Alternative RNA splicing of the human endothelin-A receptor generates multiple transcripts

Yoshihiro MIYAMOTO, Takaaki YOSHIMASA*, Hiroshi ARAI, Kazuhiko TAKAYA, Yoshihiro OGAWA, Hiroshi ITOH and Kazuwa NAKAO

Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606, Japan

In order to elucidate the regulatory mechanisms of expression of the human endothelin-A receptor (hET-AR) gene, we characterized hET-AR transcripts using reverse transcriptase (RT)-PCR analysis in a variety of human tissues. RT-PCR of lung mRNA using a set of primers from exons 2 and 5 showed two lower-molecular-mass transcripts in addition to the expected fragment. When RT-PCR with primers from exons 4 and 8 was performed, no transcripts other than the expected one were detected. PCR cloning utilizing a set of primers from exons 2 and 8 which covered the entire coding sequence revealed that the cDNA clones corresponding to the two novel transcripts contained deletions of 199 bp and 327 bp respectively compared with the previously described hET-AR cDNA. Comparison of their sequences with that of the hET-AR gene showed that the deleted sequences correspond exactly to exon 4 and exons 3 and 4 respectively, indicating that these lower-molecular-mass ET-AR transcripts result from alternative RNA splicing (designated ET-ARΔ4 and ET-ARΔ3,4 respectively). Alternative splicing of exon 4 results in a transcript which would be translated into a C-terminal truncated protein containing the first, second and third transmembrane domains, while the splicing out of exons 3 and 4 would produce a protein with five membrane-spanning domains but lacking the third and fourth domains present in the ET-AR protein. An RNase protection assay revealed that ET-ARΔ4 and ET-ARΔ3,4, as well as ET-AR, transcripts were observed in various human tissues, including the lung, aorta, atrium, kidney and placenta, which are known to express ET-AR abundantly. Thus we have isolated the cDNAs of novel transcripts of hET-AR which are generated by alternative RNA splicing, and these results suggest that this alternative RNA splicing might contribute to the regulation of ET-AR gene expression.

INTRODUCTION

Endothelin-1 (ET-1) was initially identified as a potent vasoconstrictor peptide comprised of 21 amino acid residues which was produced by cultured porcine aortic endothelial cells [1]. Cloning and sequence analysis of the ET genes revealed that ETs comprise a peptide family consisting of three isopeptides, ET-1, ET-2 and ET-3 [2–5]. Pharmacological and physiological studies have suggested the existence of multiple ET receptor (ET-R) subtypes [6–8]. In 1990 we succeeded in cloning the cDNA encoding an ET-1-selective ET receptor, the ET-A receptor (ET-AR), from a bovine lung cDNA library using the *Xenopus* oocyte expression system [9], while Sakurai et al. reported the cDNA cloning of the non-isopeptide-selective ET-B receptor (ET-BR) from rat lung using the COS-7 cell expression system [10]. Subsequently we have isolated the cDNAs for the human ET-AR (hET-AR) and the human ET-BR (hET-BR) from a human placenta cDNA library [11,12].

In order to elucidate the transcriptional regulation of ET-R genes and to analyse possible genetic disorders of ET-Rs, we have isolated and analysed the genes encoding the human ET-R family [13,14]. Southern blot hybridization analysis using human genomic DNA revealed that distinct single genes encode the hET-AR and hET-BR. Using a human–rodent somatic hybrid cell DNA, the genes for the hET-AR and hET-BR were assigned to human chromosomes 4 and 13 respectively.

Many genes encoding G-protein-coupled receptors, such as the β_2-adrenergic receptor [15], lack introns, but a number of exceptions are known. Both the hET-AR and hET-BR genes belong to the latter group. As shown schematically in Figure 1, the hET-AR gene consists of eight exons and seven introns spanning more than 40 kb, while the hET-BR gene spans 24 kb and contains seven exons and six introns. The first and second transmembrane domains of the hET-AR are encoded by exon 2, and each of other membrane-spanning domains is coded by one of exons 3–8 respectively. Similarly, exons 2–6 of the hET-BR encode the third to seventh transmembrane domain respectively, and exon 1 encodes both the first and second membrane-spanning domains.

