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Intronic enhancement of angiotensin II type 2 receptor transgene expression in vitro and in vivo

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Abstract

The angiotensin II type 2 receptor (AT2R) can influence a variety of intracellular signaling molecules and cellular functions. However, its physiological functions and the roles of introns in the regulation of its expression have not been well determined. We first demonstrated that both intron 1 and intron 2 of AT2R could significantly enhance AT2R overexpression. Thus, we have provided some new prerequisites for further studies on the mechanisms that control AT2R gene expression. Next, we established a highly efficient method of delivering this receptor in vitro and in vivo using an AT2R recombinant adenoviral vector containing two introns of the AT2R. The vector may be useful in helping to uncover AT2R physiological functions and also for gene therapy related to AT2R. Moreover, we determined the important role of introns in gene expression cassettes and the inconsistency of expression between the targeted gene and the reporter gene.

Keywords: Angiotensin II type 2 receptor; Introns; CMV promoter; Adenoviral vector; Enhancement of AT2R overexpression

Recent investigations have implicated roles for the angiotensin II type 2-receptor (AT2R) in cardiovascular, brain, and renal function, as well as in the modulation of various biological processes involved in development, cell differentiation, tissue repair, and apoptosis [1]. However, the AT2R-mediated physiological functions and intracellular signaling pathways are still far from being completely understood. One factor that has impeded progress in understanding the physiological roles of AT2R is that their expression within adult tissues is relatively low (compared with that of angiotensin II type 1-receptors [AT1R]), and that the AT2R antagonist may cross-react with AT1R [2].

The AT2R is a G-protein coupled receptor, whose gene is located on the X chromosome and spans approximately 5 kb. The AT2R gene is composed of two small non-coding exons, two introns of 152 bp (intron 1) and 1207 bp (intron 2), and exon 3 which contains the entire protein-coding region [3]. Intronic polymorphisms in the human AT2R gene have been suggested to have a genetic relationship with some diseases [4,5], but the roles of AT2R introns in the regulation of expression of this Ang II receptor subtype have not been well determined.

Highly effective expression of foreign transgenes within organs, tissues, and cells is an important issue in the study of gene therapy and gene function. Adenoviral (Ad) vectors are promising gene therapy vehicles due to their in vivo stability and efficiency, but their potential utility is compromised by the immunological response against viral proteins produced by infected cells and their restricted tropism [6–8]. Gutless adenoviruses and targeting strategies have been devised to address this issue, but less importance has been placed on improving the expression efficiency of each Ad particle unit [7,9,10].

Gene expression can be modulated at many levels, including transcription, post-transcriptional modification

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of RNA, RNA export and stability, and translation [6,11,12]. It has been demonstrated that higher transgene expression can be obtained via optimization of transcriptional regulatory elements such as the promoter, enhancer, intron and P(A) sequence, and post-transcriptional regulatory elements, such as Woodchuck hepatitis virus post-transcriptional regulation element (WPRE) [6,13].

In this study, we first demonstrated that both intron 1 and intron 2 of AT2R contributed to the significantly enhanced AT2R overexpression in different cell lines (C2C12, NIH/3T3, and CATH.a). We also found that AT2R introns increased overexpression of a heterogeneous gene, rat macrophage migration inhibitory factor (MIF). In order to establish a highly efficient method of delivering AT2R to tissues, we constructed an AT2R first generation recombinant adenoviral vector, Ad-G-AT2R-EGFP, containing genomic AT2R (G-AT2R) DNA with two introns. AT2R overexpression mediated by this vector was compared in vitro and in vivo to that obtained with Ad-C-AT2R-EGFP, which contains AT2R cDNA (C-AT2R). Finally, the mRNA level of AT2R and EGFP produced from the same kind of expression cassette was compared and analyzed. The results obtained should offer important insight into the development of highly efficient vectors.

