal sequence cleavage [18], the N-terminal 20 amino acid residues probably constitute a signal sequence. Hydropathicity analysis of the amino acid sequence indicates that there are 7 hydrophobic clusters of 22–26 residues, each separated by stretches of hydrophilic residues, indicating that the cDNA encodes a protein

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                                                                                                                     GAAT   -481
TCGCGGCCGCCTCTTGCGGTCCCAGAGTGGAGTGGAAGGTCTGCAGCTTTGGGAGGAGACGGGGAGGACAGACTGGAGGCGTGTTCCTCCGGAGTTTTCTTTTTCGTGCGAGCCCTCGCG   -361
CGCGCGTACAGTCATCCCGCTGGTCTGACGATTGTGGAGAGGCGGTGGAGAGGCTTCATCCATCCCACCCGGTCGTCGCCGGGGATTGGGGTCCCAGCGACACCTCCCCGGGAGAAGCAG   -241
TGCCCAGGAAGTTTTCTGAAGCCGGGGAAGCTGTGCAGCCGAAGCCGCCGCCGCGCCGGAGCCCGGGACACCGGCCACCCTCCGCGCCACCCACCCTCGCTTTCTCCGGCTTCCTCTGGC   -121
CCAGGCGCCGCGCGGACCCGGCAGCTGTCTGCGCACGCCGAGCTCCACGGTGAAAAAAAAAGTGAAGGTGTAAAAGCAGCACAAGTGCAATAAGAGATATTTCCTCAAATTTGCCTCAAG     -1
 1                                                    20                                                                40
MetGluThrLeuCysLeuArgAlaSerPheTrpLeuAlaLeuValGlyCysValIleSerAspAsnProGluArgTyrSerThrAsnLeuSerAsnHisValAspAspPheThrThrPhe
ATGGAAACCCTTTGCCTCAGGGCATCCTTTTGGCTGGCACTGGTTGGATGTGTAATCAGTGATAATCCTGAGAGATACAGCACAAATCTAAGCAATCATGTGGATGATTTCACCACTTTT    120
                                                      60                                                                80
ArgGlyThrGluLeuSerPheLeuValThrThrHisGlnProThrAsnLeuValLeuProSerAsnGlySerMetHisAsnTyrCysProGlnGlnThrLysIleThrSerAlaPheLys
CGTGGCACAGAGCTCAGCTTCCTGGTTACCACTCATCAACCCACTAATTTGGTCCTACCCAGCAATGGCTCAATGCACAACTATTGCCCACAGCAGACTAAAATTACTTCAGCTTTCAAA    240
                                    I                100                                                               120
TyrIleAsnThrValIleSerCysThrIlePheIleValGlyMetValGlyAsnAlaThrLeuLeuArgIleIleTyrGlnAsnLysCysMetArgAsnGlyProAsnAlaLeuIleAla
TACATTAACACTGTGATATCTTGTACTATTTTCATCGTGGGAATGGTGGGAATGCAACTCTGCTCAGGATCATTTACCAGAACAAATGTATGAGGAATGGCCCCAACGCGCTGATAGCC    360
                  II                                 140                                                               160
SerLeuAlaLeuGlyAspLeuIleTyrValValIleAspLeuProIleAsnValPheLysLeuLeuAlaGlyArgTrpProPheAspHisAsnAspPheGlyValPheLeuCysLysLeu
AGTCTTGCCCTTGGAGACCTTATCTATGTGGTCATTGATCTCCCTATCAATGTATTTAAGCTGCTGGCTGGGCGCTGGCCTTTTGATCACAATGACTTTGGCGTATTTCTTTGCAAGCTG    480
                             III                     180                                                               200
PheProPheLeuGlnLysSerSerValGlyIleThrValLeuAsnLeuCysAlaLeuSerValAspArgTyrArgAlaValAlaSerTrpSerArgValGlnGlyIleGlyIleProLeu
