Efficient transcription of the human angiotensin II type 2 receptor gene requires intronic sequence elements

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To investigate mechanisms of human angiotensin II type 2 receptor (hAT2) gene regulation we functionally characterized the promoter and downstream regions of the gene. 5'-Terminal deletion mutants from -1417/+100 to -46/+100 elicited significant but low functional activity in luciferase reporter gene assays with PC12W cells. Inclusion into the promoter constructs of intron 1 and the transcribed region of the hAT2 gene up to the translation start enhanced luciferase activity 6.7 ± 1.6 -fold and 11.6 ± 1.7 -fold (means $\pm$ S.E.M.) respectively, whereas fusion of the promoter to the spliced 5' untranslated region of hAT2 cDNA did not, which indicated an enhancement caused by intronic sequence elements. Reverse transcriptase-mediated PCR confirmed that the chimaeric hAT2-luciferase mRNA was regularly spliced in PC12W cells. A Northern blot analysis of transfected cells showed levels of luciferase mRNA expression consistent with the respective enzyme activities. Mapping of

intron 1 revealed that a 12 bp sequence in the centre of the intron was required for the increase in promoter activity, whereas the 5' adjacent intronic region mediated a decrease in luciferase activity. Mutation of the 12 bp region led to altered protein binding and markedly decreased luciferase activity. Cloned into a promoterless luciferase vector, a 123 bp intron 1 fragment was able to direct reporter gene expression to the same activity as occurred in conjunction with the 5' flanking region. These results indicate that sequence elements in intron 1 are necessary for efficient transcription of hAT2. In reporter gene assays, intron 1 might by itself function as a promoter and initiate transcription from an alternative start point.

Key words: *cis*-active DNA element, promoters, reporter gene assay.

INTRODUCTION

The octapeptide angiotensin II (Ang II), as the primary effector molecule of the renin-angiotensin system, is an important regulator of blood pressure, fluid balance and electrolyte balance and seems to be involved in the pathogenesis of cardiovascular disorders such as hypertension and cardiomyopathies. Two seven-transmembrane Ang II receptor subtypes have so far been identified in humans. Most of the known effects of Ang II are mediated by the G-protein-coupled Ang II type 1 receptor (AT1). The function, regulation and signal transduction of the Ang II type 2 receptor (AT2) are still far from being understood. AT2 receptors are abundantly expressed in fetal tissues but are rapidly down-regulated after birth, which suggests a role in development and differentiation [1,2]. In the adult, AT2 expression is restricted to a few tissues (adrenals, uterus, ovary, brain, kidney, heart and endothelium) and is up-regulated under pathological circumstances such as neointima formation after vascular injury, cardiac remodelling and infarction [3–5]. AT2 exerts anti-proliferative effects in rat coronary endothelial cells [6] and vascular smooth-muscle cells [7], and induces apoptosis in AT2-expressing rodent cells [8,9]. These effects, as well as other AT2 receptor-mediated responses, are thought to be mediated by the activation of protein tyrosine phosphatases [10,11]. In AT2 knock-out mice, disruption of the AT2 gene increased the basal blood pressure and the Ang II-induced, AT1-mediated rise in blood pressure respectively [12,13].

Results on the transcriptional regulation of the AT2 gene are based almost exclusively on the rodent genes owing to the lack of AT2-expressing human cell lines [14–22]. The mouse AT2 promoter was activated by a high intracellular ratio of interferon regulatory factors 1 and 2 [16]. In rat aortic vascular smooth-muscle cells and rodent AT2-expressing R3T3, PC12 and PC12W cells, AT2 mRNA levels were down-regulated by various peptide growth factors, glucocorticoids and factors that activate the protein kinase C pathway [17–21], whereas insulin, insulin-like growth factors and interleukin 1 β up-regulated AT2 [20,22]. Transcriptional and post-transcriptional mechanisms contributed to the described regulations. Because differently spliced AT2 mRNA species have been demonstrated [5], translational regulation by different 5' untranslated regions (5' UTRs) as described for AT1 mRNA species [23] could be a further regulatory mechanism.

