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Alternative Transcript of the Nonselective-Type Endothelin Receptor from Rat Brain

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SUMMARY

A novel cDNA encoding the nonselective type of endothelin (ET) receptor was isolated from a rat brain cDNA library. The cloned cDNA encoded a 442-amino acid protein with seven putative transmembrane domains. Nucleotide sequence analysis showed that the rat brain cDNA differed from the cloned rat lung nonselective ET receptor (ET_B) cDNA by three extra nucleotides in its coding regions, which produced an encoded protein with four amino acid substitutions. In addition, both the 5′ and 3′ noncoding sequences of the rat brain cDNA were divergent from those of rat lung cDNA. Expression of the rat brain cDNA in COS-1 cells demonstrated that the encoded receptor displayed equal affinity toward the three ET isopeptides. However, Southern blot analysis indicated a single-copy gene for the rat ET_B receptor. Further genomic cloning and sequence analysis demonstrated that rat brain cDNA encoded the authentic protein sequences of the rat ET_B receptor. Moreover, the 5′ noncoding sequences in rat brain cDNA that were divergent from those in rat lung cDNA were encoded by a distinct region, an upstream exon, in the rat ET_B genome. All the findings suggest that rat brain cDNA represents an alternative transcript of the rat ET_B gene. Preliminary Northern blot analysis indicated that the expression of this ET_B cDNA sequence might be not only in the brain but also in other tissues, whereas its expression might be somehow tissue-specifically regulated.

ETs are a family of 21-amino acid vasoactive peptides consisting of three isopeptides, ET-1, ET-2, and ET-3 (1, 2). Among them, ET-1 is the most potent mammalian vasoconstrictor peptide known to date (1). It has been suggested that ET-1 may play an important role in regulating system blood pressure and perhaps local blood flow and that the disturbance of this regulatory mechanism could contribute to pathological states of hypertension (3) or vascular spasm. ET-1 also demonstrated a wide variety of pharmacological effects in various other tissues (1). The diverse pharmacological activities of ET-1 suggest the existence of subtypes of ET-1 receptors. Recently, two cDNAs encoding ET receptors have been cloned from bovine lung and rat lung by Arai *et al.* (4) and Sakurai *et al.* (5), respectively. The receptor cloned from bovine lung, with the affinity ET-1 > ET-2 > ET-3, is referred to as the selective-type ET receptor (ET_A), whereas that from rat lung, which has the same affinity toward the ET isoforms, is the nonselective-type ET receptor (ET_B). Each receptor contains seven transmembrane domains, suggesting that the receptors belong to the superfamily of G protein-coupled receptors.

ETs are also neuropeptides found in mammalian brain. Both ET-1 and ET-3 have been identified in porcine spinal cord and brain homogenate (6, 7). In addition, an *in situ* hybridization study (8) revealed widespread distribution of ET mRNA in neurons of the brain, suggesting that ET in brain may play a fundamental role in regulating nervous system function. In this communication, we report the molecular cloning of a novel cDNA encoding the nonselective ET receptor (ET_B) from rat brain. Our findings differ from the published report with regard to the sequences encoding the amino terminus of the ET_B receptor and both the 5′ and 3′ noncoding sequences. Several experiments provide persuasive evidence that the rat brain cDNA represents an alternative transcript of the rat ET_B gene. In addition, the coding sequences in this cloned DNA encode the authentic protein sequences of the rat ET_B receptor.

Experimental Procedures

Materials. ET-1, ET-2, ET-3, and BQ123 were from Peninsula Laboratories (Belmont, CA). GeneAmp DNA amplification reagent was from Perkin-Elmer Cetus (Norwalk, CT). A rat brain cDNA library and a rat liver genomic DNA library were purchased from Stratagene (La Jolla, CA). Sequenase version 2.0 sequencing kit was obtained from United States Biochemical Corp. (Cleveland, OH). ^{125}I-ET-1 (specific activity, 2000 Ci/mmol) was supplied by Amersham International

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ABBREVIATIONS: ET, endothelin; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SSC, standard saline citrate; bp, base pair(s); kb, kilobase(s).