TTCCCCTTTTTGCAGAAGTCCTCGGTGGGGATCACCGTCCTCAACCTCTGCGCTCTTAGTGTTGACAGGTACAGAGCAGTTGCCTCCTGGAGTCGTGTTCAGGGAATTGGGATTCCTTTG    600
                                           IV        220                                                               240
ValThrAlaIleGluIleValSerIleTrpIleLeuSerPheIleLeuAlaIleProGluAlaIleGlyPheValMetValProPheGluTyrArgGlyGluGlnHisLysThrCysMet
GTAACTGCCATTGAAATTGTCTCCATCTGGATCCTGTCCTTTATCCTGGCCATTCCTGAAGCGATTGGCTTCGTCATGGTACCCTTTGAATATAGGGGTGAACAGCATAAAACCTGTATG    720
                                                     260                   V                                           280
LeuAsnAlaThrSerLysPheMetGluPheTyrGlnAspValLysAspTrpTrpLeuPheGlyPheTyrPheCysMetProLeuValCysThrAlaIlePheTyrThrLeuMetThrCys
CTCAATGCCACATCAAAATTCATGGAGTTCTACCAAGATGTAAAGGACTGGTGGCTCTTCGGGTTCTATTTCTGTATGCCCTTGGTGTGCACTGCGATCTTCTACACCCTCATGACTTGT    840
                                                     300                                                           VI  320
GluMetLeuAsnArgArgAsnGlySerLeuArgIleAlaLeuSerGluHisLeuLysGlnArgArgGluValAlaLysThrValPheCysLeuValValIlePheAlaLeuCysTrpPhe
GAGATGTTGAACAGAAGGAATGGCAGCTTGAGAATTGCCCTCAGTGAACATCTTAAGCAGCGTCGAGAAGTGGCAAAAACAGTTTTCTGCTTGGTTGTAATTTTTGCTCTTTGCTGGTTC    960
                                                     340                                                          VII  360
ProLeuHisLeuSerArgIleLeuLysLysThrValTyrAsnGluMetAspLysAsnArgCysGluLeuLeuSerPheLeuLeuLeuMetAspTyrIleGlyIleAsnLeuAlaThrMet
CCTCTTCACTTAAGCCGTATATTGAAGAAAACTGTGTATAACGAAATGGACAAGAACCGATGTGAATTACTTAGTTTCTTACTGCTCATGGATTACATCGGTATTAACTTGGCAACCATG   1080
                                                     380                                                               400
AsnSerCysIleAsnProIleAlaLeuTyrPheValSerLysLysPheLysAsnCysPheGlnSerCysLeuCysCysCysCysTyrGlnSerLysSerLeuMetThrSerValProMet
AATTCATGTATAAACCCCATAGCTCTGTATTTTGTGAGCAAGAAATTTAAAAATTGTTTCCAGTCATGCCTCTGCTGCTGCTGTTACCAGTCCAAAAGTCTGATGACCTCGGTCCCCATG   1200
                                                     420               427
AsnGlyThrSerIleGlnTrpLysAsnHisAspGlnAsnAsnHisAsnThrAspArgSerSerHisLysAspSerMetAsn
AACGGAACAAGCATCCAGTGGAAGAACCACGATCAAAACAACCACAACACAGACCGGAGCAGCCATAAGGACAGCATGAACTGACCACCCTTAGAAGCACTCCTCGGTACTCCCATAATC   1320
CTCTCGGAGAAAAAAATCACAAGGCAACTGTGACTCCGGGAATCTCTTCTCTGATCCTTCTTCCTTAATTCACTCCCACACCCAAGAAGAAATGCTTTCCAAAACCGCAAGGTAGACTGG   1440
TTTATCCACCCACAACATCTACGAATCGTACTTCTTTAATTGATCTAATTTACATATTCTGCGTGTTGTATTCAGCACTAAAAAATGGTGGAGCTGGGGAGAATGAAGACTGTTAAAT   1560
GAAACCAGAAGGATATTTACTACTTTTGCATGAAAATAGAGCTTTCAAGTACATGGCTAGCTTTTATGGCAGTTCTGGTGAATGTTCAATGGGAACTGGTCACCATGAAACTTTAGAGAT   1680
