Upstream Stimulatory Factors 1 and 2 Mediate the Transcription of Angiotensin II Binding and Inhibitory Protein*

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Background: Regulation of angiotensin II type 1 receptor-interacting and inhibitory protein (ATRAP/Agtrap) is important in pathophysiology.

Results: Gene knockdown in cells and unilateral ureteral obstruction in mice indicate that Usf1 decreases and Usf2 increases Agtrap expression.

Conclusion: Interplay between E-box and Usf1/Usf2 is important for Agtrap regulation.

Significance: A strategy of modulating the E-box-Usf1/Usf2 interaction may have novel therapeutic potential.

The angiotensin II type 1 receptor (AT1R)-associated protein (ATRAP/Agtrap) promotes constitutive internalization of the AT1R so as to specifically inhibit the pathological activation of its downstream signaling yet preserve the base-line physiological signaling activity of the AT1R. Thus, tissue-specific regulation of Agtrap expression is relevant to the pathophysiology of cardiovascular and renal disease. However, the regulatory mechanism of Agtrap gene expression has not yet been fully elucidated. In this study, we show that the proximal promoter region from −150 to +72 of the mouse Agtrap promoter, which contains the X-box, E-box, and GC-box consensus motifs, is able to elicit substantial transcription of the Agtrap gene. Among these binding motifs, we showed that the E-box specifically binds upstream stimulatory factor (Usf) 1 and Usf2, which are known E-box-binding transcription factors. It is indicated that the E-box-Usf1/Usf2 binding regulates Agtrap expression because of the following: 1) mutation of the E-box to prevent Usf1/Usf2 binding reduces Agtrap promoter activity; 2) knockdown of Usf1 or Usf2 affects both endogenous Agtrap mRNA and Agtrap protein expression, and 3) the decrease in Agtrap mRNA expression in the afflicted kidney by unilateral ureteral obstruction is accompanied by changes in Usf1 and Usf2 mRNA. Furthermore, the results of siRNA transfection in mouse distal convoluted tubule cells and those of unilateral ureteral obstruction in the afflicted mouse kidney suggest that Usf1 decreases but Usf2 increases the Agtrap gene expression by binding to the E-box. The results also demonstrate a functional E-box-Usf1/Usf2 interaction in the human AGTRAP promoter, thereby suggesting that a strategy of modulating the E-box-USF1/USF2 binding has novel therapeutic potential.

Evidence has been accumulating that the activation of angiotensin II (Ang II) type 1 receptor (AT1R) through the tissue renin-angiotensin system plays a pivotal role in the pathogenesis of cardiovascular remodeling and renal injury (1, 2). The intrarenal activation of AT1R has also been proposed to play a role in the regulation of sodium and water reabsorption through the constriction of the glomerular arteries, hence a direct effect on renal tubular transport function, and to evoke excessive sodium retention, resulting in hypertension, when thus inappropriately stimulated (3, 4). The C-terminal portion of the AT1R is involved in the control of AT1R internalization independent of G protein coupling, and it plays an important role in linking receptor-mediated signal transduction with the specific biological response to Ang II (5, 6). The AT1R-associated protein (ATRAP/Agtrap) was identified as an interacting molecule with the C-terminal domain of AT1R (7, 8), and previous in vitro and in vivo studies showed that Agtrap promotes constitutive internalization of the AT1R so as to specifically inhibit the pathological activation of its downstream signaling and yet preserve base-line physiological signaling activity (2, 9–17).