The AT2 gene is located on the X chromosome and spans approx. 5 kb. The gene comprises 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 [24]. The size of exon 1 depends on the transcriptional start site used. Exon 2 spans 59 bp. Until now, no functional results on human AT2 (hAT2) promoter regulation have been available. Because the cardiac expression patterns and regulation of angiotensin receptors differ between species, and AT2 represents the pre-dominant receptor subtype in the human heart [5,25,26], the functional characterization of the hAT2 promoter is indispens-

Abbreviations used: Ang II, angiotensin II; AP-1, activator protein 1; AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor; CMV, cytomegalovirus; hAT2, human AT2; RT-PCR, reverse transcriptase-mediated PCR; SV40, simian virus 40; UTR, untranslated region.

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able in the elucidation of the pathophysiological role of the AT2 in cardiovascular diseases. In the present study we cloned the hAT2 gene promoter and demonstrated its functional activity in PC12W cells by using luciferase reporter gene assays. We found that efficient transcriptional activity required intronic sequence elements, whereas the 5' flanking region alone exhibited only weak promoter activity.

EXPERIMENTAL

Materials

All cell culture reagents were purchased from Gibco BRL Life Technologies (Eggenstein, Germany). Luciferase and β -galactosidase enzyme assay systems were from Promega (Boehringer Ingelheim, Heidelberg, Germany) and restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). Oligonucleotide primers were synthesized by TIB Molbiol (Berlin, Germany), Applied Biosystems (Weiterstadt, Germany) and Invitex (BioTeZ Berlin, Germany). The promoter region was numbered in accordance with the transcription start site identified in the present paper. The numbering of nucleotide positions in the transcribed region follows that of Martin and Elton [24].

Primer extension analysis of the 5' end of the AT2 mRNA

A primer (+104/+79, 5'-GTGCTTACTCGTTCTCTCAGACGCTG-3') binding to the first exon of the AT2 gene was end-labelled with [γ - 32 P]dATP (DuPont NEN; specific radioactivity 3000 Ci/mmol) with T4 polynucleotide kinase (MBI Fermentas). The primer was purified from non-incorporated nucleotides by using Sephadex G-25 spin columns. To 100 μ g of human uterus total RNA prepared with TRIzol Reagent (Gibco BRL), 0.1 pmol of primer was hybridized for 15 min at 62 °C before extension at 42 °C for 30 min with Superscript RNase H⁻ Reverse Transcriptase (Gibco BRL). Extension products and a corresponding sequencing ladder were analysed on 6% (w/v) polyacrylamide/urea gels followed by autoradiography.

Construction of hAT2 promoter luciferase reporter gene plasmids

The promoter was cloned from a genomic library (Human genomic Library Lambda Fix II vector; Stratagene) (EMBL database accession number X87722) [27]. Six 5'-terminal deletion mutants of the 5' flanking region were generated with *Nhe*I and *Nde*I restriction sites and PCR respectively, with downstream primers introducing a *Sac*I site (constructs AH, BH, CH and DH). The fragments were cloned in pGL2basic (Promega). Nucleotide positions are indicated in Figure 2. The 3' end of the promoter fragments was the *Eco*RI restriction site in the first exon (+100 according to the identified transcription start site, +106 according to [26]; see Figures 1B and 2).

Reporter gene constructs, which extended further into the transcribed region of the hAT2 gene, were PCR-generated by the use of the AT2-specific Lambda DNA as the template, the downstream primer AT2SacIB (-271/-248, the same as used for construct BH) and the following upstream primers: AT2Ex3rev (+1593/+1575, 5'-CTTAAGCTTGAAATGTCAGCAGCTCC-3'), AT2Ex2 (+356/+332, 5'-GAAGCTTACTGGTTGTTGAAGTTTAAAGC-3'), AT2Int1 (+293/+273, 5'-AAAAGCTTCCTGTAAGAGAAACAGCAGC-3'), AT2Int1a (+193/+174, 5'-TTAAGCTTAATGTATGTTCTACCTTC-3'), AT2Int1/202 (+202/+183, 5'-AAAGCTTATTTCATTTAATGTATG-3'), AT2Int1/209 (+209/+194, 5'-CAAGCTTAAAAAATATTTTCAT-3'), AT2Int1/217 (+217/+196, 5'-AAAGCTTCATCCATAAAAAATATTTTC-3') AT2-