Expression of the hET-R gene can be modulated at several levels. Alterations in transcription and mRNA stability might influence hET-AR gene expression. In fact, it has been shown that hET-AR gene expression can be modulated at the transcriptional level [16]. In the present study, in order to elucidate the regulatory mechanisms of hET-AR gene expression, we have characterized the hET-AR transcripts in a variety of human tissues using reverse transcriptase (RT)-PCR and demonstrated the alternative splicing of one or two exons to generate two novel hET-AR transcripts.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Superscript reverse transcriptase, RNase A and RNase T_1 from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.; oligo$(dT)_{15}$ primers from Promega Biotech., Madison, WI, U.S.A.; Gene Amp DNA amplification reagent kit and Taq polymerase from Perkins-Elmer Cetus, Norwalk, CT, U.S.A.; GeneScreen Plus

Abbreviations used: ET, endothelin; ET-R, ET receptor; (h)ET-AR, (human) ET-A receptor; (h)ET-BR, (human) ET-B receptor; RT, reverse transcriptase.

* To whom correspondence and reprint requests should be addressed.

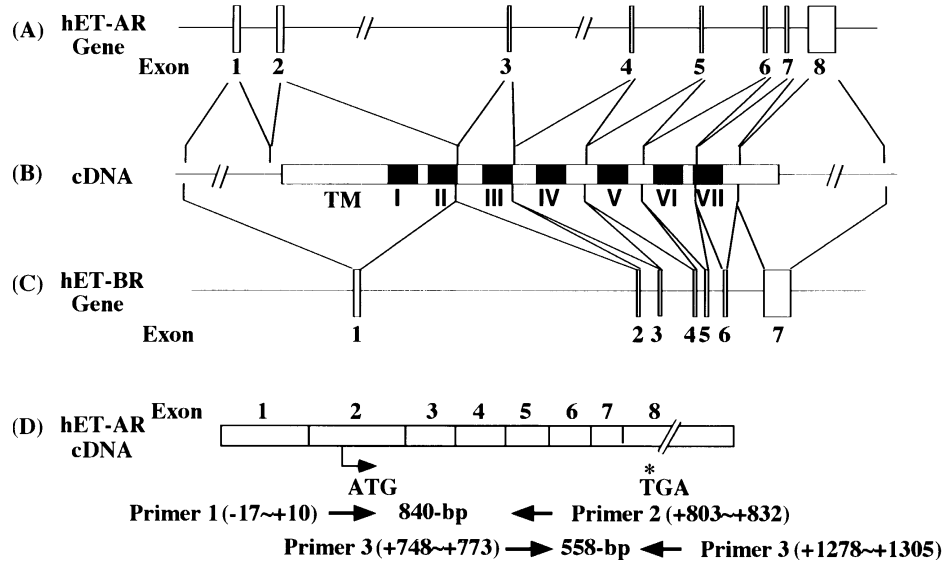

Figure 1 Detection of the three ET-AR mRNA isoforms in human tissues and human umbilical vein endothelial cells

Schematic representations are shown of the hET-AR gene (**A**) and of the hET-BR gene (**C**) and cDNA (**B**). Exons are represented by open boxes and are numbered (**A**, **C**). The regions encoding the membrane-spanning domains are represented by closed boxes and are numbered (**B**) TM, transmembrane domain. (**D**) Schematic representation of the structure of the mature 8-exon hET-AR mRNA. The translation start (ATG) and stop (TGA) codons are indicated in exons 2 and 8 respectively. The region of exons 2–5 was amplified using PCR with primers 1 and 2 as indicated. Similarly, the region of exons 5–8 was amplified using primers 3 and 4. The expected sizes of the PCR products are 840 bp and 558 bp respectively.

paper from NEN Products, Wilmington, DE, U.S.A.; restriction endonuclease and T_3 RNA polymerase from Toyobo, Osaka, Japan; pBluescript from Stratagene, La Jolla, CA, U.S.A.; Sequenase from United States Biochemical, Cleveland, OH, U.S.A.; pCDM8 from Invitrogen, San Diego, CA, U.S.A.; ET-1 and ET-3 from Peptide Institute, Minoh, Japan; ^{125}I-ET-1 and ^{125}I-ET-3 from Amersham International, Amersham, Bucks, U.K. Other reagents were obtained as described previously [13,14].