Although Agtrap is abundantly expressed in the renal nephron tubules, it is also widely expressed in many other cell types and tissues in addition to the kidney. Thus, it is important to elucidate the molecular mechanism of the cell type- and tissue-specific regulation of Agtrap gene expression to determine...
the regulatory machinery for the tissue Agtrap level and/or Agtrap activity under both physiological and pathological conditions. The balance of the endogenous expression of Agtrap and AT1R in local tissues is important for the regulation of tissue AT1R signaling. Down-regulation of Agtrap and/or up-regulation of AT1R at local tissue sites together with the resultant pathological activation of the tissue renin-angiotensin system are pathogenetic mechanisms that may be responsible for cardiovascular and renal disease. For example, in Ang II-infused mice and genetically hypertensive rats, the development of hypertension and organ injury, such as cardiac hypertrophy and renal fibrosis, was reportedly accompanied by a decrease in the tissue Agtrap expression without altered AT1R expression (2, 15–19). In addition, we previously showed that serum starvation stimulates Agtrap gene expression in mouse distal convoluted tubule cells (mDCT cells) and that Runx3, one of the Runt-related transcription factors, is involved in the transcriptional activation of Agtrap gene expression (20). However, the regulatory mechanism of Agtrap gene expression in relation to organ injury needs further investigation to elucidate the relationship of the regulation of Agtrap expression with the pathophysiology of cardiovascular and renal disease at the molecular level.

The transcription factors upstream stimulatory factor (USF/Usf) 1 and USF2/Usf2 were originally identified in HeLa cells by biochemical analysis (21, 22). The human cDNA cloning of USF1 and USF2 revealed that the USFs belong to the c-Myc-related family of DNA-binding proteins, which have a helix-loop-helix motif and a leucine repeat, and that USF interacts with its target DNA as a dimer (23). Previous examination of the tissue and cell type distribution of USF1 and USF2 revealed that although both are ubiquitously expressed, different ratios of USF homo- and heterodimers are found in different tissues and cell types (24). The results of mouse Usf1 cDNA cloning showed a high level of sequence homology between the mouse and human USF1 genes (25). Previous studies that were undertaken to assign a physiological role to the Usfs in vivo, including the disruption of Usf1 and Usf2 genes in mice, revealed that Usf1 and Usf2 play a role in the modulation of glucose and lipid metabolism by modulation of their trans-activating efficiency (26–29). Subsequent studies also showed that Usf1 and Usf2 are involved in the pathophysiology of several metabolic disorders, including familial hypercholesterolemia and diabetic nephropathy (30–33). In this study, we show that the proximal promoter region (−72 to −43) of the mouse Agtrap gene contains an “E-box (CANNTG)” sequence, which is a putative binding site for Usf1 and Usf2 that interacts with these transcription factors. It is shown both in vitro and in vivo that Usf1 decreases and Usf2 increases the Agtrap gene expression through their binding to the E-box.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The mDCT cells were kindly provided by Dr. Peter A. Friedman (University of Pittsburgh School of Medicine). These cells have been shown to have a phenotype of a polarized tight junction epithelium along with both morphological and functional features retained from the parental cells (14, 34–36). The mDCT cells also express the endogenous AT1R and Agtrap (14). Human embryonic kidney-derived 293 (HEK293) cells were cultured according to the American Type Culture Collection (ATCC) protocol, as described previously (37, 38).

**Animals and Treatment**—Adult C57BL/6 mice were purchased from Oriental Yeast Kogyo (Tokyo, Japan). The procedure of unilateral ureteral obstruction (UUO) was performed using C57BL/6 mice, as described previously (20, 39). Briefly, with the mice under anesthesia, the left ureter was ligated with 4–0 silk at two locations and then cut between the ligatures to prevent retrograde urinary tract infection. Mice that were operated on were sacrificed under anesthesia 7 days after UUO. Sham operation was also performed in which the ureters were manipulated but not ligated. Seven days after the sham operation, mice were sacrificed to obtain control kidneys. The procedures were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the Animal Studies Committee of Yokohama City University.

**Plasmid Construction and Transcriptional Mouse Agtrap and Human AGTRAP Promoter Assay**—For the analysis of the mouse Agtrap promoter, 5022-, 2943-, 2090-, 1272-, 972-, 613-, 453-, 374-, and 222-bp mouse Agtrap promoter fragments (−4950, −2871, −1200, −900, −541, −381, −302, and −150 to +72 of the putative transcriptional start site, respectively) were amplified from C57BL/6 genomic DNA, using the pair of primers indicated in Table 1, and subcloned into the multicloning sites of pBluescript. A 613-bp Agtrap promoter fragment (−541 to +72 of the putative transcriptional start site)-containing plasmid was used as a template to construct mutations in the X-box, E-box, and GC-box by oligonucleotide (ODN)-directed mutagenesis (40–42). The sequences of the oligonucleotide used to create the mutated X-box (X-box mt), mutated E-box (E-box mt), mutated GC-box (GC-box mt), and mutated X- and E-boxes (X/E-box mt) are also shown in Table 1. To normalize transfection efficiency, we employed the Dual-Luciferase Assay System (Promega) for the transcriptional Agtrap promoter assay using pGGL3-basic plasmid-based luciferase constructs, as described previously (20, 36).

