Identification of Cis-elements of the Human Endothelin-A Receptor Gene and Inhibition of the Gene Expression by the Decoy Strategy*

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Previously, we succeeded in molecular cloning of the cDNA and the gene for human endothelin-A receptor (ET-AR). In the present study, we define cis-elements in the 5' flanking region of the ET-AR gene. Deletion analyses were performed in A7r5 cells, rat vascular smooth muscle cell line, and Chinese hamster ovary cells using ET-AR promoter-luciferase gene constructs including 5 kilobases of the 5' flanking region. These analyses demonstrated the existence of one negative regulatory element (−2.0 kilobases to −857 bases) and two positive regulatory elements (−137 to −53 and −53 to +251). Gel mobility shift assay revealed a nuclear protein binding to the region (−104 to −78) (R1). DNase I footprinting analysis showed a footprint spanning from −91 to −83 whose sequence is CCCCACCTT (ETA-P1). When a plasmid including R1 fragments (R1 decoy) was co-transfected into A7r5 cells with ET-AR (−137 to +251)-luciferase gene construct, it significantly reduced the luciferase activity in a dose-dependent manner. Moreover, R1 decoy down-regulated the endogenous ET-AR mRNA in A7r5 cells by a maximum of 75%. Thus, we identified cis-elements that regulate basal transcriptional activity of the ET-AR gene and proved the feasibility to suppress the expression of the ET-AR gene by the DNA decoy strategy using the positive regulatory element we identified.

Endothelins (ETs) are a vasoactive peptide family consisting of three isopeptides, ET-1, ET-2, and ET-3 (1). ETs exhibit strong vasoconstrictive activity and a variety of biological effects via multiple subtypes of ET receptor (ET-R). At least two subtypes of ET-R have been isolated to date: an ET-1 selective ET receptor, ET-A receptor (ET-AR), and a non-isopeptide selective ET receptor, ET-B receptor (ET-BR). We have succeeded in molecular cloning of the cDNA encoding an ET-AR from a bovine lung cDNA library (2), and Sakurai et al. (3) reported the cDNA cloning of ET-BR from a rat lung cDNA library. Subsequently, we isolated the cDNAs for human ET-AR and ET-BR (4, 5). In humans, both subtypes of ET-R were expressed in a wide variety of tissues. The ET-AR mRNA is distributed at the highest level in the aorta and high levels in the lung, atrium, colon, and placenta (4). On the other hand, the ET-BR mRNA is expressed at the highest level in the cerebral cortex and cerebellum and at high levels in the placenta, lung, kidney, adrenal, colon, and duodenum (5). In the vascular wall, vascular smooth muscle cells (VSMC) express ET-AR and/or ET-BR, both of which can mediate the direct vasoconstrictive action of ETs, and endothelial cells express ET-BR that mediates vasorelaxation via endothelin-induced activation of nitric oxide synthase and synthesis of prostacyclin.

ETs are postulated to have potential physiological and pathophysiological roles. Mainly, they are thought to be involved in the maintenance of a vascular tone. ET-1 is produced from endothelial cells and causes potent and long acting vasoconstriction via ET-AR or ET-BR expressed on VSMC. In some pathological conditions, such as systemic and pulmonary hypertension, atherosclerosis, acute myocardial infarction, congestive heart failure, vasospasm due to subarachnoidal hemorrhage, and postischemic renal failure, ET-1 is thought to have potential roles (6). Recent introductions of ET-R antagonists have provided the evidence for the pathophysiological significance of ETs. Infusion of BQ-123, a selective ET-A antagonist, reduced the extent of experimentally induced myocardial infarction by 40% (7). Similarly, BQ-123 prevented the early cerebral vasospasm following subarachnoidal hemorrhage (8) and prevented acute renal failure following renal ischemia in rat (9). Moreover, long term treatment with BQ-123 reduced cardiac hypertrophy and improved long term survival in heart failure (10). Recent studies on ETs or ET receptor-deficient mice indicated a new aspect for their biological functions. ET-1 or ET-A-deficient mice had a craniofacial abnormality due to defects in the development of first pharyngeal arch-derived tissues (11). ET-3 or ET-B-deficient mice showed aganglionic megacolon and pigmentary disorders caused by developmental abnormality of neural crest-derived cell lineage (12, 13). The elucidation of the molecular mechanisms for the regulation of the ET-AR gene expression should provide a clue to understanding of such a physiological and pathophysiological role of ETs.