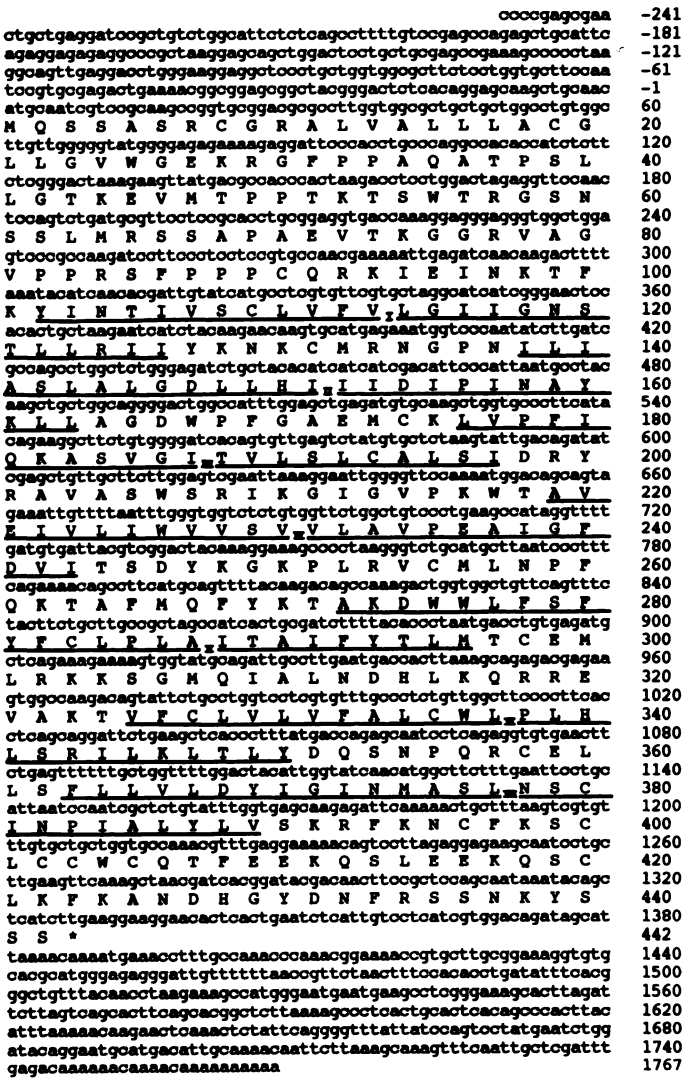

Fig. 1. Nucleotide and deduced amino acid sequences of the rat brain cDNA. Nucleotides are numbered beginning with the first residue of the ATG initiation codon. Amino acids are numbered from the amino terminus. The single-letter amino acid code is used. *Black bars*, seven predicted transmembrane domains.

(Amersham, Buckinghamshire, UK). GeneScreenPlus hybridization transfer membrane was from DuPont-New England Nuclear (Boston, MA). DOTAP (*N*-[1-(2,3-dioleoyloxyl)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) transfection reagent was purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Restriction enzymes were supplied by Promega (Madison, WI). The RNA extraction kit and mRNA isolation kit were from Pharmacia (Uppsala, Sweden).

Molecular cloning of rat brain ET receptor cDNA. A rat brain cDNA library constructed in λZapII vector was screened by the PCR (9) differentiation method using degenerate sense oligonucleotide A_1 (5′-AACAAGTGCATGAG(GA)AATGG-3′, located in the first cytoplasmic loop) and antisense oligonucleotide A_2 (5′-GTCAT(GT)-AGGGTGTAAAAGAT-3′, located in the sixth transmembrane region), which were highly homologous sequences between bovine lung ET_A cDNA (7) and rat lung ET_B cDNA (8). The cDNA library was divided into fractions, each containing 30,000 individual cDNA clones, and amplified by the plate lysate method (10). Aliquots of phages eluted from each plate lysate were analyzed by PCR with 30 cycles of 1 min at 94°, 2 min at 45°, and 3 min at 72°. The positive fractions whose PCR product contained a DNA fragment with the expected size of about 500 bp were further stepwise fractionated into pools of 3000, 300, and 30 and were reassayed until a single positive clone was isolated. The isolated λZapII phage clones, λRETB-27 and λRETB-39, were rescued as Bluescript plasmids, pRETB-27 and pRETB-39, respectively, using the *in vivo* excision process. Nucleotide sequences of the plasmid cDNAs were determined on both strands by the dideoxynucleotide chain termination method (11), using Sequenase version 2.0. Most of the sequence was determined by synthesizing successive oligonucleotide primers every 200–250 bp along the DNA, whereas the remainder was deduced using the Bluescript T3 and T7 primers (Stratagene).