For analysis of the human AGTRAP promoter, 575-bp AGTRAP promoter fragments (−480 to +95 of the putative transcriptional start site, NC_000001.9) containing two adjacent wild-type or mutated E-box motifs, were gene-synthesized (Eurofins MWG Operon). The human AGTRAP promoter assay using the Dual-Luciferase Assay System (Promega) was performed using pGL3- and pGL4.1-basic plasmid-based luciferase constructs (20, 36).

**Real Time Quantitative RT-PCR Analysis**—Total RNA was extracted and purified using the RNeasy kit (Qiagen), and cDNA was synthesized using SuperScript VILO (Invitrogen). Real time quantitative RT-PCR was performed by incubating the RT product with the TaqMan Universal PCR Master Mix and designed TaqMan FAM™ dye-labeled probes for Usf1, Usf2, and Agtrap (Applied Biosystems), and a TaqMan VIC dye-labeled probe as the internal control (18 S rRNA Endogenous Control, Applied Biosystems) in the same reaction mixture (CFX96 system, BIO-RAD), essentially as described previously (20).
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Immunoblot Analysis—A 14-amino acid synthetic peptide corresponding to amino acids 148–161 of the C-terminal tail of mouse (DBA/2J) ATRAP was used for the generation of a polyclonal anti-ATRAP antibody (7), and the characterization and specificity of the anti-ATRAP antibody were described previously (9, 15, 43). Antibodies for USF1 (C-20 sc-229, Santa Cruz Biotechnology), USF2 (ab32616, Abcam), TATA-binding protein (ab818[1TBP18], Abcam), and α-tubulin (ab40742 Abcam) were also used. Immunoblot analysis was performed as described previously (9, 15, 43), and the images were analyzed using a FUJI LAS3000mini Image Analyzer (FUJI Film, Tokyo, Japan).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from mDCT cells (70–80% confluent, a 15-cm diameter dish) were prepared with a modification of the protocols of Dignam et al. (44) and Swick et al. (45). The final protein concentration was adjusted to 1 mg/ml. EMSA was performed essentially as described previously (46, 47). Briefly, single-stranded ODN sequences were biotin-labeled at 3'-ends by the manufacturer, annealed to each other, and used as the probe. The ODN sequences for the E-box and mutated E-box (E-box mt) are shown in Table 1. Nuclear extracts (2 μg) were incubated on ice in a 20-μl EMSA binding reaction mixture containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, pH 8.0, 4% glycerol, 1 μg of BSA, and 1 μg of double-stranded poly(dI-dC) in the presence or absence of a specific double-stranded competitor DNA and biotin-labeled DNA probe. The incubation mixture was loaded onto a 5% polyacrylamide mini (7.5 9.0 cm) gel in 0.5× TBE and electrophoresed at 350 V for 25 min, followed by transfer of DNA from the gel onto nylon membranes (Hybond-N+, GE) by cross-linking the transferred DNA to the membrane and rinsing with the TN buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl). After blocking the incubation with Blocking Reagent (FP1020, PerkinElmer Life Sciences), incubating with streptavidin-horseradish peroxidase (HRP) conjugate (NEL750, PerkinElmer Life Sciences), and washing to remove unreacted excess reagent with PBST (0.05% Tween 20/PBS), the biotin-labeled DNA was visualized by chemiluminescence (Immobilon Western Detection Reagent, Millipore) and analyzed using an LAS3000mini Image Analyzer (FUJI Film, Japan).