Int1b (+229/+211, 5'-AAAGCTTGAAAAACAGAAGTT-CATCC-3'), and the mutagenesis primers AT2Int1b/mut I (+229/211, 5'-AAAGCTTGAAAAACCGCCGTTTCATCC-3') and AT2Int1b/mut II (+229/+211, 5'-AAAGCTTGCCCCCAGAAGTTCATCC-3') (underlining indicates a generated *Hind*III site, italics a nucleotide sequence not identical with the hAT2 sequence, and underlined bold type represents mutated nucleotides). PCR products were cloned in pGL2basic. Schematic drawings of the resulting constructs are shown in Figures 3 and 5.

For the construction of reporter gene plasmids, which comprised the hAT2 promoter (-271/+100) and the 5' UTR of hAT2 cDNA, reverse transcriptase-mediated PCR (RT-PCR) products with exon compositions 1+2+3 and 1+3 were reamplified with primers AT2Ex1for (+104/+127, 5'-CA GAATTCAAAGCATTCTGCAGCC-3'; underlined is the endogenous *Eco*RI site) and AT2Ex3rev and fused to the promoter construct BH (-271/+100; see Figure 3). To test the possible promoter activity of the first intron, a 123 bp (+106/+229) PCR product was cloned in pGL2 basic (see Figure 7). All plasmid constructs were controlled by sequencing both strands. For transfections, at least two different preparations of each plasmid were used.

DNA transfection and determination of luciferase activity

Rat pheochromocytoma PC12W cells were maintained in RPMI 1640/10% (v/v) horse serum/5% (v/v) fetal bovine serum/2 mM L-glutamine/50 μ g/ml gentamicin. Transfections were performed in accordance with a modified standard electroporation protocol [28]. hAT2 promoter luciferase reporter gene construct (10 μ g), 2 μ g of a β -galactosidase vector [pCMV β -gal (in which CMV stands for cytomegalovirus); Stratagene], and 18 μ g of pBSSK⁺ plasmid DNA were transfected into 3×10^6 PC12W cells in 300 μ l of culture medium by electroporation (cuvette width 4 mm, 250 V, 1200 μ F; Easyject Plus[™] Electroporator Equibio; Eurogentec). The cell survival rate and transfection efficiency were 65% and 20% respectively. The hAT2 promoter constructs were compared with control vectors (pGL2basic, pLuc [29]) and a reporter gene plasmid that comprised 513 bp (-513/+90) of the human AT1 promoter [30]. At 48 h after transfection, cells were lysed in 80 μ l of lysis buffer. The assay was performed on 50 μ l of the cleared lysate and 50 μ l of luciferase assay reagent (Promega). Enzyme activities were measured over a 10 s window after a delay of 0.5 s in the Lumat LB 9501 (Berthold). Cell lysate (20 μ l) was subjected to a β -galactosidase assay (Promega). Luciferase activities were normalized with reference to the respective β -galactosidase activities.

RT-PCR

RNA was prepared from transfected cells as described [31] and digested with RNase-free DNase I (Boehringer) followed by a TRIzol extraction. Reverse transcription was performed on 0.5 μ g of RNA with Superscript[™] RNase H⁻ reverse transcriptase (Gibco/BRL) followed by PCR with one-fifth of the reverse transcriptase product, primers AT2Ex1for and AT2Ex3rev and *Taq* DNA polymerase (Gibco BRL) (35 cycles of 1 min at 93 °C, 30 s at 60 °C and 1 min at 72 °C). Amplified RNA was analysed on a 1% (w/v) agarose gel.

Northern blot analysis

RNA from two separate transfections with eight replicates each was prepared as described above and pooled. Each RNA (20 μ g)

was separated on a 1% (w/v) denaturing agarose gel and transferred to a Hybond N⁺ membrane (Amersham). The membrane was hybridized with a digoxigenin-labelled luciferase cDNA and the signals were detected with an alkaline phosphatase coupled anti-digoxigenin conjugate and the chemiluminescence substrate CDP-Star[®] (Boehringer). The membrane was exposed to a Hyperfilm enhanced chemiluminescence system (Amersham) for 5 min.