Preparation of RNA

Human tissues were obtained at autopsy or operation. Informed consent was obtained from each family. Human umbilical vein endothelial cells were cultured as previously reported [17]. Total RNA was extracted by the guanidinium thiocyanate method, and poly(A)-enriched RNA was prepared with an oligo(dT)–cellulose column. This study was approved by the Ethical Committee on Human Research, Kyoto University (No. 61-98).

RT-PCR analysis

A 10 μg sample of total RNA was reverse transcribed with Superscript RT and oligo(dT)$_{15}$ primers. A fraction (20 %) of the resulting single-stranded cDNA sample was used in the PCR. The hET-AR cDNA in the region of exons 2–5 and exons 4–8 was amplified with two sets of primers. Two pairs of primers with either a *Hin*dIII or *Xho*I restriction site at the 5′-end had the following sequences: primer 1, 5′-CTCAAGCTTGCCTCAAGATGGAAACCC-3′, −17 to +10 (sense); primer 2, 5′-TGACTCGAGAGAAGATCGCAGTGCACACCA-3′, +803 to +832 (antisense); primer 3, 5′-TTCAAGCTTGATGTAAAGGACTGGTG-3′, +748 to +773 (sense); primer 4, 5′-GATCTCGAGTTCTAAGGGTGGTCAGTTC-3′, +1278 to +1305 (antisense), as indicated by arrows in Figure 1(D). Complementary DNA templates were amplified with 1 unit of Taq polymerase in 50 μl of a reaction mixture containing 10 mM Tris, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml BSA, 3 mM dithiothreitol and 0.2 mM each of the nucleotides dATP, dCTP, dGTP and dTTP. The mixture was overlaid with 50 μl of mineral oil and subjected to 30 cycles of amplification (30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C), followed by a final extension for 5 min at 72 °C.

Southern-blot analysis

Amplified DNA fragments were size-fractionated on 1.5 % agarose gels and transferred to a nylon membrane. The membrane was hybridized with the ^{32}P-labelled oligonucleotide (hET-AR cDNA sequence-specific oligonucleotide) probes overnight at 37 °C in 6 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1 % SDS, 10 × Denhardt's solution, 20 μg/ml tRNA and 50 μg/ml denatured salmon sperm DNA. The membrane was washed using standard conditions with a final wash at 42 °C in 6 × SSC/0.1 % SDS. Autoradiography was performed for 6 h at −70 °C using Konica X-ray films with enhancing screens.

Isolation of cDNA encoding hET-AR isoforms

Since initial studies demonstrated that the PCR products of hET-AR transcripts in the region of exons 2–5 contained two smaller (approx. 640 bp and 510 bp) transcripts as well as the expected 840 bp transcript (see Figure 2), the portion of an agarose gel corresponding to the smaller transcripts was recovered from the gel, purified and subcloned into the plasmid Bluescript II SK$^+$. As the two smaller transcripts turned out to be novel isoforms which are generated by alternative splicing of exon 4 and exons 3 and 4 respectively, as detailed in the Results section, RT-PCR was performed using a set of primers in exons 2 and 8 which covered the coding sequence of the hET-AR cDNA, and the amplified fragments were subcloned into the plasmid Bluescript II SK$^+$. The sequence was determined by the dideoxy chain-termination method using Sequenase version 2.0. In addition to

T_3 and T_7 primers, sequence-specific oligonucleotides were synthesized using a DNA Synthesizer 381A (Applied Biosystems). Sequencing was carried out on both strands on denatured plasmid templates.

RNase protection analysis

To generate antisense cRNA probes, a 372 bp *Eco*RV–*Bam*HI fragment spanning from exon 2 to exon 4 of the hET-AR was subcloned into the *Eco*RV and *Bam*HI sites of the plasmid Bluescript SK^+. A ^{32}P-labelled antisense cRNA probe was transcribed from this construct with T_3 RNA polymerase after digestion with *Eco*RV; the total length of this probe, including the attached vector sequence, was 425 nucleotides. Synthetic cRNA corresponding to the sense strand of the alternative clone (hET-ARΔ3,4) was obtained from plasmid hET-ARΔ3,4 with T_3 RNA polymerase after digestion with *Xho*I, yielding a sense strand of approx. 1048 bases, of which 78 bases were derived from the plasmid vector.