Streptavidin-Biotin Complex Assay—Streptavidin-biotin complex assay was performed using 3'-biotin-labeled oligonucleotides corresponding to the Agtrap E-box and X-box (Table 1), essentially as described previously (28, 48, 49). The streptavidin that was immobilized on agarose CL-4B (85881, Sigma) was pretreated with TN buffer containing 1% BSA and incubated with 50 μg of nuclear extracts from mDCT cells on ice in a 200-μl EMSA binding buffer for 20 min. After five washing steps with EMSA binding buffer, the streptavidin-biotin-DNA complex was eluted with SDS buffer, and a one-fifth volume was used for immunoblot analysis.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed essentially according to the manufacturer’s protocol (Active Motif) (50, 51). Briefly, mDCT or HEK293 cells were treated with formalin to cross-link the protein-DNA complexes, and glycine was added to stop the reaction. The cells were lysed with 300 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1% SDS, protease inhibitor mixture; P8340, Sigma), and the lysates were sonicated using the Bioruptor Sonication System (250 watts, 30 s on and 30 s off/30 cycle; Bioruptor UCD-250, COSMO BIO, Tokyo, Japan) to reduce the DNA fragments. Subsequently, the sonicated lysates were divided into three equal aliquots for immunoprecipitation with specific antibodies, immunoprecipitation with control IgG (rabbit anti-HA antibody; 561, MBL, Japan), and input reference. After immunoprecipitation with an anti-USF1 antibody (C-20 sc-229, Santa Cruz Biotechnology), anti-USF2 antibody (C-20 sc-862, Santa Cruz Biotechnology), anti-SREBP1 antibody (H-160 sc-8984, Santa Cruz Biotechnology), anti-BMAL1 antibody (ab3350, Abcam), or control IgG, DNA was purified from the antibody-bound and unbound input fractions. The anti-USF1 antibody and anti-USF2 antibody used in the ChIP assay were characterized in detail in a previous study (28). Enrichment of the mouse Agtrap promoter sequences in the ChIP assay was characterized in detail in a previous study (28).

For the ChIP analysis of human AGTRAP, HEK293 cells were treated with formalin to cross-link the protein-DNA complexes, and then the cells were lysed with lysis buffer and sonicated to reduce the DNA fragments. After immunoprecipitation with an anti-USF1 antibody, anti-USF2 antibody, anti-SREBP1 antibody, anti-BMAL1 antibody or control IgG, DNA was purified from the antibody-bound and unbound input fractions. Enrichment of the AGTRAP promoter or exon3 sequences in the bound fractions was estimated by quantitative PCR with the SsoFast EvaGreen system (Bio-Rad) using the primers shown in Table 1 to detect the 134-bp fragment (−65 to +69 of the transcriptional start site).

RESULTS

Determination of the Minimal Mouse Agtrap Promoter—To determine the minimal region required for basal activity of the core promoter of the Agtrap gene, the 5-kb promoter region upstream of its transcriptional start site was isolated. Then, we generated a series of luciferase reporter plasmids containing the various Agtrap proximal promoter regions, which are illustrated in Fig. 1A. To determine the minimal Agtrap promoter, we transfected these plasmids into mDCT cells, and luciferase activity was measured. Although the luciferase activity was gradually increased by the deletion from −4950 to −541, further deletion, i.e. from −381 to −150, resulted in a decrease in the luciferase activity of the Agtrap reporter constructs (Fig. 1A). Consistent with this finding, this region contains two important Agtrap regulatory elements, the SMAD-binding element (−261 to −257) and the Runt-binding element (−246 to −241) (20). Intriguingly, the promoter region from −150 to +72 maintained the luciferase activity of the Agtrap reporter constructs. This suggested that this region contains important
regulatory elements for Agtrap gene transcription. To identify the candidate transcription factors involved in Agtrap gene transcription, we next performed a computational sequence analysis of the Agtrap proximal promoter region using TFSEARCH: Searching Transcription Factor-binding Sites software and identified the consensus binding motifs for several transcription factors (Fig. 1B).