Transfection of DNA into COS-1 cells. The 2.0-kb *Pvu*II/*Sal*I restriction fragment of the pRETB-39 insert was blunt-ended with S1 nuclease and ligated into the blunt-ended *Eco*RI site of the expression vector pMT2 (12). The correct orientation of the insert in pMT2 was confirmed by restriction enzyme analysis. The recombinant DNA and control plasmid DNA were then used to transfect monolayers of COS-1 cells, which were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. About 60% confluent COS-1 cells were transfected with 40 μg of DNA/150-mm plate, using the lipofection method (13). Two days after transfection, the crude membranes of the cells transfected with DNA were isolated for binding assays.

Radioligand binding assay. A binding assay mixture (0.5 ml) containing 10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 3 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 20 μg/ml bacitracin, 0.1% bovine serum albumin, and 4 μg/ml membrane protein, plus various concentrations of unlabeled ET-1, ET-2, ET-3, or BQ123, was incubated with 5 pM ^{125}I-ET-1 for 2 hr at 25°. Nonspecific binding was determined in the presence of 1 μM nonradioactive ET-1. The receptor-^{125}I-ET-1 complex was separated from free ^{125}I-ET-1 by filtration through a Whatman GF/C glass filter (14). The filters were washed twice with 10 ml of cold buffer. The radioactivity of the filter was counted with a γ counter.

Southern blot hybridization. Approximately 20 μg of rat genomic DNA isolated from rat liver were digested with *Bam*HI and *Eco*RI, separated on a 0.7% agarose gel, and transferred to a GeneScreenPlus nylon membrane. A 147-bp DNA fragment spanning the amino-terminal coding sequence of the ET_B receptor (nucleotides +78 to +225) was amplified from pRETB-39 cDNA by PCR and was prepared as a radioprobe. After hybridization, the membrane was successively washed twice for 5 min in 2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) at 25°, twice for 30 min in 2× SSC/1.0% sodium dodecyl sulfate at 65°, and finally twice for 30 min in 0.1× SSC at 25°.

Screening of rat genomic library. A rat liver genomic DNA library constructed in λDASH vector was screened by plaque hybridization using the same ^{32}P-labeled pRETB-39 cDNA probe (nucleotides +78 to +225) as described for Southern blot analysis. Of 1×10^6 recombinants screened, three positive clones were identified and further purified.

Preparation and analysis of RNA. Total RNA was extracted from the various tissues of adult male Sprague Dawley rats with 4 M guanidine isothiocyanate and was isolated through a CsTFA (cesium trifluoroacetate) cushion following the manufacturer's suggested protocol. After further purification by oligo(dT)-cellulose column chromatography, poly(A)$^+$ RNA was analyzed by Northern blotting. About 5 μg of poly(A)$^+$ RNA were separated by formaldehyde-agarose gel electrophoresis and capillary transferred to a nylon membrane. The membrane was hybridized with the radioprobe prepared from the coding region of ET_B cDNA (nucleotides +78 to +225). In addition, the same Northern membrane was reprobed with a 35-mer corresponding to the brain-specific 5′ noncoding region (nucleotides −157 to −191 of rat brain ET_B cDNA), which was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Results

Isolation and sequence analysis of rat brain ET receptor cDNA. Using the PCR differentiation screening method, we isolated from rat brain cDNA library two distinct cDNA