Materials and methods

Preparation of recombinant adenoviral constructs

The rat genomic AT2R, which contains two introns, two small non-coding exons, and the third exon (the coding region), was derived from pcDNA3-AT2R, kindly provided by Dr. N. Fukuda (National Zentsuji Hospital, Kagawa, Japan). The rat AT2R cDNA was derived from pTYF-EF1-AT2R [14]. Both AT2R cDNA and AT2R genomic DNA were identified by sequencing and cloned into the shuttle vector pAdtrack-CMV using standard methods [15,16]. The foreign genes G-AT2R (genomic AT2R), C-AT2R (AT2R cDNA), and EGFP were driven by a separate CMV promoter within the transfer vectors pAdtrack-C-AT2R-EGFP and pAdtrack-G-AT2R-EGFP. The AdEasy Vector System was used for construction of two AT2R first generation recombinant adenoviral vectors: Ad-G-AT2R-EGFP containing G-AT2R DNA and Ad-C-AT2R-EGFP containing the AT2R encoding region [17]. The recombinant viruses were purified through two cesium chloride gradients and purified virus was then desalted by dialysis at 4 °C against 10 mM Tris–hydrochloric acid buffer with 4% sucrose and stored in aliquots at −80 °C. The titer of the viral preparations was determined using an Adeno-X Rapid Titer Kit (BD Biosciences, Palo Alto, CA).

Cloning of the AT2R introns and construction of plasmids containing the introns

Four primers were designed as follows, according to the sequence of rat AT2R introns:

IN1-F: 5′ TAAGTCAGAACAATTTAC 3′,
IN1-R: 5′ TGTAAGAGAAACAGCGGTT 3′,
IN2-F: 5′ TAAGTCTTTAGTGGGATTTA 3′,
IN2-R: 5′ TGGGGGAGAAAAAAGAGA 3′.

Intron 1, Intron 2 or Introns 1 and 2 were amplified by PCR using pcDNA-AT2R as a template with primers IN1-F and IN1-R, IN2-F and IN2R or IN1-F and IN2-R. The PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). They were then cloned between the CMV promoter and the AT2R cDNA of pAdtrack-CMV-C-AT2R vector, which were identified by DNA sequencing.

Rat MIF cDNA, derived from pShuttle-SYN-MIF which was constructed in our laboratory [18], was cloned into pAdtrack-CMV vector. The obtained recombinant plasmid was named pCMV-MIF. pCMV-AT2R/Int-MIF was constructed by cloning the AT2R Introns 1 and 2 between the CMV promoter and MIF cDNA.

Cell cultures, viral transduction, and plasmid transfections

Cell lines. C2C12 cells (mouse muscle myoblasts) and HEK 293 cells (human kidney epithelial cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NIH/3T3 cells (mouse embryo fibroblasts) were grown in DMEM plus 10% FBS. CATH.a cells (mouse locus coeruleus tumor cells) were maintained in growth medium consisting of RPMI 1640 supplemented with 8% horse serum and 4% FBS. PC12W (pheochromocytoma) cells were generously provided by Dr. Tad Inagami (Vanderbilt University, Nashville, TN). PC12W cells were grown in DMEM containing 10% fetal calf serum and 5% horse serum. All other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured using the protocol provided by the company. Sera and media were purchased from Invitrogen (Carlsbad, CA). For the plasmid transfection or viral transduction, cells (1×10^5) were seeded into 12-well Nunc tissue culture plates (Naperville, IL). On the following day, cells were transfected with plasmids plus lipofectamine 2000 according to the manufacturer's instructions or were transduced with Ad vectors (1×10^7 infectious units per well). Forty-eight hours later, AT2R expression in the cells was determined using an ^{125}I-(Sar^1, Ile^8)-Ang II receptor binding assay or real-time RT-PCR. Transfection or transduction was performed in triplicate and repeated at least once.

Primary cell cultures. Primary cortical neuronal cultures were prepared from newborn Sprague–Dawley (SD) rat pups (Charles River Farms, Wilmington, MA) exactly as described previously [19]. Ad-G-AT2R-EGFP or Ad-C-AT2R-EGFP viral particles were added directly to the media surrounding the cultures at 7–10 days after plating (1×10^7 infectious units per well or per dish), and the expression of AT2R was analyzed 3 days later. Each experimental number (n) represents data obtained from neuronal cultures prepared from separate litters of pups.

Adenoviral-mediated gene transduction in vivo

Adenoviral vectors (6×10^9 infectious units) were injected intracardially (left ventricle) into 6-week-old male SD rats. Three days later, rats were euthanized, the liver, heart, and kidney were removed, and the expression of AT2R or EGFP in each tissue was determined by real-time PCR.