Electrophoretic mobility-shift assays

Nuclear extracts from PC12W cells were prepared as described [32]. Double-stranded oligonucleotides corresponding to the primer AT2Int1b (+211/+229) and the mutagenesis primers AT2Int1b/mut 1 and AT2Int1b/mut II were used as probes. Binding reactions (25 µl) were performed at room temperature for 15 min in 10 mM Tris/HCl (pH 7.5)/50 mM NaCl/0.5 mM dithiothreitol/5 mM EDTA/0.25 mM MgCl₂/3 µg of poly(dI-dC)·poly(dC-dI) (Sigma)/4% (v/v) glycerol with 5 µg of nuclear extract and 25000 c.p.m. (approx. 50 fmol) of the labelled probe. In competition experiments a 100-fold molar excess of the unlabelled probes was included. DNA-protein complexes were separated on non-denaturing 4% (w/v) polyacrylamide gels under circulating buffer conditions at 160 V and 4 °C. The gels were exposed to a PhosphorImager screen overnight and analysed in a Fujix Bas II PhosphorImager (Fuji).

Statistics

Data are given as means ± S.E.M. Statistical analysis was performed with the unpaired two-tailed Student's *t* test. Values of *P* < 0.01 were considered significant.

RESULTS

The transcriptional start site was localized 34 bp upstream of the TATA box

Primer extension analysis, which used human uterus RNA and a radiolabelled primer specific for the first exon, resulted in two bands, which indicated transcriptional start sites at 34 bp (S1) and 10 bp (S2) upstream of the most 3' located TATA box (Figure 1A, lane 2). The G nucleotide 34 bp upstream of the TATA box was designated +1 (Figure 1B). This nucleotide position corresponds to +7 in the sequence published by Martin and Elton [24] and to position 1419 in the sequence submitted to the EMBL database (accession no. U20860) by those authors. For reasons of clarity we use here the numbering introduced in [24] for the transcribed region of the hAT2 gene.

hAT2 promoter fragments from -1417/+100 to -46/+100 exhibited weak but significant promoter activity in PC12W cells

All six 5'-terminal deletion mutants of the hAT2 promoter ranging from -1417/+100 to -46/+100 proved to be functionally active (*n* = 6, *P* < 0.01) (Figure 2). Construct BH (-271/+100) was set to 100%. The shortest promoter segment AH (-46/+100) exhibited 51.6 ± 18.1% of the activity of BH, which indicated positive regulatory *cis* DNA elements between -47 and -271. The plasmids NdeH (-1032/+100) and NheH (-564/+100) tended to express the highest promoter activities (141.2 ± 27.6% and 140.7 ± 18.1% respectively) but the differences between the four constructs NdeH, NheH, CH and DH were not significant. Luciferase activity was approx. 0.8% of the activity of the simian virus 40 (SV40) promoter, which drove luciferase gene expression of the plasmid pBLuc, and 2.4 ± 1.1%

Figure 1 Transcriptional start sites of the hAT2 gene as determined by primer extension analysis

(A) A radiolabelled primer [see (B)] was hybridized to human uterus total RNA and extended with reverse transcriptase. The sequencing reaction was performed with the same primer and the plasmid NdeH as template. The two bands in lane 2 were reproduced in three independent experiments. Lane 1, primer extension reaction with labelled primer and *Escherichia coli* tRNA. (B) Proximal promoter region with transcription factor binding sites (FACTOR program of HUSAR package; DKFZ, Heidelberg, Germany) and transcriptional start sites (arrows S1 and S2). S1 was designated position +1. The sequence of the cDNA cloned by rapid amplification of cDNA ends PCR is framed [27]. The broken arrow indicates the position of the primer.

of a comparable human AT1 promoter (-513/+90) construct (results not shown). The promoterless pGL2 basic expressed $5.4 \pm 2\%$ of BH (-271/+100) activity.