Growth of VSMC plays a critical role in the development of atherosclerosis and neointimal hyperplasia after balloon injury. In response to endothelial injury, the migration of VSMC from the medial layer to the intima triggers abnormal VSMC proliferation. Several growth factors are thought to be involved
in the migration and increase cell number (proliferation) of cultured VSMC (14). In addition to the vasoconstrictive effect, ET is shown to have a mitogenic effect on VSMC (15), suggesting its contribution to the processes of atherosclerosis and neointimal hyperplasia. Therefore, inhibition of the expression of either ET or ET-R may inhibit these processes and become a candidate molecule for a gene therapy of a proliferative vascular lesion.

There are some ways to modulate the expression of the selected gene using gene transfer techniques, i.e. antisense nucleic acids, ribozymes, transdominant mutation proteins, and suicide genes. “Decoy” strategy is one of the ways to regulate the gene expression at transcriptional level. Once introduced, short DNA fragments corresponding to the sequence of the cis-element, namely DNA decoy, will compete for the binding of transcription factors to their physiological target cis-element on the gene and then will modulate the transcription of the gene. Elucidation of the molecular mechanisms for transcriptional regulation of the gene and identification of the cis-element are essential for the DNA decoy strategy.

In the present study, to elucidate the molecular mechanisms for transcriptional regulation of the human ET-AR gene, we performed the analysis of the 5'-flanking region of the gene and identified positive and negative regulatory elements. In addition, using one of the positive regulatory elements as a DNA decoy, we showed that the DNA decoy can actually inhibit the ET-AR gene expression in A7r5 cells, which verify the feasibility of a DNA decoy strategy to specifically regulate the ET-AR gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from Toyobo (Osaka, Japan). Bacterial alkaline phosphatase, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Takara Shuzo (Shiga, Japan). Plasmid pBluescript was purchased from Stratagene. Gene Amp DNA amplification reagent kit and Taq polymerase were purchased from Perkin-Elmer. γ-32P]ATP was obtained from Amersham Life Science. BandShift kit and SureTrack Footprinting kit were purchased from Pharmacia Biotech Inc. Other reagents were purchased from standard commercial suppliers.

**Plasmid Construction**—We previously isolated a human genomic clone, hETAR1, that contained the 5'-end of the human ET-AR gene and 5 kilobases (kb) of the immediate upstream sequence (16). The 5'-half of the hETAR1 insert (from a SalI site in the multiple cloning site to an EcoRI site in the intron 2) was first subcloned into the plasmid vector pBluescript. Then various lengths of ET-AR 5'-flanking sequences were excised from the plasmid and were inserted into Picagene Basic vector (Toyobo, Tokyo, Japan) upstream of the luciferase reporter gene (17). A construct containing SalI-SacI fragment (from -5.0 kb to +426 bases, relative to the transcription initiation site) was named ETA-5.0k LUC. In the same way, serial 5'-truncated constructs were named ETA-2.0k LUC (HindII-SacI), ETA-857 LUC (BamHI-SacI), ETA-137 LUC (EcoRI-SacI), ETA-53 LUC (HindII-SacI), and ETA-251 LUC (Smal-SacI). Picagene Control Vector (Toyobo Inc., Tokyo, Japan) which has the SV40 promoter and enhancer upstream of the luciferase gene was used as a positive control.

**Cell Culture**—Rat vascular smooth muscle cell line (A7r5) was a generous gift of Dr. Fujimoto (Shionogi, Osaka, Japan) (18). A7r5 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Chinese hamster ovary (CHO) cells were maintained at 37 °C in ES medium supplemented with 10% fetal calf serum (19). The day before transfection, the cells were plated in 6-cm diameter dishes.

**Transfection and Luciferase Assay**—The ET-AR promoter-luciferase fusion plasmids were purified by CsCl/ethidium bromide gradients (19). The plasmids were introduced into the cells (~80% confluence) by the lipofection method using Genetransfer (20) (Wako Pure Chemical, Osaka, Japan). Cells were incubated in Eagle's medium with 0.3 μg of the plasmid that contained β-galactosidase gene downstream of the actin promoter (pAct-β-gal) for transfection efficiency. Cells were incubated for 16 h with the liposome-plasmid DNA, washed, and supplemented with fresh medium. After incubation for an additional 36 h at 37 °C, the cells were harvested and extracted in 100 μl of 0.25 M Tris-HCl (pH 7.5) by freezing-thawing. The cell extracts were assayed for luciferase activity using Luciferase Assay System (Toyo Ink, Tokyo, Japan) and for β-galactosidase activity (21). For the luciferase assays, the cell extract (20 μl) was mixed with 100 μl of the luciferase assay reagent which was automatically injected by the LB9501 Luminescence spectrofluorometer (Berthold Biomat, Berthold Analytical Instruments Inc, Nashua, NH). The ratio of luciferase activity to β-galactosidase activity was used to correct for transfection efficiency.
RESULTS