AT2R binding assay

This was performed as described previously [20]. Transduced cells in 12-well plates were washed with phosphate-buffered saline (pH 7.4) and incubated for 1 h at room temperature with 0.1 nM ^{125}I-(Sar^1, Ile^8)-Ang II (2176 Ci/mmol; University of Mississipi, Oxford, MS) in the absence or presence of unlabeled Ang II (Sigma, St. Louis, MO) to assess non-specific binding or the AT2R-selective blocker (PD123319,

Sigma, St. Louis, MO) to assess AT2R-specific binding. Cells were washed four times with phosphate-buffered saline, lysed in 0.5 M NaOH, and the bound ^{125}I-(Sar^{1}, Ile^{8})-Ang II in the lysate under each condition was quantified in a gamma counter (LKB-Wallac Clini-Gamma 1272, Turku, Finland) [21].

Real-time RT-PCR for the detection of AT2R, EGFP or MIF mRNA

Oligonucleotide primers for specific rat AT2R , EGFP or MIF were designed from the GenBank databases using Primer Express (Applied Biosystems, Foster City, CA). The primer sequences were as follows:

AT2R-F: 5′-CCGCATTTAACTGCTCACACA-3′,
AT2R-R: 5′-ATCATGTAGTAGAGAACAGGAATTGCTT-3′,
EGFP-F: 5′-GAGCTGAAGGGCATCGACTT 3′,
EGFP-R: 5′-CTTGTGCCCCAGGATGTTG-3′,
MIF-F: 5′-CAAGCCGGCACAGTACAT-3′,
MIF-R: 5′-GCTCGAGCCACTAAAAGTCATG-3′.

Total RNA was prepared from the transduced cultures or tissues using an RNeasy kit (Qiagen , Valencia, CA). Two-step RT-PCR was performed according to the protocols of the manufacturer with an ABI Prism 7000 HT Detection System (Applied Biosystems) using SYBR Green PCR Master Mix. Relative quantification was performed using the comparative method as described in Applied Biosystems User Bulletin 2 using ribosomal RNA (18S) as an endogenous control. No reverse transcriptase and no template controls were used to monitor any contaminating amplification.

A standard curve was generated with primers for EGFP or AT2R using a 5 log spanning serial dilution of the vector plasmid pAdtrack-C-AT2R-EGFP containing one EGFP and AT2R per plasmid molecule. Serial dilution ranged from 0.01 to 100 pg of the vector plasmid. The standard curves were calculated by regression of the crossing points of the PCR curves from the dilution series of the vector plasmid.

Animal procedures

All animal procedures were approved by the University of Florida Institutional Animal care and Use Committee.

Results

Intronic enhancement of AT2R expression in vitro

In the first series of experiments, we examined whether the presence of AT2R introns can influence the expression of AT2R in vitro. The following AT2R recombinant plasmids were constructed: pAT2R-INTRON1-cDNA, pAT2R-INTRON2-cDNA, pAT2R-INTRON1&2-cDNA, and pAT2R-anti-INTRON1&2-cDNA, which contain (respectively) AT2R-intron 1, -intron 2, -introns 1 and 2, and antisense introns 1 and 2 situated between the CMV promoter and AT2R cDNA. As shown in Fig. 1, pAT2R-INTRON1-cDNA, pAT2R-INTRON2-cDNA, and pAT2R-INTRON1 and 2-cDNA and pAdTrack-G-AT2-EGFP generated 1.6- to 6.5-fold higher levels of AT2R specific binding in transfected cell lines (NIH/3T3, C2C12, and CATH.a) than did pAdtrack-C-AT2R-EGFP(C-AT2R). Moreover, there was no significant difference