Inclusion of the two hAT2 gene introns in luciferase constructs increased reporter gene expression substantially

To examine the transcribed region of the hAT2 gene for the presence of *cis*-acting elements, a set of reporter gene constructs containing different 3'-terminally deleted segments of the transcribed region in addition to the proximal promoter (-271/+100) were used. Inclusion of the first intron in the promoter constructs (plasmid Int1, -271/+293) induced a 6.7 ± 1.6 -fold increase in luciferase activity compared with the control construct BH (-271/+100) (Figure 3). Integration of intron 1 and the untranslated exon 2 (plasmid Ex2, -271/+356) did not alter luciferase activity significantly compared with Int1. A plasmid containing the complete transcribed region through the translational start codon in exon 3 (plasmid Ex3, -271/+1593) expressed an 11.6 ± 1.7 -fold higher luciferase activity than the control plasmid BH, which suggested the presence of positively regulating elements in intron 2 (Figure 3). To confirm that the observed increases in the promoter activity

Figure 2 Activity of 5'-terminal deletion mutants of the hAT2 promoter

Six 5'-terminal deletion mutants (–1417/+100, –1032/+100, –807/+100, –564/+100, –271/+100 and –46/+100) of the promoter were cloned into the pGL2 basic luciferase reporter gene vector. hAT2 promoter construct (10 µg) and 2 µg of a pCMV β-gal vector were transiently transfected into PC12W cells by electroporation. After 48 h, luciferase activities were determined and normalized with reference to the respective β-galactosidase activities. The activity of BH (–271/+100) was set to 100%. Luciferase activities are means ± S.E.M. for six experiments with four replicates per construct. The pBLuc control vector comprised the SV40 promoter. **P* < 0.01 compared with BH.

were due to the presence of the intron sequences in the reporter gene constructs, the cDNA species of the alternatively spliced 5' UTRs of the hAT2 mRNA were also fused to the proximal promoter (plasmids Ex1/2/3 and Ex1/3). These constructs exhibited similar promoter activities to those of the control plasmid BH. When compared with the construct Ex1/3, luciferase expression was moderately decreased to 68.9 ± 19.3% by the presence of exon 2 in the reporter gene construct (plasmid Ex1/2/3; Figure 3).

The 5' UTR of the chimaeric hAT2–luciferase mRNA was spliced as the endogenous hAT2 mRNA

RT-PCR was performed to exclude the possibility that the chimaeric hAT2–luciferase mRNA was not spliced regularly in transfected PC12W cells, thus generating different 5' leader sequences with possibly different translationally regulatory properties. RNA from transfections with construct Ex3 (–271/+1593), which contained the complete unspliced downstream region, yielded the same amplification product as RNA from cells transfected with the construct Ex1/2/3, which lacked the introns (Figure 4A, lanes 1 and 3). Thus the chimaeric hAT2–luciferase mRNA of plasmid Ex3 was spliced in PC12W cells like the endogenous hAT2 mRNA in the human heart, in which the most abundant AT2 transcript is composed of exons 1, 2 and 3 [5]. The splice product with exon composition 1 and 3 was not detected in Ex3-transfected cells (Figure 4A, lane 1), just as it was not detectable in human atria [5].

Luciferase activities corresponded to hAT2–luciferase mRNA levels in transfected cells. To confirm that the observed effect on

Figure 3 Increased activity of promoter constructs including the transcribed region of the hAT2 gene and comparison with constructs lacking the intron sequences

Reporter gene constructs that contained the promoter (–271/+100) and the downstream region of the hAT2 gene inclusive of intron 1 (Int1), exon 2 (Ex2) and intron 2 plus exon 3 up to the translational start (Ex3), were compared with construct BH (–271/+100) and reporter gene constructs that contained the spliced and thus intronless downstream region (Ex1/2/3, Ex1/3). Luciferase activities of these constructs 48 h after transfection into PC12W cells are shown. Results are means ± S.E.M. for four experiments with four replicates per construct. **P* < 0.01 compared with BH.