Functional Analysis of the Human ET-AR Promoter—Northern blot analysis revealed that A7r5 cells exclusively expressed the ET-AR gene, whereas the ET-AR mRNA was undetectable in CHO cells. These results were confirmed by the binding assay (data not shown). To analyze the transcriptional activity of different regions of the ET-AR promoter, various length of 5′-flanking sequences were fused to the luciferase reporter gene and introduced into A7r5 cells by transfection. The luciferase activities were normalized for transfection efficiency using the mean luciferase activity of the ETA-5.0k LUC as arbitrarily defined as 1.0, i.e., the mean luciferase activity of the ETA-5.0k LUC is arbitrarily defined as 1.0, and presented are the representatives of at least two separate experiments.

Northern Blot Analysis—Total RNA was prepared from cultured A7r5 cells using TRIzol reagent (Life Technologies, Inc.). RNA samples (10 µg) were separated on 1% formamide/agarose gels, transferred to a nylon membrane, Biodyne (PALL, Austria), and then hybridized with the ET-AR cDNA probe. The probe was labeled previously by random priming with [α-32P]dCTP. The blot was washed finally with 0.1 M NaCl/0.1% sodium dodecyl sulfate (SSC) at 60 °C. The same membranes were rehybridized with the 443-bp HindIII fragment of human β-actin genome probe (Wako Pure Chemical, Osaka, Japan). Imaging and quantification of ET-AR mRNA expression were also made by the image analyzer BAS2000.

Statistical Analysis—All results were expressed as mean ± S.D. with n = 3–6. Statistical analysis of the data was performed using analysis of variance. p < 0.05 was considered significant. The experiments presented are the representatives of at least two separate experiments.

Gel Mobility Shift Assay—Gel mobility shift assays were performed to detect proteins in A7r5 nuclear extract that associate with the region of the positive regulatory element. The 96-base pair (bp) fragment (F1) (+139 to –44 bases) was synthesized by PCR and end-labeled with [32P]ATP. This probe formed four prominent retarded bands upon incubation with A7r5 nuclear extract (Fig. 2), suggesting that the protein-DNA complexes were formed between F1 and A7r5 nuclear proteins. Two of the retarded bands (a and b) disappeared by adding an excess of the same unlabeled fragment. The formation of these protein-DNA complexes was not competed by an excess of the unrelated DNA (φX174 DNA digested by HaeIII). These results demonstrate that there exist some nuclear factors that specifically bind to the region.

To investigate the binding sites of A7r5 nuclear proteins in the region, seven 20-mer oligonucleotides were synthesized that cover the region from –139 to –44 bases as shown in Fig. 3a, and analogous gel mobility shift assays were performed using these oligonucleotides as competitors (C1–C7). As shown in Fig. 3b, addition of a 100-fold molar excess of either unlabeled C3 or C6 to the reaction mixture of nuclear extracts and the labeled F1 led to the disappearance of the uppermost band (band a) on the polyacrylamide gel. Taken together with the fact that these C3 and C6 are overlapping each other and spanning from –104 to –78 bases, these results suggest that a cis-element lies in the region between –104 and –78 bases (R1).

DNase I Footprinting Assay—To identify the actual binding site of A7r5 nuclear proteins, the labeled 300-bp fragment deletion to –53 bases (ETA-53 LUC) reduced promoter function by 70% (p < 0.001). This result shows that there exists a positive regulatory element between –137 and –53 bases. The luciferase activity became negligible with deletion to +251 bases (ETA-251 LUC), being compatible with the fact that the putative promoter, GC box, and the site of transcription-initiation (position +1) lie between –53 and +251 bases.

When the fusion plasmids were introduced into CHO cells, the levels of expression were comparable with those found in A7r5 cells (Fig. 1b). The profile of the 5′-deletion analysis in CHO cells was quite similar to that in A7r5 cells.