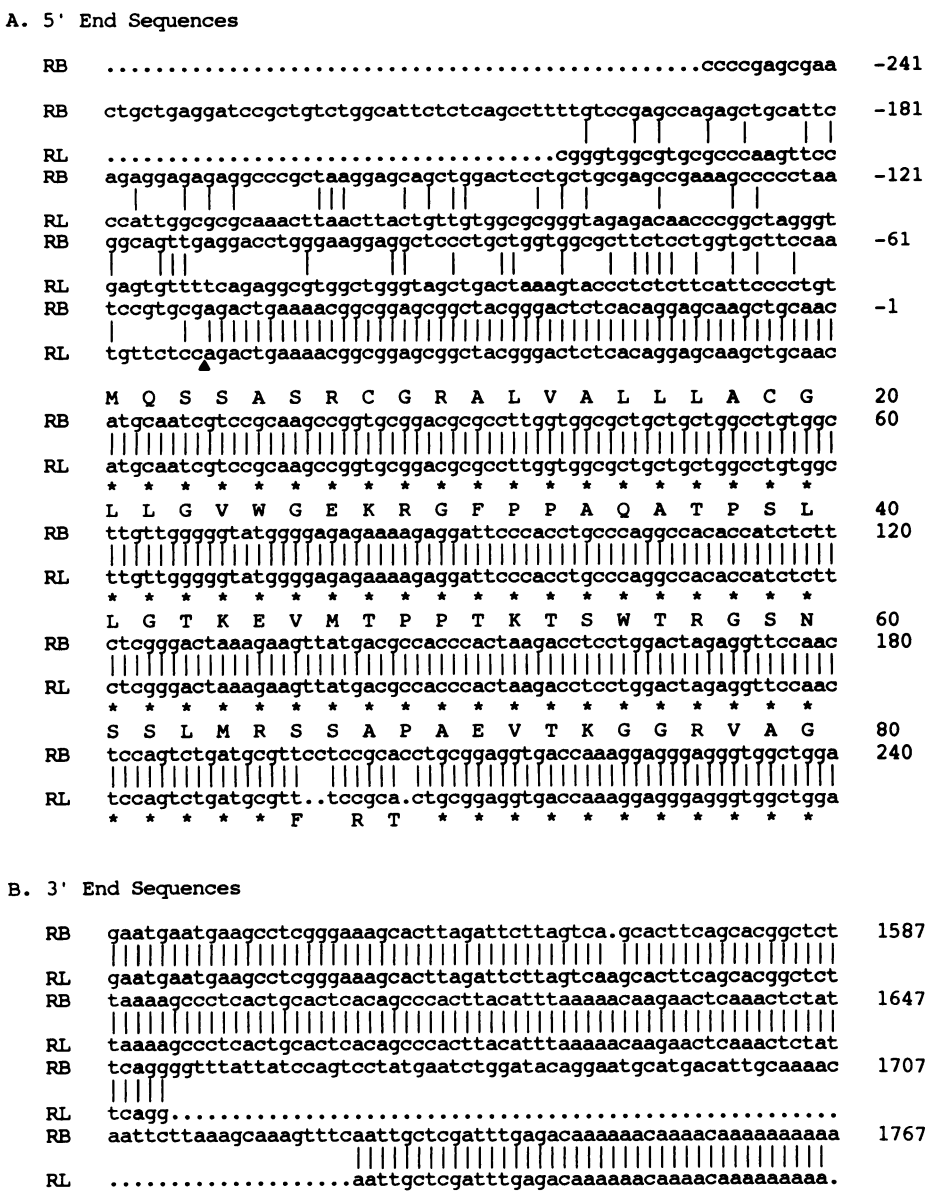

Fig. 2. Comparative nucleotide and amino acid sequence analysis of rat brain cDNA and rat lung cDNA. Displayed is partial 5′-end (A) and 3′-end (B) sequence information for rat brain (*RB*) and rat lung (*RL*) ET_B cDNA clones. *Arrowhead*, mapped point of divergence between the 5′ flanking sequences of these two cDNA clones (A, position −52). *Dots* were inserted to maximize homology. The deduced amino acids from rat brain cDNA are indicated *above their nucleotides*; amino acid residues from rat lung cDNA are *below the nucleotides* and are marked (*) if their sequences are identical to the corresponding portions in rat brain ET_B cDNA.

clones, λRETB-27 and λRETB-39. Sequence analysis revealed that pRETB-39, with a 2017-bp insert, contained the longest 1329-bp open reading frame, encoding 442 amino acid residues. The entire nucleotide and deduced amino acid sequences of pRETB-39 are shown in Fig. 1. The encoded protein contains seven transmembrane domains and belongs to the G protein-coupled receptor family. The other clone, pRETB-27, contained a 1480-bp insert beginning at position +287 of the open reading frame of the pRETB-39 insert. The cDNA sequences of pRETB-27 and pRETB-39 were identical in the other regions.