Figure 4 RT-PCR demonstrating that the chimaeric hAT2-luciferase RNA of construct Ex3 is regularly spliced in PC12W cells, and Northern blot analysis of transfected PC12W cells showing the regulated expression of hAT2-luciferase transcripts

(A) RT-PCR on RNA from transfected PC12W cells. In lanes 1 and 2, cells were transfected with plasmid Ex3 (−271/+1593), which harboured the complete downstream region of the AT2 gene (including both introns) up to the translational start; in lanes 3 and 4, cells were transfected with construct Ex1/2/3 (−271/+1593), which lacked the two introns. Lanes 1 and 3, with reverse transcription (RT); lanes 2 and 4, without RT; lane 5, untransfected cells; lane 6, water control; lane 7, DNA control (plasmid Ex3); lane 8, DNA control (plasmid Ex1/2/3); lane M, 100 bp DNA ladder. One of three identical experiments is shown. (B) Northern blot analysis of 20 µg of RNA from transfected PC12W cells (16 replicates each to compensate for varying electroporation efficiencies), demonstrating that the increase in luciferase activity corresponds to an increase in luciferase mRNA level. Panel I: 1% (w/v) agarose gel; 18 S and 28 S rRNA indicated. PC12W cells were transfected as follows: lane 1, BH; lane 2, Ex3; lane 3, Ex1/3; lane 4, Int1; lane 5, Int1a. Panel II: transferred RNA was hybridized with a digoxigenin-labelled luciferase cDNA. One of two identical experiments is shown.

reporter gene expression was paralleled by a concomitant increase in luciferase mRNA levels, we performed a Northern blot analysis (Figure 4B). Because approx. 20% of PC12W cells were transfected, the hybridization signals obtained with the luciferase cDNA probe were expected to be relatively weak. Cells transfected with Ex3 (Figure 4BII, lane 2) and Int1 (Figure 4BII, lane 4) gave distinct hybridization signals, whereas luciferase mRNA species from cells transfected with BH (lane 1), Ex1/3 (lane 3) and Int1a (lane 5) were below the detection limit. This pattern corresponded to the luciferase activities of the respective promoter constructs, which suggests that the regulation occurred at the mRNA level.

A 12 bp region in the centre of intron 1 is required for the intronic enhancer activity

Mapping of the intron 1 with the 3'-terminally deleted reporter gene constructs Int1a (−271/+193), Int1/202 (−271/+202), Int1/209 (−271/+209), Int1/217 (−271/+217) and Int1b (−271/+229) revealed that a 12 bp region in the centre of the

intron (+218/+229) was necessary to increase luciferase activity markedly. The construct Int1b, which differed from the construct Int1/217 only by the 12 bp sequence, expressed an approx. 5-fold higher luciferase activity than both the construct Int1/217 and the control construct BH (−271/+100) (Figure 5; for primer positions see also Figure 7B). Intriguingly, the activities of constructs Int1a (−271/+193), Int1/202 (−271/+202) and Int1/209 (−271/+209) were even significantly lower than the activity of the control plasmid BH, which suggested the presence of negative regulatory elements upstream of the 12 bp enhancer region. Mutation of the 12 bp sequence CTTCTGTTTTC by the replacement of three or five T nucleotides by G nucleotides (Int1b/mut I, CGGCGGTTTTC; Int1b/mut II, CTTCTG-GGGGC) led to significant decreases in reporter gene activity to 4% (Int1b/mut I) and 1.2% (Int1b/mut II) of Int1b activity (Figure 5). This indicated that the mutations destroyed the *cis*-active DNA element within the 12 bp region, which mediated the enhancing effect on promoter activity.

Electrophoretic mobility-shift assays performed with PC12W cell nuclear extracts and the double-stranded oligonucleotide probe AT2Int1b, which comprised the 12 bp sequence, revealed three protein–DNA complexes (Figure 6, arrows at the left). Two of the complexes were eliminated by competition with the unlabelled probe (Figure 6, third lane), whereas an unrelated activator protein 1 (AP-1) oligonucleotide probe did not compete for the protein binding (results not shown), which demonstrated the specificity of the binding reaction. When using mutated double-stranded probes, which corresponded to the mutagenesis primers AT2Int1b/mut I and AT2Int1b/mut II, the lower specific complex was eliminated (probe Int1b/mut I) and new bands appeared that could not be completely competed out with the unlabelled probes (probe Int1b/mut II; Figure 6, arrows at the right). This suggested that the introduced nucleotide substitutions altered the protein-binding characteristics of the mutated probes, which explains the decrease in promoter activity of the respective reporter gene constructs.