Fig 2. Gel mobility shift analysis of 5′-flanking region of the human ET-AR gene. An end-labeled probe was incubated with the A7r5 cell nuclear extract with or without unlabeled specific or nonspecific competitor. Probe, PCR fragment spanning position –139 to –44, end-labeled with (γ-32P)ATP (0.5 ng); nuclear extract, A7r5 cell nuclear extract (20 µg); specific competitor, unlabeled PCR fragment (30 ng); nonspecific competitor, φX174 DNA digested by HaeIII (30 ng). The specific protein-DNA complexes were designated as a and b.
ET-AR mRNA expression in A7r5 cells. Either 0–17.5 decoy strategy, we examined the effect of R1 decoy on the endogenous ET-AR gene expression by the competitive gel mobility shift analysis. An end-labeled probe was incubated in the presence of 20-mer competitors. Specific protein-DNA complexes demonstrated by the previous gel mobility shift analysis (Fig. 2) are designated as a and b. Probe, PCR fragment spanning position −139 to −44, end-labeled with [γ-32P]ATP (0.5 ng); nuclear extract, A7r5 cell extract (20 μg); 20-mer competitor, C1 to C7 (see “Experimental Procedures”).

Specific Binding of a Nuclear Protein in A7r5 to ETA-P1—To confirm the specific binding of a nuclear protein to ETA-P1, additional gel mobility shift assays were performed. The 27-base pair fragment containing ETA-P1 (F2) was synthesized and used as a probe and the specific competitor. Three fragments (m1–3) that contained mutated nucleotides in various regions of ETA-P1 were also synthesized and utilized as competitors (Fig. 5a). As shown in Fig. 5b, the incubation of the labeled F2 and nuclear extract from A7r5 revealed a single retarded band indicated by arrow (band c). The addition of a 30-fold molar excess of unlabeled F2 fragment as the specific competitor to reaction mixture resulted in compete disappearance of band c, whereas the addition of unlabeled mutated fragments (m1–3) showed much weaker competition on the band c. The densities of band c with the addition of a 30-fold molar excess of m1, m2, and m3 were approximately 98, 79, and 99%, respectively, compared with the band without competitor, and those with the addition of 90-fold molar excess of competitor were approximately 94, 61, and 65%, respectively. This result suggested the existence of the actual and specific binding of a nuclear protein in A7r5 to ETA-P1.

Competitive Co-transfection Assays—The function of ETA-P1 as a positive regulatory element was examined by competitive co-transfection assays. The plasmid ETA-137 LUC and various amounts of either R1 decoy plasmid or pBluescript were cotransfected into A7r5 cells. As shown in Fig. 6, R1 decoy reduced the promoter activity of the ETA-137 LUC in a dose-dependent manner. When 120-fold molar excess of R1 decoy was introduced, the luciferase activity was significantly decreased by about 30%. This result suggests that ETA-P1 acts as a positive regulatory element in A7r5 cells.

Inhibition of the ET-AR Gene Expression by Introducing R1 Decoy into A7r5 Cells—Finally, to demonstrate the feasibility to suppress the endogenous ET-AR gene expression by the decoy strategy, we examined the effect of R1 decoy on the ET-AR mRNA expression in A7r5 cells. Either 0–17.5 μg of R1 decoy plasmid or control pBluescript was introduced into A7r5 cells cultured in 10-cm diameter dishes by the lipofection method. When R1 decoy was introduced, the ET-AR mRNA level was decreased by 75% compared with the level in cells transfected with a control pBluescript at 48 h (Fig. 7). On the other hand, β-actin mRNA expression in R1 decoy-introduced A7r5 cells and pBluescript-transfected ones revealed no difference, suggesting that the introduction of R1 decoy suppressed the endogenous ET-AR gene expression specifically.

FIG. 3. Competitive gel mobility shift analysis of the human ET-AR promoter using 20-mer competitors. a, the 5′-flanking sequence of the human ET-AR gene. 5′-Ends of the 5′-deletion mutant in ETA-137 LUC and ETA-53 LUC are indicated by hooked arrows. The regions corresponding to the sequences of 20-mer competitors are indicated by arrows and numbered from 1 to 7. b, the results of competitive gel mobility shift analysis. An end-labeled probe was incubated in the presence of 20-mer competitors. Specific protein-DNA complexes demonstrated by the previous gel mobility shift analysis (Fig. 2) are designated as a and b. Probe, PCR fragment spanning position −139 to −44, end-labeled with [γ-32P]ATP (0.5 ng); nuclear extract, A7r5 cell extract (20 μg); 20-mer competitor, C1 to C7 (see “Experimental Procedures”).