The nucleotide and deduced amino acid sequences of pRETB-39 were compared with those of rat ET receptors described previously (5, 15). The coding regions of the rat brain cDNA were essentially identical to those of the ET_B receptor determined from rat lung, except for three additional bases in pRETB-39 that caused its encoded protein to have four amino acid substitutions and to be one amino acid longer than the rat lung ET_B receptor (Figs. 1 and 2A), which was reported to contain 441 amino acid residues. These four amino acid substitutions (^{60}Ser-Ser-Ala-Pro instead of Phe-Ala-Thr in the rat lung ET_B receptor) were localized at the amino terminus of the receptor protein. On the other hand, both the 5′ and 3′ noncoding sequences in the rat brain cDNA clone were divergent from those in rat lung ET_B cDNA (Fig. 2). The 5′ flanking sequences at nucleotide −52 upstream from the translation initiation codon (ATG) were found to be distinct between these two cDNA clones (Fig. 2A). In addition, the 3′ nontranslated region of pRETB-39 was shorter by one nucleotide at position +1570 but had 75 extra bases (from position +1653 to position +1727) that the rat lung cDNA clone did not contain (Fig. 2B). All these observations indicate that the cDNA of pRETB-39 from rat brain is distinct from the rat lung ET_B cDNA.

Functional expression assay. The cDNA insert of pRETB-39 was further subcloned into a mammalian expression vector, pMT2. Incubation of ^{125}I-ET-1 with membranes derived from COS-1 cells transfected with pMT2-RETB-39 revealed saturable binding of ligand (Fig. 3). Scatchard analysis (Fig. 3, *inset*) showed a single high affinity binding site for ET-1, with

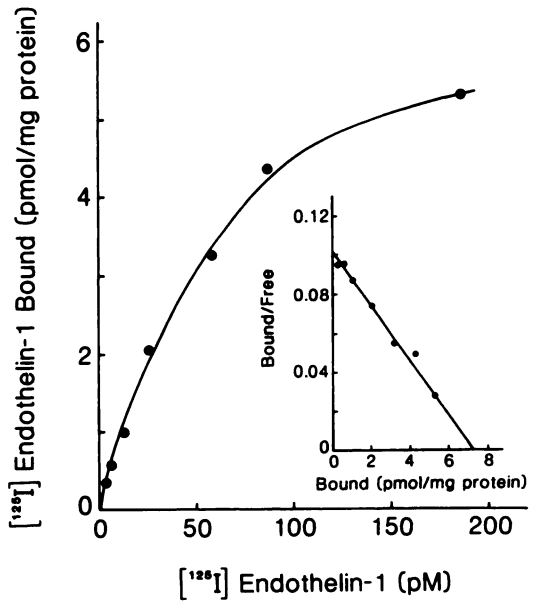

Fig. 3. Saturation isotherm. *Inset*, Scatchard plot of ^{125}I-ET-1 binding to membranes of COS-1 cells transfected with pMT2-RETB-39.

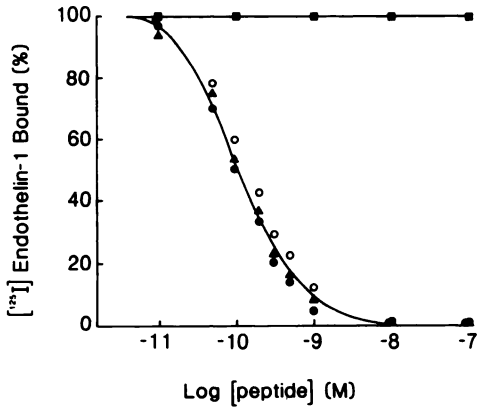

Fig. 4. Competitive binding of ^{125}I-ET-1 to membranes of COS-1 cells transfected with pMT2-RETB-39. About 5 pM ^{125}I-ET-1 was incubated with transfected cell membranes (4 μg/ml) and the designated concentrations of ET-1 (●), ET-2 (▲), ET-3 (○), or BQ123 (■) at 25° for 2 hr. Results are expressed as percentage of the maximal specific ^{125}I-ET-1 binding. Each *point* represents the mean of three separate experiments, each done in triplicate.

a dissociation constant (K_d) of 70 pM and a B_{max} value of 7.5 pmol/mg of membrane protein. In addition, the displacement of ^{125}I-ET-1 specific binding from the transfected cell membrane by unlabeled ET-1, ET-2, and ET-3 revealed that the expressed receptor had equal affinity toward the three isopeptides (Fig. 4). Moreover, BQ123, an antagonist of the ET_A receptor (16), did not significantly affect the specific binding of ^{125}I-ET-1 to transfected cell membranes even at a concentration of 1 μM. No specific binding was observed in cells transfected with control plasmid pMT2 (data not shown). All these results indicated that the cloned cDNA from rat brain encoded a nonselective subtype of ET receptor.