The +106/+229 intron 1 fragment is able to direct transcription in the absence of the 5' flanking region

Fused to the promoterless pGL2basic vector a fragment of intron 1 (+106/+229) exhibited luciferase activities in PC12W cells comparable to the construct ultInt1b (−271/+229), which also comprised the core promoter (Figure 7A). Thus the first intron was able to direct transcription initiation by itself, independently of the 5' flanking region. A database search [33] revealed several putative TATA boxes, CAAT boxes and octamer motifs in the intron able to direct transcription initiation within this region (Figure 7B).

DISCUSSION

In the present study we functionally characterized the promoter and downstream region of the hAT2 gene and localized a positive regulatory *cis*-element in the first intron. Furthermore, we identified two transcriptional start sites upstream of the TATA box and in close vicinity to the 5' end of the previously cloned (by rapid amplification of cDNA ends PCR) hAT2 cDNA [27]. The physiological implications of these alternative starts are not yet known, but the resulting 5' UTRs of the mRNA species might regulate the efficiency of translation initiation.

Reporter-gene assays were performed in rat PC12W cells, which had been shown to express AT2 mRNA and protein in pilot experiments. PC12W cells represent one of the few cell lines known to express the AT2 at high levels and are frequently used for studies on AT2 regulation and function [9,10]. Until now, no

Figure 5 Mapping of the first intron in reporter gene assays reveals a centrally located 12 bp region that mediates the increase in promoter activity

Promoter activities of reporter gene constructs containing different 3'-terminally deleted intron 1 fragments were compared with Int1 and the reference construct BH. The 12 bp region (+217/+229) included in Int1b, but not in Int1/217, was necessary to mediate the increase in activity. The low activity of Int1a, Int1/202 and Int1/209 indicated negative regulatory elements upstream of +194. Constructs Int1b/mut I and Int1b/mut II, which contained nucleotide substitutions (arrows) within the 12 bp region, expressed significantly lower luciferase activities than the Int1b wild-type construct. Luciferase activities are means $\pm$ S.E.M. for four to eight experiments with four replicates per construct. * $P < 0.01$ compared with BH.

Figure 6 The 12 bp region specifically binds PC12W cell nuclear protein; mutations within this region alter the protein binding pattern

The 32 P-labelled double-stranded oligonucleotide probe Int1b, which comprised the hAT2 region +211/+229, yielded one non-specific and two specific protein-DNA complexes (arrows at the left). The latter were competed for by a 100-fold molar excess of the unlabelled probe (third lane). Mutations within the 12 bp region led to a decrease in the lower specific complex (probe Int1b/mut I) and to band shifts (arrows at the right) and an increase in non-specific protein binding (Int1b/mut II).

hAT2-expressing cell line has been available, and in primary cells the receptor is rapidly down-regulated. Because the proximal promoters (-200/+50) of the human and the rodent AT2 genes exhibit approx. 90 % nucleotide sequence identity, we considered that PC12W cells would support the hAT2 promoter too.

The 5' flanking region of the hAT2 gene exhibited low luciferase activity, whereas inclusion of the downstream region with the two introns increased reporter gene expression substantially, which indicated the activity of intronic enhancers. Northern blot analyses confirmed that the increases in luciferase activity were paralleled by mRNA increases. Because Northern blot analyses reflect steady-state mRNA levels, the different abundances of reporter gene mRNA species might be caused by either transcriptional regulation or alterations of mRNA degradation rates, or both. Because the 3' UTRs as well as the protein coding regions are identical in the different hAT2-luciferase mRNA species, it is unlikely that the regulation of mRNA degradation contributes to the observed differences in mRNA levels. On the basis of the respective luciferase activities, the hAT2-luciferase mRNA expression of cells transfected with the construct Ex3, which comprised both introns, was expected to be 1.7-fold higher than hAT2-luciferase mRNA expressed from construct Int1. This difference was not detected in the Northern blot, probably because of the low sensitivity of this assay when using transiently transfected cells. However, the possibility cannot be excluded that this increase in luciferase activity was caused by the splicing of intron 2, which might lead to an increased efficiency of mRNA processing and luciferase expression.