TAACGACAAGATTTTCTACTTTTTTTAAGTGATTTTTTGTCCTTCAGCCAAACACAATATGGGCTCAGGTCACTTTTATTTGAAATGTCATTTGGTGCCAGTATTTTTTAACTGCATAAT   1800
AGCCTAACATGATTATTTGAACTTATTTACACATAGTTTGAAAAAAAAAGACAAAAATAGTATTCAGGTGAGCAATTAGATTAGTATTTTCCACGTCACTATTTATTTTTTTAAAACAC   1920
AAATTCTAAAGCTACAACAAATACTACAGGCCCTTAAAGCACAGTCTGATGACACATTTGGCAGTTTAATAGATGTTACTCAAAGAATTTTTTAAGAACTGTATTTTATTTTTTAAATGG   2040
TGTTTTATTACAAGGGACCTTGAACATGTTTTGTATGTTAAATTCAAAAGTAATGCTTCAATCAGATAGTTCTTTTTCACAAGTTCAATACTGTTTTTCATGTAAATTTTGTATGAAAAA   2160
TCAATGTCAAGTACCAAAATGTTAATGTATGTGTCATTTAACTCTGCCTGAGACTTTCAGTGCACTGTATATAGAAGTCTAAAACACACCTAAGAGAAAAAGATCGAATTTTTCAGATGA   2280
TTCGGAAATTTTCATTCAGGTATTTGTAATAGTGACATATATATGTATATACATATCACCTCCTATTCTCTTAATTTTTGTTAAAATGTTAACTGGCAGTAAGTCTTTTTTGATCATTCC   2400
CTTTTCCATATAGGAAACATAATTTTGAAGTGGCCAGATGAGTTTATCATGTCAGTGAAAAATAATTACCCACAAATGCCACCAGTAACTTAACGATTCTTCACTTCTTGGGGTTTTCAG   2520
TATGAACCTAACTCCCCACCCCAACATCTCCCTCCCACATTGTCACCATTTCAAAGGGCCCACAGTGACTTTTGCTGGGCATTTTCCCAGATGTTTACAGACTGTGAGTACAGCAGAAAA   2640
TCTTTTACTAGTGTGTGTGTGTATATATATAAACAATTGTAAATTTCTTTTAGCCCATTTTTCTAGACTGTCTCTGTGGAATATATTTGTGTGTGTGATATATGCATGTGTGTGATGGTA   2760
TGTATGGATTTAATCTAATCTAATAATTGTGCCCCGCAGTTGTGCCAAAGTGCATAGTCTGAGCTAAAATCTAGGTGATTGTTCATCATGACAACCTGCCTCAGTCCATTTTAACCTGTA   2880
GCAACCTTCTGCATTCATAAATCTTGTAATCATGTTACCATTACAAATGGGATATAAGAGGCAGCGTGAAAGCAGATGAGCTGTGGACTAGCAATATAGGGTTTTGTTTGGTTGGTTGGT   3000
TTGATAAAGCAGTATTTGGGGTCATATTGTTTCCTGTGCTGGAGCAAAAGTCATTACACTTTGAAGTATTATATTGTTCTTATCCTCAATTCAATGTGGTGATGAAATTGCCAGGTTGTC   3120
TGATATTTCTTTCAGACTTCGCCAGACAGATTGCTGATAATAAATTAGGTAAGATAATTTGTTGGGCCATATTTTAGGACAGGTAAAATAACATCAGGTTCCAGTTGCTTGAATTGCAAG   3240
GCTAAGAAGTACTGCCCTTTTGTGTGTTAGCAGTCAAATCTATTATTCCACTGGCGCATCATATGCAGTGATATATGCCTATAATATAAGCCATAGGTTCACACCATTTTGTTTAGACAA   3360
TTGTCTTTTTTTCAAGATGCTTTGTTTCTTTCATATGAAAAAAATGCATTTTATAAATTCAGAAAGTCATAGATTTCTGAAGGCGTCAACGTGCATTTTATTTATGGACTGGTAAGTAAC   3460
TGTGGTTTACTAGCAGGAATATTTCCAATTTCTACCTTTACTACATCTTTTCAACAAGTAACTTTGTAGAAATGAGCCAGAAGCCAAGGCCCCTGAGTTGGCAGTGGCCCATAAGTGTAAA   3600
ATAAAAGTTTACAGAAACCTT-poly(dA)                                                                                                3621
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Fig. 1. Nucleotide and deduced amino acid sequences of the human ET-1 receptor cDNA. Nucleotides are numbered at the end of each line, and amino acids are numbered above the sequence. Positions of the putative transmembrane domains I–VII are indicated above the sequence. Triangles, potential *N*-glycosylation sites; circles, serine residues potentially able to be phosphorylated. The ATTTA sequences are underlined. The potential polyadenylation signal is dashedly underlined.