Functional Involvement of X-box, E-box, and GC-box in the Proximal Mouse Agtrap Promoter Activity—Among the consensus binding motifs of the transcription factors listed in Fig. 1B, there are highly homologous sequences of the X-box (5'-GGCCGAAGTG-3') (52), E-box (5'-CAGGCTG-3' or 5'-GGCCGAGGG(A/C)) (3'), which are highly conserved in mammals (Fig. 2A). To examine the functional role of these conserved elements in the regulation of Agtrap gene transcription, we mutated the core binding sequences of the X-box, E-box, and GC-box in the Agtrap promoter, X-box mt, E-box mt, and GC-box mt (Fig. 2B). Although the promoter region from -541 to +72 of the putative transcriptional start site of the Agtrap gene exhibited substantial luciferase activity in mDCT cells, site-directed mutations of the X-box, E-box, or GC-box decreased the luciferase activity to 39.7% (X-box mt), 48.2% (E-box mt), and 51.2% (GC-box mt) of that achieved with the wild-type promoter, respectively (Fig. 2C). Mutations of any two of the three consensus motifs further decreased the luciferase activity to 39.7% (X-box mt), 48.2% (E-box mt), and 51.2% (GC-box mt) of that achieved with the wild-type promoter, respectively (Fig. 2C).
erase activity (E-box/GC-box mt, 25.3 ± 1.4%; X-box/GC-box mt, 24.7 ± 1.3%; X-box/E-box mt, 28.6 ± 2.4%) relative to that achieved with the wild-type promoter, whereas mutation of all three motifs reduced the luciferase activity almost to the background reference level (X-box/E-box/GC-box mt, 7.2 ± 0.3%).

These results indicate that the three binding motifs of the X-box, E-box, and GC-box are important for the basal transcriptional activity directed by the minimal Agtrap promoter and suggest that these binding motifs independently modulate the promoter activity of the Agtrap gene.

Identification of the E-box as a Transcription Factor-binding Site in the Mouse Agtrap Promoter—Among the X-box, E-box, and GC-box in the Agtrap proximal promoter, the canonical E-box is a target for many genes involved in pathophysiological conditions such as diabetic nephropathy and fibrotic disease (33, 54, 55). Therefore, we focused on the functional characterization of the E-box in the regulation of the Agtrap promoter. To determine whether the E-box is capable of binding transcription factors, nuclear extracts were prepared from mDCT cells (Fig. 3A), and EMSA analysis was performed with an Agtrap promoter fragment (−72 to −43) probe containing the E-box but not the X-box or GC-box (Table 1). The E-box probe formed a DNA-protein complex (Fig. 3B, lanes 8 and 12), and the formation of the complex was completely impaired by the addition of an excess amount of the unlabeled probe with a wild-type sequence (Fig. 3B, lanes 5–7), but not by a mutated probe (Fig. 3B, lanes 9–11). These results indicate that there are nuclear factors that bind to the E-box sequence of the Agtrap promoter.

Specific Binding of Usf1 and Usf2 to the E-box of the Mouse Agtrap Promoter—Several candidate transcription factors, including Usf1, Usf2, BMAL1/Arnt1, and Srebf1, are reported to be capable of binding to the E-box sequence. Among these factors, Usf1, Usf2, and BMAL1/Arnt1, but not Srebf1 mRNA, were detectably expressed on RT-PCR and immunoblot analyses in mDCT cells (data not shown). We then examined whether Usf1, Usf2, and/or BMAL1 interact with the E-box of the Agtrap promoter using a biotin-labeled E-box probe and X-box probe. These biotin-labeled probes were individually mixed with the nuclear extracts of mDCT cells and pulled down using streptavidin-agarose. The results showed that substantial amounts of Usf1 (43 kDa) and Usf2 (44 kDa) proteins from nuclear extracts were pulled down with the biotin-labeled E-box, but not the X-box, of the Agtrap promoter (Fig. 4A). However, no binding of BMAL1 to the biotin-labeled E-box or X-box in the Agtrap promoter was observed.
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We next performed ChIP analysis to determine whether Usf1 and Usf2 physiologically interacted with the Agtrap promoter region. As shown in Fig. 4B, the 134-bp E-box containing the sequence from −65 to +69 of the transcriptional start site of the Agtrap promoter was recovered from mDCT cells after immunoprecipitation of sheared genomic DNA with an anti-USF1 antibody and anti-USF2 antibody but not after immunoprecipitation with an anti-SREBP1 antibody or anti-BMAL1 antibody. Quantitative PCR analysis confirmed that Usf1 and Usf2 are present in the Agtrap E-box promoter region, and the corresponding genomic DNA was enriched with both an anti-USF1 antibody (*, p < 0.05, versus IgG control) and anti-USF2 antibody (**, p < 0.01, versus IgG control) but not with an anti-SREBP1 antibody or anti-BMAL1 antibody. These data provide evidence for the occupancy by Usf1 and Usf2, but not Srebf1 or BMAL1, of the mouse Agtrap promoter E-box in vivo.