Samples of 10 μg of total RNA from several human tissues and 10 pg of synthetic RNA were hybridized with a radiolabelled cRNA probe for 10 h at 60 °C in 80 % (w/v) formamide, 400 mM NaCl, 1 mM EDTA and 40 mM Pipes, pH 6.0, in a final volume of 30 μl. Following hybridization, the reactions were diluted 10-fold with a solution containing 300 mM NaCl, 10 mM Tris/HCl, pH 7.6, 5 mM EDTA, 20 μg/ml RNase A and 10 units/ml RNase T_1, and incubated for 30 min at 30 °C. The reaction solutions were then incubated for 30 min at 37 °C after adding 20 μl of 10 % SDS and 10 μl of 10 mg/ml Proteinase K. The protected riboprobes were purified by phenol extraction, precipitated with ethanol, dissolved in 5 μl of 80 % formamide containing 0.1 % Bromophenol Blue, 0.1 % xylene cyanol and 1 mM EDTA, and separated by electrophoresis in a 3.5 % polyacrylamide gel containing 7.5 M urea. After electrophoresis, the gels were dried and exposed to Konica X-ray films for 1–4 days at −70 °C with an intensifying screen.

Expression of hET-ARΔ3,4 in COS-7 cells

The *Hin*dIII–*Xho*I fragment of phET-ARΔ3,4 was subcloned into the eukaryotic expression vector CDM8 (pCDM8-hET-ARΔ3,4). The *Xho*I–*Not*I fragment of phET-AR subcloned into CDM8 [11] was used as a positive control. COS-7 cells were transfected with pCDM8-hET-ARΔ3,4 (20 μg of DNA per 100-mm dish) using the calcium phosphate method [12].

Competitive binding assay

At 60 h after transfection, COS-7 cells were incubated for 60 min at 37 °C in 1 ml of Hank's balanced salt solution containing 0.1 % BSA with a saturable concentration of either ^{125}I-ET-1 or ^{125}I-ET-3 (200 pM and 150 pM respectively) in the presence or absence of 0.1 μM unlabelled ET-1 and ET-3 (Peptide Institute, Minoh, Japan) respectively. Following the incubation, cells were solubilized in 0.5 ml of 1 M NaOH and associated radioactivity was measured in an Autogamma counter (Aloka, Tokyo, Japan).

RESULTS

cDNA isolation and sequence analysis of multiple ET-AR transcripts

Southern blot analysis of PCR-amplified hET-AR cDNA in the region of exons 2–5 showed the expected 840 bp segment in all human tissues examined. The transcript was detected at highest abundance in the aorta and cerebellum, at high levels in the lung, atrium and cerebral cortex, and at modest levels in the placenta, kidney, adrenal gland, duodenum, colon, ventricle and liver. No transcript was detected in human umbilical vein endothelial cells (Figure 2). These results are compatible with our previous study [13]. In addition, two lower-molecular-mass fragments were detected at approx. 640 bp and 510 bp respectively in almost all tissues, as shown in Figure 2. The same two transcripts were observed by RT-PCR using polyadenylated RNA from ventricle, lung and liver as template (results not shown). When RT-PCR utilizing a set of primers in exons 4 and 8 was performed, no transcripts other than the expected 558 bp fragment were detected (results not shown).

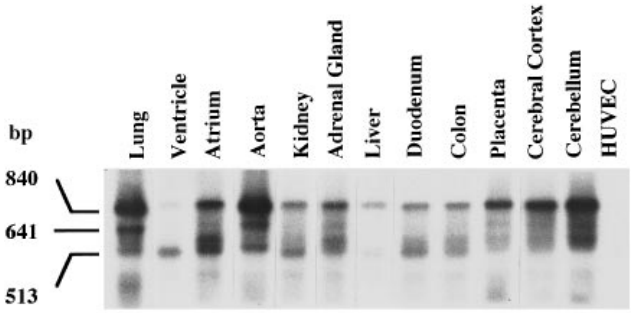

Figure 2 Expression of hET-AR transcripts in human tissues and human umbilical vein endothelial cells (HUVEC)

The PCR-amplified hET-AR transcripts obtained using primers 1 and 2 (Figure 1D) were evaluated by agarose gel electrophoresis and Southern blot hybridization. The positions on the gel of the 840, 641 and 513 bp fragments are indicated on the left.