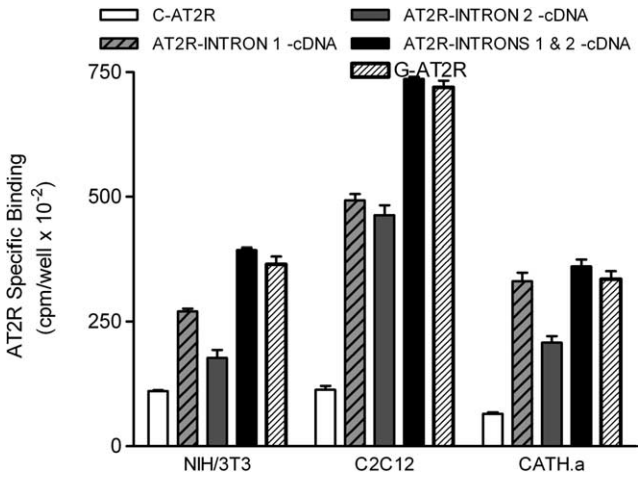

Fig. 1. AT2R receptor expression in cell cultures transfected with AT2R recombinant plasmids. NIH/3T3, C2C12, and CATH.a cells were transfected with 400 ng of the AT2R recombinant plasmids pAdtrack-C-AT2R-EGFP (C-AT2R), pAT2R-INTRON1-cDNA, pAT2R-INTRON2-cDNA, pAT2R-INTRON1&2-cDNA or pAdtrack-G-AT2R-EGFP(G-AT2R) for 48 h. Incubations were followed by analysis of AT2R receptor-specific binding as described in the Materials and methods. Bar graphs show the level of AT2R-specific binding in each case. Data are means ± SEM are from three experiments.

in AT2R overexpression between pAT2R-INTRON1&2-cDNA and pAdTrack-G-AT2R-EGFP. In descending order, the effectiveness of the AT2R introns in enhancing the expression of AT2R in vitro were: INTRONS1&2 > INTRON1 > INTRON2. pAT2R-anti-INTRON1&2-cDNA did not produce any AT2R expression in the transduced cells (not shown).

Next, we determined whether the presence of AT2R introns could enhance the expression of an unrelated gene, rat MIF. PC12W cells were transfected with pCMV-MIF or pCMV-AT2R/Int-MIF, and MIF mRNA was assessed 48 h later via real-time RT-PCR. The data presented in Fig. 2 demonstrate that pCMV-

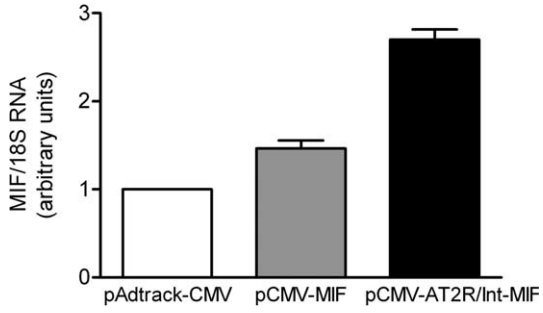

Fig. 2. Levels of MIF mRNA in PC12W cells transfected with pCMV-MIF or pCMV-AT2R/Int-MIF. PC12W cells were transfected with 400 ng of plasmids pAdtrack-CMV, pCMV-MIF or pCMV-AT2R/Int-MIF for 48 h, followed by analysis of MIF mRNA by real-time RT-PCR as described in the Methods. Data are means ± SEM from 3 experiments , normalized to 18S RNA and presented as arbitrary units.

AT2R/Int-MIF elicited a 3.2-fold higher increase in MIF expression than did pCMV-MIF.

Ad-G-AT2R-EGFP-mediated AT2R expression in vitro and in vivo

For these studies, we constructed two first generation AT2R recombinant adenoviral vectors: Ad-G-AT2R-EGFP containing genomic AT2R (G-AT2R) DNA with introns 1 and 2, and the encoding region, and Ad-C-AT2R-EGFP containing the encoding region of the AT2R cDNA (C-AT2R) (Fig. 3). Transduction of CATH.a neural cells, rat primary neuronal cultures, NIH/3T3 fibroblasts, and C2C12 myoblast cells with Ad-G-AT2R-EGFP led to respective 2.2-, 41.6-, 100.2-, and 231.4-fold higher increases in AT2R expression than those obtained with Ad-C-AT2R-EGFP, according to receptor binding analyses (Fig. 4). Consistent with this, real-time RT-PCR analysis demonstrated that the AT2R mRNA level in neuronal cultures transduced with Ad-G-AT2R-EGFP was 40.8-fold greater than with the Ad-C-AT2R-EGFP construct (Fig. 5A).