FIG. 4. DNase I footprinting assay of the 5′-flanking region of the human ET-AR gene. Probe, PCR fragment spanning position −239 to 60, end-labeled with [γ-32P]ATP, and then digested 3′-end by BglII (1–2 × 104 cpm/lane); nuclear extract, various amounts of A7r5 nuclear extract. The sequence of the footprint, CCCCCACCTT, is indicated.
and m3.

The addition of 10-, 30-, and 60-fold molar excess of unlabeled F2, respectively, to the complexes is designated as a gel mobility shift assay using labeled F2 fragment as a probe. Specific protein-DNA complexes are formed in vitro in a sequence-specific fashion in A7r5 cells. The competition experiment using the excess of unlabeled F2 or m1–3, lanes 3–5, shows the results of gel mobility shift assay using labeled F2 fragment as a probe. Specific protein-DNA complexes are designated as band c (arrow). Nuclear extract, A7r5 nuclear extract (20 μg). Probe, F2 fragment end-labeled with [γ-32P]ATP (0.5 μg). Competitor, various amounts of unlabeled F2 or m1–3. Lane 1, probe only; lane 2, probe with nuclear extract; lanes 3–5, addition of 10-, 30-, and 60-fold molar excess of unlabeled F2, respectively; lanes 6–11, addition of 30- and 90-fold molar excess of m1, m2, and m3.

+251 bases. We previously analyzed the nucleotide sequence downstream of the position −858 bases in the 5′-flanking region of the human ET-AR gene and reported the existence of putative cis-acting elements including E box, GATA motif, CRE-like sequence, CArG box, and inverted acute phase reagent regulatory element upstream of GC box (16), but none of these elements has been demonstrated to be actually functional. As shown in Fig. 8, all of these consensus sequences were mapped in the region upstream of the position −137 bases, and the deletion from −857 to −137, which includes all these motifs, caused no changes in the luciferase activity. These results suggest that none of these cis-elements actually regulates the promoter activity of the ET-AR gene, and some novel cis-elements that exist in the downstream of −137 bases should confer the degree of the ET-AR promoter activity. The complete reduction of the luciferase activity after the deletion of the region from −53 to +251 bases suggests that cis-elements that define the core ET-AR promoter activity should exist in this region. As the putative promoter, GC box, and the transcription initiation site lie in the region, the reduction of the promoter activity was thought to be caused by the deletion of these regions that are essential for initiation of the transcription. But the possibility of the existence of some other novel positive regulatory elements except GC box in that region could not be excluded.

As reported (17, 18), A7r5 cells express ET-AR abundantly, whereas the expression of ET-AR mRNA is hardly detectable in CHO cells. Somewhat unexpectedly, the levels of the luciferase expression of the fusion plasmid in these cells were comparable to those found in the A7r5 cells. It is true that it is impossible to compare directly the luciferase expression in A7r5 cells and CHO cells, because the half-lives of the luciferase mRNA and/or protein may be different between the two cell lines. However, the ratio of the luciferase activity of the ET-AR chimera construct to that of Picagene Control Vector, which expresses luciferase gene driven by SV40 promoter and enhancer, should become one of the indicators to make a comparison of transcriptional activities in different cells. That is, the ratio of the luciferase activity of the ET-AR chimera construct to that of the Picagene Control Vector in A7r5 and CHO is 8.1 and 12.4%, respectively (data not shown), which strongly suggests that promoter activities of the constructs are comparable between these cells. In addition, the qualitative pattern of the expression in CHO cells is quite similar to that found in A7r5 cells. These results suggest that the positive and negative elements we identified may be employed in a wide variety of cells. The tissue-specific regulatory element should present outside the region we evaluated (i.e. downstream of exon 1 or far upstream of the 5′-flanking region) and might suppress the expression of the endogenous ET-AR gene in CHO cells and contribute to the tissue-specific expression of the ET-AR gene. Existence of tissue-specific regulatory element outside the 5′-flanking regions have been reported for many genes, i.e. apolipoprotein E/CI/CII and B (24, 25), c-myc (26), κ-immunoglobulin (27), and CD4 (28).