with 7 membrane-spanning domains, an extracellular N tail and a cytoplasmic C tail, which is consistent with the superfamily of G protein-coupled receptors.

As shown in Fig. 1, there are several potential sites for post-translational modification: two consensus sites for *N*-glycosylation; six cysteine residues in the N terminus of the cytoplasmic C tail, one of which may be palmitoylated as in the β_2-adrenergic receptor [19]; and serine residues potentially able to be phosphorylated by serine/threonine kinases [20].

3.2. *Ligand selectivity of human ET-1 receptor expressed in COS-7 cells*

To confirm that the cDNA clone we have obtained indeed encodes a receptor for ET-1, we constructed the expression vector CDM8-phET1R. The ligand binding property of the receptor expressed by the CDM8-phET1R was determined by competitive binding of ^{125}I-ET-1 in the presence of unlabeled ET-1, ET-2 or ET-3 (Fig. 2). The amount of ^{125}I-ET-1 binding in COS-7 cells transfected with control plasmid CDM8 was indistinguishable from the level of non-specific ^{125}I-ET-1 binding in the presence of excess unlabeled ET-1 (data not shown). Competition for ^{125}I-ET-1 binding with unlabeled ET-1 gave an IC_{50} of 3.0×10^{-9} M. IC_{50} of ET-2 was 6.1×10^{-9} M and approximately two times larger than IC_{50} of ET-1. IC_{50} of ET-3 was more than 1×10^{-6} M. Thus, the rank order of the binding affinity to the human ET-1 receptor was ET-1 $\geqslant$ ET-2 $\gg$ ET-3.

The relative potency of the binding of ET-2 to human ET-1 receptor was slightly different from that to bovine ET-1 receptor [8]. This difference might be explained in part by the difference of the binding assay method. In our previous study, we used membrane preparation for the binding assay, but in the present study, we used intact transfected COS-7 cells.

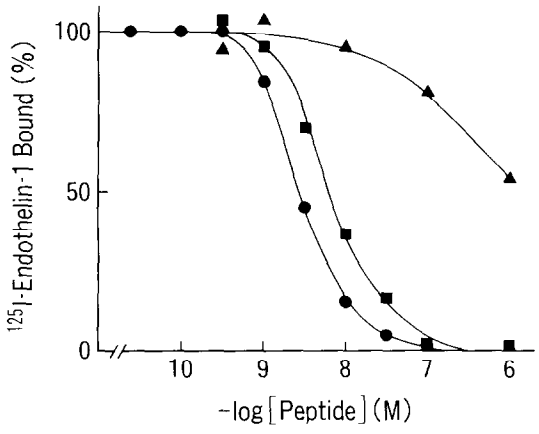

Fig. 2. Competitive binding of ^{125}I-ET-1 to COS-7 cells transfected with CDM8-phET1R. ^{125}I-ET-1 (50 pM) was incubated in the absence or presence of various concentrations (10^{-10}–10^{-6} M) of ET-1 (●), ET-2 (■) or ET-3 (▲). Data are expressed as the percentage of specific ^{125}I-ET-1 binding and values are the means of duplicate determinations.

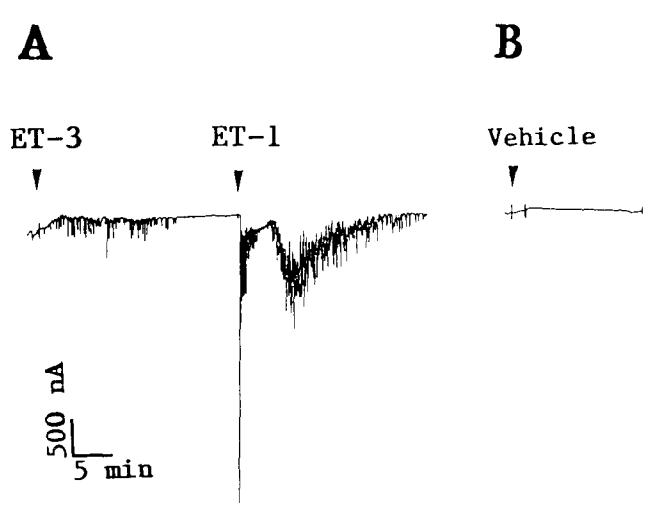

Fig. 3. Current trace recorded from *Xenopus* oocytes injected with the in vitro synthesized human ET-1 receptor mRNA. 1×10^{-7} M ET-1, 1×10^{-7} M ET-3 (A) and vehicle (B) were applied. Downward deflection indicates inward current.