Three days after intracardiac injection into adult SD rats, expression of AT2R mRNA was detected in the liver, heart, and kidney via real-time RT-PCR. Ad-G-AT2R-EGFP led to a 369.2-fold higher AT2R expression in rat liver compared with Ad-C-AT2R-EGFP (Fig. 5B). A significant increase of AT2R mRNA levels was also detected in the hearts and kidneys of rats injected with Ad-G-AT2R-EGFP compared with control rats, while there was no detectable increase in AT2R mRNA in the rats injected with Ad-C-AT2R-EGFP (data not shown).

Comparison of AT2R and EGFP mRNA levels following overexpression in vitro and in vivo

To assess whether the expression of the reporter gene EGFP was consistent with that of the foreign gene (AT2R), which is located in the same expression cassette as EGFP, levels of AT2R and EGFP mRNAs were

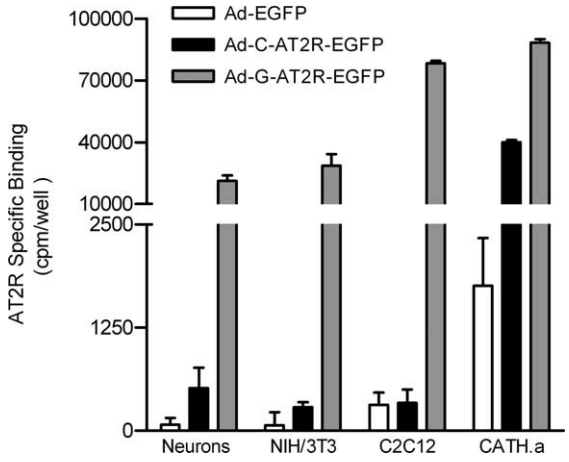

Fig. 4. Adenoviral-mediated AT2R receptor expression in primary neuronal cultures and cell lines. Cell cultures were transduced with 1×10^7 ifu per well/dish of either Ad-EGFP, Ad-G-AT2R-EGFP or Ad-C-AT2R-EGFP for 48 h (NIH/3T3; C2C12 or CATH.a cells) or 72 h (primary cortical neurons; neurons). This was followed by analysis of AT2R receptor-specific binding as described in the Materials and methods. Bar graphs show the level of AT2R-specific binding in each case. Data are means ± SEM are from three experiments.

detected and compared using real-time PCR in rat primary neuronal cultures or SD rats transduced with the Ad vectors Ad-G-AT2R-EGFP and Ad-C-AT2R-EGFP. We first determined the standard curves generated with primers for EGFP or AT2R using a serial dilution of the vector plasmid pAdtrack-C-AT2R-EGFP. The results demonstrated that the standard curves overlapped, and so the amplification efficiency of PCR using primers for AT2R was the same as that obtained with the primers for EGFP. Thus, we could directly compare the levels of AT2R and GFP mRNAs by real-time RT-PCR. Ad-G-AT2R-EGFP produced the same level of EGFP and AT2R mRNA in transduced neuronal cultures or rat livers, but there was much lower expression of AT2R mRNA compared with EGFP mRNA following transduction with Ad-C-AT2R-EGFP. Both Ad-G-AT2R-EGFP and Ad-C-AT2R-EGFP elicited the same