Southern blot analysis of the rat ET_B gene. To determine the number of genes encoding the rat ET_B receptor, restriction digests of rat genomic DNA were analyzed by Southern hybridization with the ^{32}P-labeled pRETB-39 cDNA (+78 to +225) as the probe. As shown in Fig. 5, a single band was

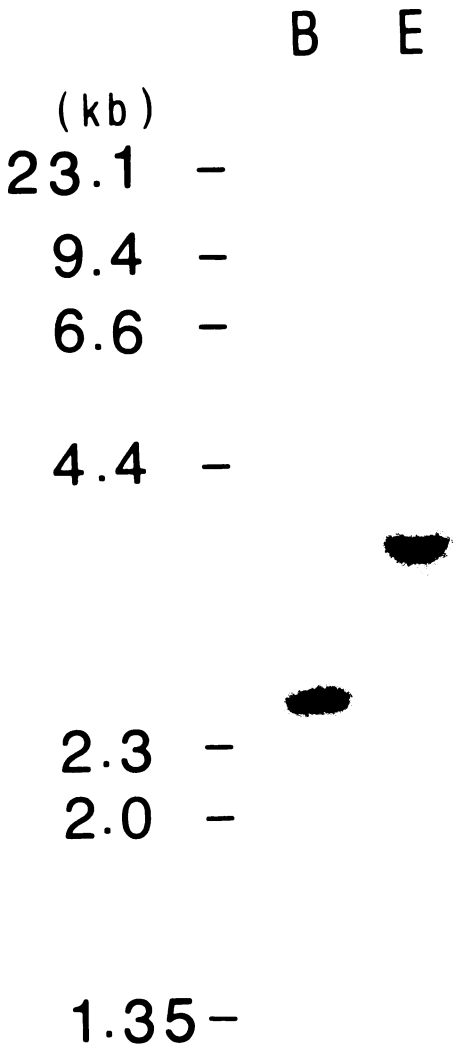

Fig. 5. Southern blot analysis of the rat ET_B gene. Rat genomic DNA (20 μg) prepared from rat liver was digested with *Bam*HI (*B*) or *Eco*RI (*E*) and hybridized with the pRETB-39 cDNA probe (nucleotides +78 to +225). Size standards are indicated on the *left*.

detected in *Bam*HI and *Eco*RI digests, indicating that the ET_B receptor gene is present as a single copy in rat genome.

Genomic cloning and analysis of the 5′ flanking sequence of the rat ET_B gene. To further elucidate the relationship between these two cDNAs, an effort was made to isolate the genomic DNA clone from a rat liver genomic DNA library constructed in the λDASH vector. Three positive clones (λRG3–1, λRG3–2, and λRG12) were isolated from 1 × 10^6 phage clones by plaque hybridization, using a DNA fragment spanning the amino-terminal coding region (nucleotides +78 to +225) as the radioprobe. Although these genomic DNA clones have not yet been well characterized, preliminary analysis of the 5′ end of the ET_B gene was done by direct sequencing of the cloned phage DNA. Fig. 6 shows the 5′-end sequence of the ET_B gene, including the partial amino-terminal coding sequence (to nucleotide +240) and its 5′ flanking region (to nucleotide −839). By comparing the genomic DNA sequences with the cloned cDNA from rat brain or from rat lung, we found that the amino-terminal coding sequences in the rat ET_B gene showed coincidence with those in rat brain ET_B cDNA. Moreover, comparison of the 5′ flanking sequences suggested