Cloning of the cDNAs of the two lower-molecular-mass transcripts detected in the lung demonstrated that they represent 641- and 513-nucleotide transcripts. The nucleotide sequence of the 641-nucleotide transcript was identical to that of the previously described ET-AR cDNA [11], with the notable exception of a 199 bp deletion located between nucleotides 549 and 747 in the coding region. Similarly, the sequence of 513-nucleotide transcript was identical to that of ET-AR cDNA with the exception of a 327 bp deletion between nucleotides 421 and 747 (see the nucleotide sequence of the ET-AR cDNA in Figure 3A). Next, to compare the nucleotide sequences of the two clones with the complete coding sequence, RT-PCR in the region of exons 2–8, which covered the coding region of the hET-AR cDNA, was performed. The two clones corresponding to the deletion clones were newly isolated (Figure 3A). Comparison of these sequences with that of the ET-AR gene [13] showed that the missing sequences of the two transcripts exactly correspond to exon 4 and exons 3 and 4 respectively, as shown schematically in Figure 3B. These data indicate that these lower-molecular-mass ET-AR transcripts may result from alternative RNA splicing. Hence these clones were designated ET-ARΔ4 and ET-ARΔ3,4 respectively. As can be seen from the splicing pattern in Figure 3(B), the nucleotide deletion in the ET-ARΔ4 clone leads to a shift in the open reading frame and to premature termination of the polypeptide because of an aberrant stop codon at the fifth amino acid position of exon 5 (as indicated by the asterisks in Figure 3B). Therefore the ET-ARΔ4 transcript would be translated to give truncated protein 240 amino acids shorter at the C-terminus than the ET-AR protein. In contrast, although the ET-ARΔ3,4 clone lacked a longer fragment, the deletion leads neither to a frame shift nor to premature termination of the polypeptide. Thus it would result in a deletion of 109 amino acids in the region from the third to the fourth transmembrane