Within intron 1 we identified a centrally located 12 bp sequence (+218/+229, CTCTGTTTTTC) that was required for the intronic enhancer activity. This sequence did not exhibit any

Figure 7 First intron of the hAT2 gene is able to direct transcription in reporter gene assays

(A) A 123 bp intron 1 fragment (primer AT2Ex1for and AT2Int1b) was cloned in the promoterless pGL2b (Int1Δprom). In PC12W cells this construct exhibited luciferase activities comparable to that of the construct Int1b, which comprised the hAT2 sequence from -271 to +229. Luciferase activities are means ± S.E.M. for four experiments with four replicates per construct. * $P < 0.01$ compared with BH. (B) Sequence of the first intron of the hAT2 gene. A database search revealed multiple transcription-factor-binding sites in the first intron, including several TATA boxes, CAAT boxes and octamer motifs. Arrows indicate the positions of primers used to generate intron 1 fragments for reporter gene constructs. Framed boxes indicate the adjacent ends of exon 1 and exon 2.

obvious sequence similarity to a known transcription-factor-binding site, but it revealed a limited similarity to the initiator (Inr) consensus sequence, which positions RNA polymerase II to the site of transcription initiation. However, when compared with the Inr consensus sequence (G/T)CA(G/T/G)(A/T/C)-(C/T)(G/T/C)(C/T) [34], the hAT2 sequence motif TCTGTTTT (underlined are nucleotides matching the Inr consensus) lacks the A nucleotide at position 3, which represents the start site and is indispensable in most genes.

One or two copies of the first intron cloned upstream of the SV40 promoter did not alter SV40 promoter activity in reporter gene assays (results not shown). The enhancing activity of intron 1 might be too low to stimulate the already high basal SV40 promoter activity. In contrast, cloned into a promoterless vector the intron was able to direct transcription to the same activity as occurred in conjunction with the 5' flanking region. Whether this potential promoter function is relevant *in vivo*, thus leading to transcripts that lack exon 1, will be addressed by an analysis of AT2 transcripts from different human tissues. Using RNA from the adult human uterus and heart we were unable to identify AT2 transcripts containing the 3' end of intron 1 ([27], and results not shown). However, the putative promoter function of the intron

might be relevant only under specific physiological circumstances, e.g. during embryonic development.

The number of genes known to be regulated by enhancer elements outside the 5' flanking region is constantly increasing. Many of them contain regulatory sequences within the first intron. These intronic enhancers often direct cell-type-specific or developmentally regulated gene expression. In the rat renin gene, intron I contains both positive and negative regulatory *cis* elements, which mediate the tissue-specific renin expression [35]. Smooth-muscle-specific gene expression driven by the smooth-muscle myosin heavy-chain promoter requires both the 5' flanking region and the first intron of the gene [36]. Transcription of the mouse TIE2 gene in embryonal endothelium is directed by the 5' flanking region, but for endothelial expression in the adult an enhancer in the first intron is necessary [37].

Alternative or cryptic promoters within introns have been identified in several genes. As enhancers they might mediate the inducibility of gene expression in a specific spatiotemporal pattern, e.g. during organogenesis and cell differentiation. In postmeiotic mouse germ cells a truncated receptor tyrosine kinase c-Kit protein is expressed from an alternative intronic promoter [38]. Endothelin-converting enzyme 1 is expressed in

two isoforms from two different promoters, one of which is located upstream of exon 3 [39]. In the hAT2 gene, transcription from the putative intronic promoter would not influence the protein coding region and thus the receptor function. However, the different 5' UTRs of the mRNA species, which result from the use of alternative promoters, might control translational efficiency and thus represent a mechanism for the regulation of receptor expression.

Because former studies on promoter regulation of the rodent AT2 genes did not include the introns [14–16], it is not yet possible to assess the functional implications of intron 1 in the rodent genes. However, similarities exist in putative *cis*-active DNA elements: TATA motifs, binding sites for ETS, AP-4 and several tissue-specific factors are present in both the rat and hAT2 intron, but only the hAT2 intron contains octamer motifs [33]. This suggests that the rodent AT2 intron might also activate AT2 gene transcription but might be regulated differently from the human gene.

With the identification of the introns of the hAT2 gene as regions with high regulatory potency, we have provided some new prerequisites for understanding AT2 promoter regulation and further studies on the mechanisms that control hAT2 gene expression in development and pathological situations.

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