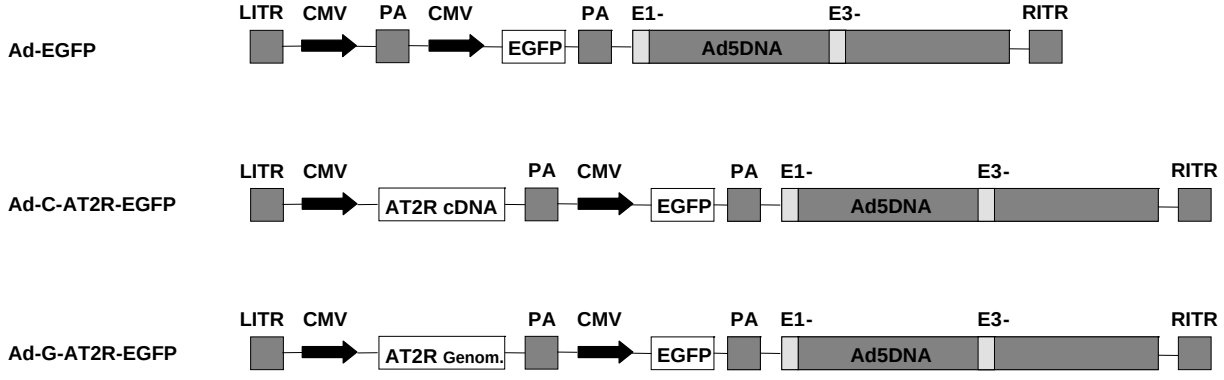

Fig. 3. Schematic representation of Ad vectors used in this study. EGFP, enhanced green fluorescence protein reporter gene; AT2R, rat angiotensin (Ang) II type 2 receptor; ITR, Ad inverted terminal repeats; CMV, human cytomegalovirus promoter; PA, SV40 polyadenylation sequence.

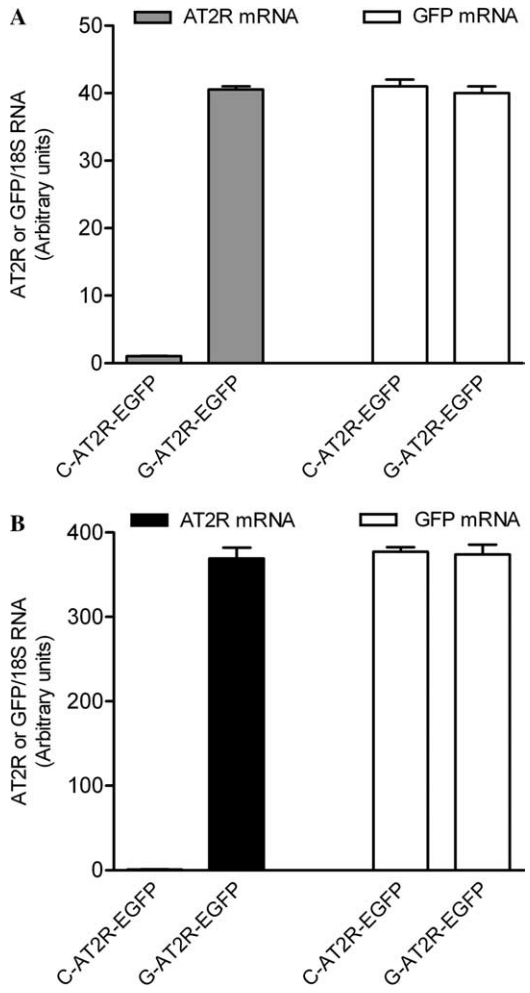

Fig. 5. Levels of AT2R or GFP mRNAs following Ad-C-AT2R-EGFP or Ad-G-AT2R-EGFP transduction in vitro and in vivo. (A) Primary cortical neuronal cultures were transduced with either Ad-G-AT2R-EGFP (G-AT2R-EGFP) or Ad-C-AT2R-EGFP (C-AT2R-EGFP) [both 1×10^7 ifu per dish] for 72 h, followed by analysis of AT2R and GFP mRNAs by real-time RT-PCR as described in the Materials and methods. (B) SD rats were injected intracardially with either Ad-C-AT2R-EGFP or Ad-G-AT2R-EGFP (both 6×10^9 infectious units). Three days later rats were euthanized, livers were removed, and levels of AT2R and GFP mRNAs were analyzed as above. Data are means ± SEM from 3 experiments (A) or rats (B) and are normalized to 18S RNA and presented as arbitrary units.

level of EGFP expression (Figs. 5A and B). This result suggests that at the transcriptional level, the high level expression of EGFP as a reporter gene may not always predict whether other foreign genes will be expressed at a similarly high level.

Discussion

The AT2R receptor gene, which is located on the X chromosome, contains two non-coding exons, two introns, and a third exon which harbors the entire uninterrupted open-reading frame of the AT2R receptor gene [3]. The structure of rat AT2R was identical to those of the mouse AT2R gene [22] and the human AT2R gene [3,23]. Recently, intron 1 of AT2R has provoked the researchers' interest in its genetics. The biallelic polymorphism G + 1675A in intron 1 of the AT2R receptor gene has been associated with left ventricular posterior, septal, and relative wall thickness, left ventricular mass index in young hypertensive males as well as congenital anomalies of the kidney and urinary tract [4,5,24]. Since we determined that intron 2 of AT2R could enhance AT2R expression as well as intron 1, it will be a potential region to be explored in studies of the genetic relationship between AT2R and certain diseases.