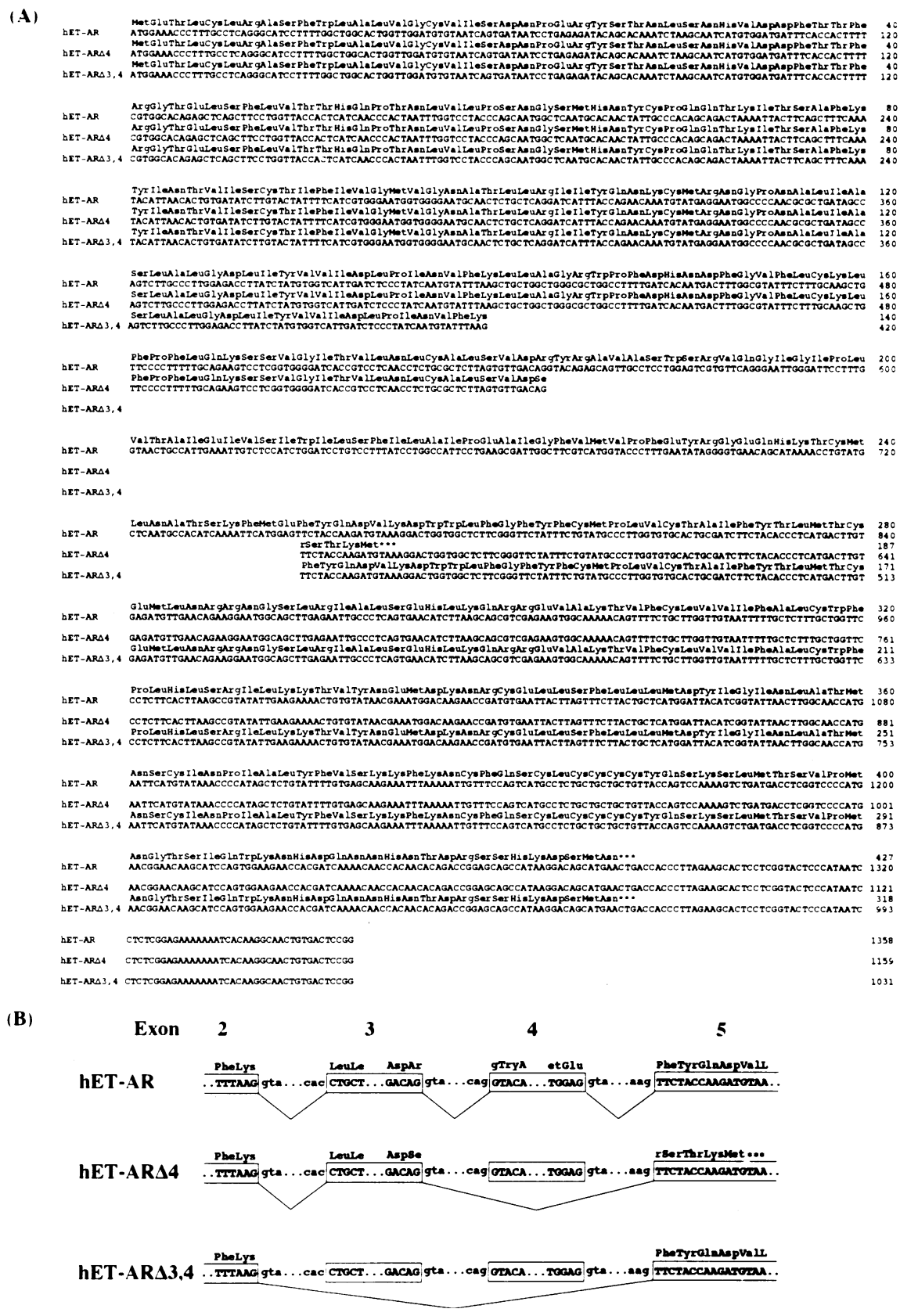

Figure 3 Nucleotide and deduced amino acid sequence of the three distinct hET-AR cDNA clones

(**A**) Alignments of the nucleotide and the deduced amino acid sequence of hET-AR, hET-ARΔ4 and hET-ARΔ3,4. Numbers to the right refer to the nucleotide sequence and the deduced amino acid residues. The region of exons 2–8 was amplified using PCR with primers 1 and 4 (see Figure 1D). The amplified fragments were subcloned and sequenced. (**B**) Organization of introns and exons spanning exons 2–5. Exon sequences are boxed and are shown in upper-case letters; intron sequences are represented in lower-case letters [13]. The splicing patterns identified in hET-AR, hET-ARΔ4 and hET-ARΔ3,4 are depicted. The deduced amino acids sequences are indicated above the boxes. The asterisks indicate an aberrant stop codon (TAA) in exon 5 for hET-ARΔ4.

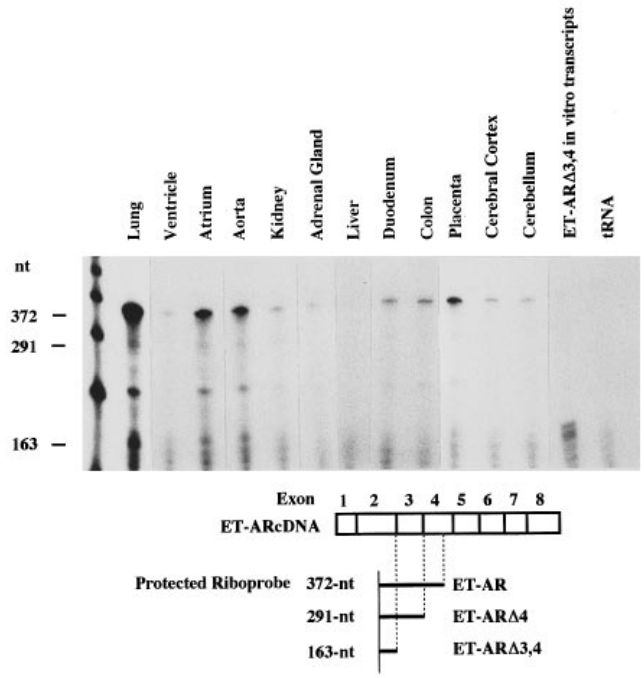

Figure 4 RNase protection analysis of hET-AR, hET-ARΔ4 and hET-ARΔ3,4 mRNAs in human tissues

The ^{32}P-labelled probe was hybridized to 10 μg samples of total RNA from human lung, ventricle, atrium, aorta, kidney, adrenal gland, liver, duodenum, colon, placenta, cerebral cortex and cerebellum. Synthetic RNA (10 pg) from clone hET-ARΔ3,4 and tRNA were used as controls. Protected fragments were resolved on a 3.5% polyacrylamide/urea gel. A schematic representaion of the expected lengths of the protected probes is presented below the autoradiograph.

domain, with an otherwise unchanged sequence compared with that of the ET-AR.