In an attempt to characterize the positive regulatory element, we performed the gel mobility shift assay using the PCR fragment (−139 to −44 bases) and the crude nuclear extract of A7r5 cells. Among four major retarded bands, upper two bands (a and b) were specifically competed by the excess of unlabeled PCR fragment (−139 to −44), suggesting that specific protein-DNA complexes are formed in vitro and demonstrate the existence of nuclear proteins that bind to the DNA segment in a sequence-specific fashion in A7r5 cells. The competition exper-
iment using 20-mer oligonucleotides revealed the existence of the protein-binding site between 2104 and 278 bases. In the competition experiment, the competition by the 20-mer competitors was observed against only the uppermost band (band a). The discrepancy of the results between the first gel mobility shift assay and the competitive experiment using 20-mer competitors might be explained by the existence of another protein-binding site outside the region between 2104 and 278 bases.

Since the weak competition against band b was observed in the presence of C5 competitor, it is possible that another protein-binding site that binds a nuclear protein with low affinity exists in the region corresponding to C5 (2124 to 2105 bases). But, in the present study, we could find no apparent footprint in that region in the following DNase I footprinting assay.

DNase I footprinting assay revealed one DNase I-protected region that covers the region from 291 to 283 bases. The sequence of the ETA-P1, CCCCACCTT, shows high homology to the positive regulatory element in the 5' flanking region of the human apolipoprotein (apo) E gene (Table I). 

The apoE B1 element was identified with positive activities upon the expression of apoE-CAT gene constructions and a footprint in the DNase I footprinting assay. Since the apoE B1 element has a positive effect on the expression in both the apoE-expressing HepG2 cells and the non-expressing HeLa cells, it is a non-tissue-specific positive regulatory element. Taking the similarities of the sequence and function between the apoE B1 element and ETA-P1, the same or quite similar nuclear protein might bind to these elements.

Additional gel mobility shift assay using F2 fragment as a probe confirmed the specific binding of a nuclear protein to the ETA-P1 region. Almost no competition with m1 (mutation of the 5' end of ETA-P1) and relatively weak competition with m3.
(mutation of the 3′-end) on the shifted band were observed, whereas moderate competition was observed with the m2 (mutation of the central portion), suggesting that both ends of ETA-P1, especially its 5′-end, are relatively more important for the specific binding of the protein to the element than the central portion of ETA-P1. Recently, 5′-flanking sequence of rat ET-AR gene was reported (30). ETA-P1 was highly conserved in rat ET-AR gene as a sequence of CCCCAGCTT, which included only one C to G nucleotide substitution in the central portion of the element. These results suggest that ETA-P1 should be critical for the regulation of ET-AR gene expression.

Competitive co-transfection assay using the fragment corresponding to the region from −104 to −78 bases (R1), the so-called decoy fragment, as a competitor shows that the decoy caused a dose-dependent reduction of the luciferase activity. The result indicates that there are trans-acting factors that interact with the cis-element in A7r5 cells, and they enhance the promoter activity of the ET-AR gene. This result also indicates the feasibility to control the expression of the endogenous ET-AR gene by introducing the decoy fragment into cells. Northern blot analysis for ET-AR in A7r5 cells after the introduction of R1 decoy demonstrated that R1 decoy could actually inhibit the endogenous ET-AR gene expression maximum by 75%, whereas the control pBluescript caused no significant change of its expression. Moreover, R1 decoy did not affect the β-actin mRNA expression in A7r5 cells. These results suggest that the decrease in the ET-AR mRNA level in A7r5 results from the inhibition of the binding of transcription factors to the endogenous ETA-P1 site by R1 decoy which lead to the transcriptional inhibition of ET-AR gene specifically. In the present study, the introduction of R1 decoy resulted in the 75% reduction of the ET-AR mRNA expression. The 75% reduction of mRNA level is thought to be enough to affect the phenotype of the host cells and individuals. In fact, recent study on the ET-BR-deficient mice demonstrate that Piebald mice, which expresses low levels of structurally intact ET-BR mRNA and protein (about 25% of control mice), produce coat color spotting (12). These results suggest that the decoy strategy provides an efficient way to regulate the ET-AR gene expression specifically.

In conclusion, we identified positive and negative regulatory elements in the 5′-flanking region of the human ET-AR gene by functional analysis. In addition, using one of the positive regulatory elements as a DNA decoy, we showed that the DNA decoy can actually inhibit the ET-AR gene expression in A7r5 cells, which proves the element is actually acting as a positive regulatory element in vivo and verifies the feasibility of the DNA decoy strategy to specifically regulate the ET-AR gene expression.

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