3.3. *Electrophysiological responses via human ET-1 receptor expressed in* Xenopus *oocytes*

Electrophysiological responses via the human ET-1 receptor to ET-1, ET-2 or ET-3 were examined in the *Xenopus* oocyte expression system coupled with the voltage clamp method as shown in Fig. 3. Twenty seconds after the application of 1×10^{-7} M ET-1, a potent inward current was recorded under voltage clamp at −60 mV in oocytes injected with the in vitro synthesized mRNA of the receptor (Fig. 3A). A similar inward current was also recorded by 1×10^{-7} M ET-2 (data not shown). In contrast, a much weaker current was induced by the application of 1×10^{-7} M ET-3 (Fig. 3A). The endothelin-evoking currents were fluctuating and long-lasting, and were thus characteristic of Ca^{2+}-activated chloride currents [21]. No currents were recorded by vehicle (vehicle was ND 96 containing 0.1% Triton X-100 and 0.1% gelatin) (Fig. 3B).

3.4. *ET-1 receptor mRNA in human tissues*

To elucidate the expression of human ET-1 receptor mRNA in various human tissues, we used a 826-bp *Eco*RV-*Eco*RI fragment of human ET-1 receptor cDNA (nucleotides 256–1081) as a probe in the Northern blotting. As shown in Fig. 4, this probe detected a single band of mRNA with a size of 4.3 kb at the highest abundance in the aorta, at high levels in the lung, atrium, colon and placenta, at moderate levels in the cerebral cortex, cerebellum, ventricle, kidney, adrenal and duodenum. We did not detect any hybridizing signal in the liver. No hybridizing band was detected in human endothelial cells of the umbilical vein.

The highest level of ET-1 mRNA in the aorta and no

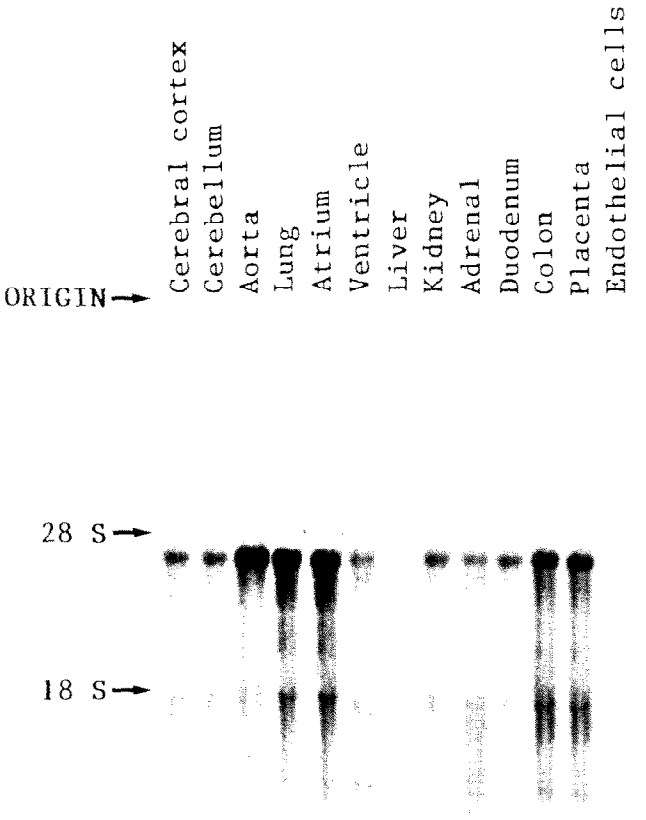

Fig. 4. Northern blot analysis of human ET-1 receptor mRNA. 20 μg of total RNA from the human cerebral cortex, cerebellum, aorta, lung, atrium, ventricle, liver, kidney, adrenal, duodenum, colon, placenta and endothelial cells of the umbilical vein were used. The presence of intact 28 S ribosomal RNA was confirmed in all lanes (not shown). The positions of 28 S and 18 S rRNA are shown on the left.

hybridizing signal in the endothelial cells suggest that the ET-1 receptor mRNA is expressed in vascular smooth muscle cells. This notion is consistent with the previous finding that ET-1 and ET-2 inhibited ^{125}I-ET-1 binding to rat A-10 vascular smooth muscle cells but ET-3 failed to compete for ^{125}I-ET-1 binding to the same cells [7]. Since no hybridizing band of the non-isopeptide-selective subtype of ET receptor mRNA was reported to be detected in the rat aorta and A-10 vascular smooth muscle cells [22], ET-1 receptor encoded by the cDNA cloned in the present study is a major subtype of the ET receptor expressed in vascular smooth muscle cells. It is conceivable that ET-1 released from the endothelial cells binds to the ET-1 receptor in vascular smooth muscle cells and works as a local regulator in cardiovascular homeostasis.

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