Subcloning and nucleotide sequence determination of the two transcripts at approx. 400 bp and 350 bp revealed that they do not have any identity with the ET-AR cDNA, indicating that they are non-specificially amplified PCR products (results not shown).

RNase protection analysis of hET-AR isoform RNA expression

Since we did not utilize quantitative PCR, we have used an RNase protection assay to determine the distribution of the two hET-AR transcripts that we have newly cloned. A 425-nucleotide radiolabelled RNA probe, spanning from exon 2 to exon 4 and including the attached vector sequence, was annealed with RNA from human tissues. As shown schematically in Figure 4, the antisense probe would protect 372 bases of hET-AR mRNA, 163 bases of hET-ARΔ3,4 mRNA and 291 bases of hET-ARΔ4 mRNA upon RNase digestion. As expected, when this probe was annealed with synthetic hET-ARΔ3,4 RNA, a fragment of 163 nucleotides was protected from RNase digestion. As shown in Figure 4, RNase protection with RNA from various human tissues generated a 372-nucleotide fragment whose distribution is compatible with that shown by RT-PCR followed by Southern blotting. In addition, the smaller 163-nucleotide fragment, which has the same size as synthetic hET-ARΔ3,4 RNA, was found in the lung, aorta, atrium, kidney and placenta, and the 291-nucleotide fragment was observed in the aorta, lung and atrium. These fragments were not detected when the RNA probe was annealed with the tRNA. Unexpectedly, an additional fragment was detected at approx. 200 nucleotides in almost all tissues. Taken together with the length and the site of the cRNA probe, this result suggests that the protected transcript might be one lacking exon 2 by alternative RNA splicing.

Expression of hET-ARΔ3,4 in mammalian cells

To further characterize the alternative hET-ARΔ3,4 isoform, either the hET-ARΔ3,4 expression vector (phET-ARΔ3,4) or a control vector (phET-AR) was transiently expressed in COS-7 cells, which were then assayed for ET isopeptide binding. Cells transfected with phET-AR specifically bound ^{125}I-ET-1 but not ^{125}I-ET-3, which is consistent with the ligand selectivity of the hET-AR [11]. No specific binding of ET-1 or ET-3 was observed in cells transfected with phET-ARΔ3,4 (results not shown).

DISCUSSION

The present study demonstrates that the hET-AR gene gives rise to at least three transcripts by alternative RNA splicing: the previously described hET-AR [13] and two novel isoforms which are generated by alternative splicing of exon 4 alone and of exons 3 and 4 (hET-ARΔ4 and hET-ARΔ3,4 respectively).

Genes for many members of the G-protein-coupled receptor superfamily, such as the β_2-adrenergic receptor [15], have been reported to contain no introns in the coding region. However, subsequent studies have revealed that, among genes for G-protein-coupled receptors, the substance K receptor, the substance P receptor, the neuromedin K receptor, the dopamine D_2, D_3 and D_4 receptors, the opsins, the luteinizing hormone receptor and the prostaglandin E receptor EP_3 subtype contain introns in the coding region [18–26]. As we reported previously [13], the hET-AR gene belongs to the latter group. The hET-AR gene contains eight exons and seven introns. Exon 1 is in a part of the 5′-non-coding region, and exon 2 encodes the first and second transmembrane domains. The other exons each encode one membrane-spanning domain. Among the G-protein-coupled receptors the use of alternative splicing to generate multiple molecular forms has been reported. It has been shown that the gene for the D_2 dopamine receptor directs the expression of two receptor isoforms, by alternative splicing of a cassette exon, which differ by the presence or the absence of a 29-amino-acid fragment located within the putative third cytoplasmic loop [27,28]. In addition, two variants of the thyrotropin-releasing hormone (TRH) receptor are produced by alternative splicing of a retained intron; one variant lacks an internal portion of a longer fusion exon presented in the TRH receptor mRNA [29]. The ET-ARΔ4 and ET-ARΔ3,4 isoforms described here are produced by alternative splicing of one or two cassette exons.