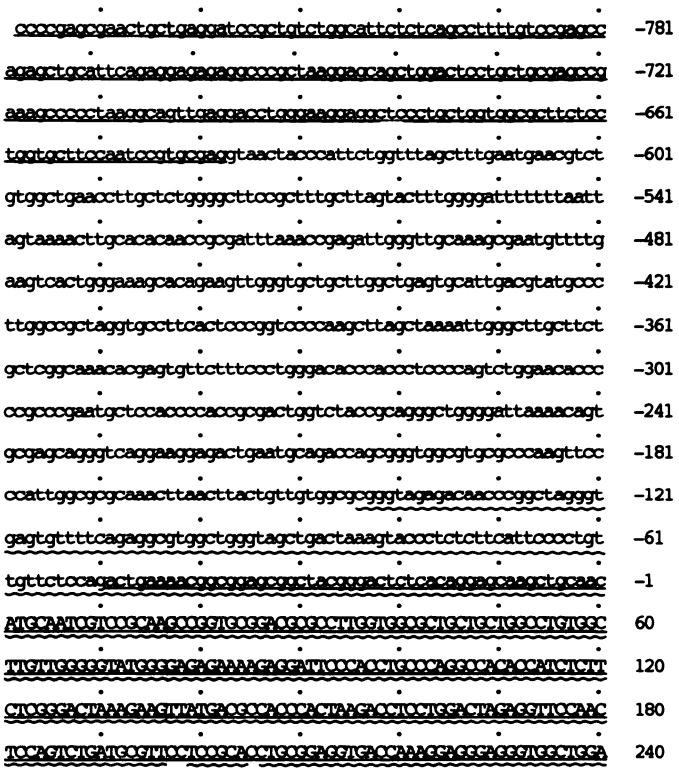

Fig. 6. Nucleotide sequence of the 5′ end of the rat ET_B gene. Nucleotides are numbered by their positions relative to the ATG initiation codon. The 5′-end sequences of the ET_B gene found in ET_B cDNA from rat brain or rat lung are underlined with *solid lines* or *wavy lines*, respectively. Every ten nucleotides is scaled by a dot.

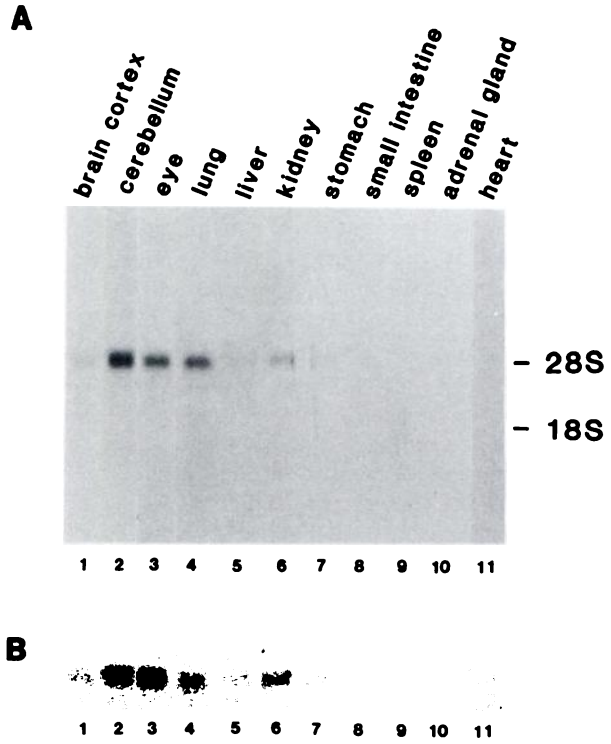

Fig. 7. Northern blot analysis of rat ET_B mRNA. About 5 μg of mRNA from the rat brain cortex, cerebellum, eye, lung, liver, kidney, stomach, small intestine, spleen, adrenal gland, and heart were used. The membrane was blotted with radioprobe prepared either from the coding region of ET_B cDNA (A) or from the brain-specific region (B). The positions of 28 S and 18 S rRNA are shown on the *right*.

that rat lung cDNA and rat brain cDNA might represent two distinct transcripts of the ET_B gene. As shown in Fig. 6, rat lung cDNA represented a transcript transcribed from nucleotide −203 of the ET_B gene, whereas rat brain cDNA represented an alternative ET_B transcript transcribed from nucleotide −839 of the ET_B gene, with a spliced region between nucleotide −51 and nucleotide −638. The fact that the nucleotide sequences surrounding nucleotides −51 and −638 fit the consensus sequences for acceptor and donor splicing sites (17), respectively, further confirms that the region from nucleotide −51 to −638 is an intron.

Expression of the ET_B gene in rat tissue. The tissue distribution of ET_B receptor mRNA was determined by Northern blot analysis using the radioprobe prepared from the coding region of ET_B cDNA. Fig. 7A shows that ET_B receptor mRNA was expressed at the highest level in the cerebellum and at substantial levels in the lung, eye, kidney, brain cortex, stomach, and liver (in decreasing order). Only one size of mRNA, corresponding to 5 kb, was detected in those tissues expressing receptor mRNA. Furthermore, the same Northern blot was rehybridized with a probe corresponding to the brain-specific 5′ noncoding region (nucleotides −157 to −191 of rat brain ET_B cDNA). A 5-kb transcript was also identified in those tissues expressing receptor mRNA (Fig. 7B), but with the highest levels in the eye and the cerebellum and moderate levels in the kidney, lung, brain cortex, stomach, and liver (in decreasing order). Obviously, the pattern of expression detected with the brain-specific sequence was different from that detected with the common coding sequence of ET_B cDNA. In particular, when carefully examining the relative levels in the lung, eye, and kidney in Fig. 7, we found that rat brain cDNA sequence was expressed much less in the lung, suggesting that the expression of this sequence might be somehow tissue-specifically regulated.