Recombinant adenoviral vectors continue to be the preferred vectors for studies in gene function and gene therapy. However, because of the immunological response against viral proteins produced by infected cells and their restricted tropism, the clinical application of Ad vectors has been limited. To alleviate this kind of immunological toxicity, one simple, but practical, method is to improve the expression efficiency of each viral particle unit so that the number of Ad particles that must be used can be decreased. In addition, the improvement of expression efficiency will be helpful in circumventing the tropism of Ad vectors and will make it possible to apply them in targeted tissues or organs where Ad vectors normally transduce with only low efficiency.

In this study, we constructed a genomic AT2R first generation recombinant adenoviral vector Ad-G-AT2R-EGFP and determined that the introns of AT2R significantly enhanced its overexpression both in vitro and in vivo compared with the AT2R cDNA recombinant adenovirus Ad-C-AT2R-EGFP. The application of the Ad-G-AT2R-EGFP vector should make the Ad vector more practical and useful in studies of AT2R gene function and gene therapy related to AT2R. AT2R transgenic and knockout animals have been used to study the role of AT2R in cardiac hypertrophy, cardiomyopathy, heart failure, and interstitial fibrosis. However, the results are conflicting, which may be due to inherent issues related to the involvement of the AT2R in the development of some tissues or organs [25–27] or to the global changes in gene expression produced in knockout or transgenic models. We believe that our highly efficient method of delivering AT2R to tissues after normal development has an advantage over the transgenic models and will circumvent these developmental and specificity issues [20].

The role of two small non-coding exons of the genomic AT2R has not been well determined. As shown in Fig. 1, both intron 1 and intron 2 of AT2R could significantly enhance AT2R overexpression and the effectiveness of the AT2R introns in enhancing the expression of AT2R in vitro was: INTRONS1 and 2 > INTRON1 > INTRON2. Moreover, pAdTrack-G-AT2-EGFP

containing the genomic AT2R and pAT2R-INTRON1 and 2-cDNA elicited a similar level of AT2R overexpression in transduced cells. Obviously, two small non-coding exons of the genomic AT2R did not significantly influence the enhancement of introns in AT2R overexpression.

EGFP is widely applied used for in vitro and in vivo studies as a reporter gene [28,29]. However, our results showed that the expressed mRNA level of the foreign gene was not always consistent with the EGFP under the control of the same kind of promoter. Similar AT2R and EGFP mRNA levels were detected in primary neuronal cultures transduced with Ad-G-AT2R-EGFP or rat tissues which were systemically injected with Ad-G-AT2R-EGFP, while the AT2R mRNA level was much less than the EGFP in Ad-C-AT2R-EGFP transduced primary neuronal cultures or infected rat tissues. The difference between AT2R and EGFP mRNA expression may be dependent on their introns. It is clear that AT2R mRNA expression is highly dependent on introns, but the EGFP expression is independent. The low level expression of some foreign genes may due to the absence of introns. Thus, the addition of introns may be able to significantly increase the expression of these genes. We have also obtained increased expression of macrophage migration inhibitory factor (MIF) by the insertion of AT2R introns between the CMV promoter and MIF.

In summary, we demonstrated that both intron 1 and intron 2 of AT2R can significantly enhance overexpression of AT2R or heterogeneous gene, rat MIF. With the identification of the introns of the rat AT2R gene as regions with high regulatory potency, we have provided some new prerequisites for further studies on the mechanisms that control rat AT2R gene expression in development and pathological situations. Furthermore, we have established a highly efficient method of delivering AT2R using a genomic AT2R recombinant adenoviral vector, Ad-G-AT2R-EGFP, which will become a powerful tool to study AT2R function and gene therapy related to AT2R. AT2R introns were determined to contribute to the significantly enhanced AT2R overexpression mediated by the vector, and in the future can hopefully be employed for optimization of transcriptional regulatory elements at the stage of Ad vector design. The highly efficient expression of the reporter gene (EGFP) may not predict the same level of expression of other foreign genes under the control of the same kind of expression cassette because of their different dependence on introns, while insertion of introns between promoter and foreign genes may significantly increase their overexpression.

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