As we reported previously [11,13], hET-AR mRNA is expressed in a wide variety of human tissues. This mRNA is detected at highest abundance in the aorta, at high levels in the lung, atrium and colon, and at moderate levels in the cerebral cortex, cerebellum, ventricle, kidney, adrenal gland and duodenum. The present study demonstrated the same distribution of the hET-AR using an RNase protection assay. In addition we detected the two novel transcripts of ET-AR mRNA, the distribution of which parallels that of the longest ET-AR transcript. These results suggest a possible role for ET-ARΔ3,4 and ET-ARΔ4 in human tissues.

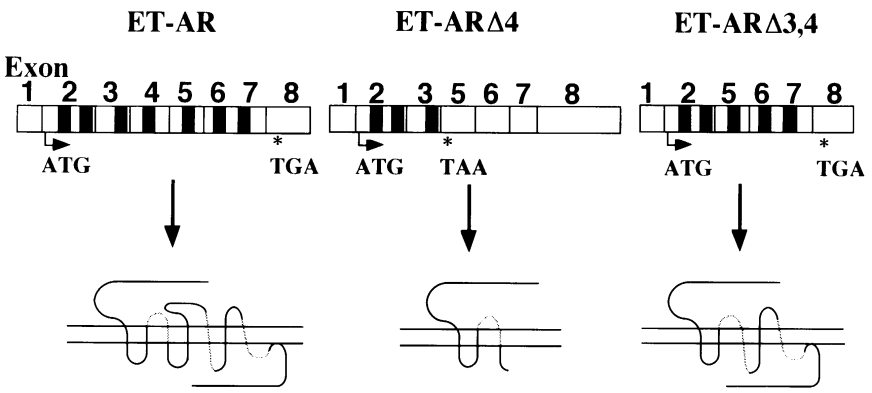

Figure 5 Schematic repesentation of putative protein structures of three isoforms generated from the hET-AR gene by alternative RNA splicing

Exons are represented by open boxes and are numbered. The regions encoding the transmembrane domains are represented by closed boxes. The translation start (ATG) and stop (TGA) codons are indicated in exons 2 and 8 respectively of the ET-AR and ET-ARΔ3,4 cDNAs, while the stop codon (TAA) of hET-ARΔ4 is located in exon 5. The ET-ARΔ4 protein is likely to have three transmembrane domains, and ET-ARΔ3,4 mRNA is putatively translated into a protein with five membrane-spanning domains.

The splicing of exons 3 and 4 in ET-ARΔ3,4 maintains an intact translation open reading frame for the downstream exons. Therefore the alternative splicing results in a transcript which would be translated into a protein with an extracellular N-terminal tail, five membrane-spanning domains, two cytoplasmic loops, two extracellular loops and a cytoplasmic C-terminal tail, as shown schematically in Figure 5. On the other hand, ET-ARΔ4 does not maintain an intact translational open reading frame for the downstream exons. Thus this alternative splicing of the ET-AR mRNA would produce a truncated form of ET-AR which contains an extracellular N-terminal tail, three membrane-spanning domains, a cytoplasmic loop, an extracellular loop and a truncated short intracellular tail (Figure 5).

What are functional properties of the novel isoforms? Radioligand binding experiments using either ^{125}I-ET-1 or ^{125}I-ET-3 demonstrated that ET-ARΔ3,4 did not show any specific ^{125}I-ET-1 or ^{125}I-ET-3 binding activity. Since the alternative transcript was identified in various human tissues, these results suggest that the apparent loss of binding capacity of ET-ARΔ3,4 could result from a defect at either the translational or the post-translational level, i.e. at any stage of translation and targeting to the plasma membrane. Indeed, unfolded or misfolded mutants of a seven-transmembrane-region receptor have been reported among the naturally occurring mutants of rhodopsin in patients with autosomal dominant retinitis pigmentosa [30]. Alternatively, there may be a loss of binding ability of the properly folded isoform in the plasma membrane, which is in accordance with a recent study showing that all seven transmembrane regions constitute the ligand binding domain [31]. Whichever alternative is correct, the high expression of the alternative transcript might alter the cellular responsiveness to ET, suggesting a regulatory role for alternative splicing in ET-AR function.

It has been reported that ET-AR and ET-BR gene expression can be modulated at the transcriptional level [16,32]. The alternative splicing of the ET-AR elucidated in the present study might represent another regulatory mechanism of ET-AR gene expression.

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