Disscussion

In the present study we have cloned a novel ET_B cDNA from rat brain. Nucleotide sequence analysis showed that the cloned rat brain cDNA differed from the previously cloned rat lung ET_B cDNA (5) by three extra nucleotides in its amino-terminal coding region and that both its 5′ and 3′ noncoding sequences were divergent from those in rat lung cDNA. However, functional expression assays demonstrated that the cDNA from rat brain encoded the ET_B receptor. Moreover, Southern blot analysis indicated that only one copy of the ET_B gene is present in the rat genome, suggesting that these two cDNAs for the ET_B receptor might be derived from the same gene.

The relationship between these two cDNAs was further investigated by genomic cloning and sequence analysis of the rat ET_B gene. After comparing the 5′-end sequence of the rat ET_B gene with the cloned cDNA from rat brain or rat lung, we found that the amino-terminal coding sequences in the ET_B gene were identical to those in rat brain cDNA. The reason why the amino-terminal coding region of rat lung ET_B cDNA contained three fewer bases is unknown thus far; however, it is possible that this disparity could be caused by a sequencing error. Moreover, a comparison of the 5′ flanking sequence suggests that rat lung cDNA and rat brain cDNA represent two distinct transcripts of the rat ET_B gene; one is derived from

nucleotide −203 of the ET_B gene, whereas the other is from nucleotide −839 of the ET_B gene, with a spliced region between nucleotides −51 and −638. Although no information is available thus far regarding the divergence of their 3′-end sequences, all the results strongly suggest that rat brain cDNA and rat lung cDNA represent two distinct transcripts of the rat ET_B gene. Whether these two transcripts are generated from a single primary transcript via alternative splicing or are transcribed initially at distinct promoters remains to be determined. Recently, structures of the human ET_A gene and the bovine ET_B gene were established by Hosoda *et al.* (18) and Mizuno *et al.* (19), respectively. Basically, their genomic organizations are very similar, except that intron 1 of the human ET_A gene occurs in the 5′ noncoding region, whereas that of the bovine ET_B gene exists in the coding region. However, in the case of the ET_B transcript from rat brain, intron 1 of the rat ET_B gene should exist in the 5′ noncoding region.

The present study also demonstrated that ET_B mRNA was expressed in a wide variety of rat tissues but only one size of ET_B mRNA was detected in the expressing tissues. In particular, there was no difference in the size of the ET_B transcripts expressed in the brain areas or in the lung. It is possible that different ET_B transcripts present in those tissues are of similar sizes and therefore indistinguishable by agarose gel electrophoresis. Preliminary Northern blot analysis demonstrated that the pattern of expression detected with the brain-specific sequence was significantly different from that of the ET_B gene, suggesting that the expression of the rat brain ET_B transcript might be tissue-specifically regulated. However, we cannot exclude the possibility that the previously cloned ET_B cDNA from rat lung might represent a transcript without splicing of the 5′ untranslated region and might also contain the brain-specific 5′ untranslated sequence in its 5′ flanking region, which was absent in the cloned rat lung cDNA due to the premature termination of reverse transcriptase activity, or the possibility that there is another unidentified ET_B transcript form that might also contain this sequence. Therefore, whether the expression pattern detected with the brain-specific sequence represents the tissue expression of the rat brain ET_B cDNA sequence remains to be determined.

In summary, the present study demonstrated that the sequence of this novel ET_B cDNA from rat brain constitutes the authentic coding sequence of the rat ET_B receptor. In addition, there are at least two distinct transcript forms of the rat ET_B gene. Whether there are more transcript forms of the ET_B gene present in other tissues and what kind of mechanism is involved in regulating their gene expression are currently under investigation. Our cloning of this novel rat ET_B cDNA not only provides a tool to study the physiological function of ET in the brain but also opens the field for study of the transcriptional regulation of this nonselective subtype of ET receptor.

Acknowledgments

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