Functional Involvement of Usf1 and Usf2 in Mouse Agtrap Promoter Activity—To determine whether Usf1 and Usf2 are involved in the transcriptional regulation of the Agtrap gene in mDCT cells, we examined the effect of Usf1 and Usf2 siRNAs transfection on endogenous Agtrap gene expression. The mRNA and protein levels of Usf1 (Fig. 5, A and D) and Usf2 (Fig. 5, B and E) were significantly decreased after transfection with their respective siRNA. In addition, although the Usf2 mRNA level was slightly increased by Usf1 knockdown (Fig. 5B), the Usf2 protein level was not affected (Fig. 5E). Intriguingly, although the siRNA reduction of Usf1 resulted in a significant increase in the levels of the Agtrap mRNA (Fig. 5C, p < 0.01, siUsf1 versus siCtrl) and protein (Fig. 5F, p < 0.01, siUsf1 versus siCtrl), Usf2 knockdown significantly decreased the Agtrap mRNA (Fig. 5C, p < 0.01, siUsf2 versus siCtrl) and protein (Fig. 5F, p < 0.01, siUsf1 versus siCtrl). These results show that Usf1 and Usf2 exert negative and positive regulatory effects on Agtrap gene expression, respectively.

Pathophysiological Relevance of Usf1 and Usf2 in Mouse Agtrap Gene Expression in the Kidney—To understand the pathophysiological roles of Agtrap in target organ injury, it is necessary to investigate the regulation of the expression of the Agtrap gene in response to pathological stimuli. UUO is a well established experimental model of progressive tubulo-interstitial fibrosis. UUO leads to changes in renal hemodynamics, inflammatory responses in the kidney, tubular hypertrophy, and interstitial fibrosis of the affected kidney by stimulating the renin-angiotensin system (39). Since we previously showed that the Agtrap mRNA level was suppressed in the affected kidney by UUO (20), we examined whether the change in Agtrap gene expression is accompanied by any modulation of the Usf1 or Usf2 gene expression in the UUO kidney. According to the results of quantitative RT-PCR analysis, while the Usf1 mRNA expression was significantly up-regulated in the affected kidney after 7 days of UUO (Fig. 6B), the Usf2 mRNA expression was significantly down-regulated in the affected kidney by UUO (Fig. 6C), with a concomitant decrease in the Agtrap mRNA expression (Fig. 6A). These results in vivo are consistent with the notion that Usf1 and Usf2 are inhibitory and stimulatory transcription factors for the Agtrap gene, respectively.

Functional Involvement of the Two Adjacent E-box Motifs in Proximal Human AGTRAP Promoter Activity—To evaluate the evolutionary and functional conservation of the regulation of AGTRAP gene expression by the E-box, we examined the activity of the AGTRAP proximal promoter with or without an E-box mutation using luciferase reporter assay. Because the promoter of the human isologous gene AGTRAP has two adjacent located E-box motifs (Fig. 2A), we analyzed both of them. As shown in Fig. 7A, the 575-bp human AGTRAP proximal promoter fragments (−480 to +95 of the putative transcriptional start site) exhibited substantial luciferase activity in human kidney-derived HEK293 cells. In addition, muta-
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DISCUSSION

Despite the accumulating evidence supporting the involvement of an altered expression of Agtrap gene at local tissue sites in the pathogenesis of hypertension and related kidney injury, little is known about the transcriptional regulation of Agtrap expression. In this study, we showed that the promoter region from −150 to +72 of the mouse Agtrap 5′-flanking sequence, which is considered to contain important regulatory elements, directs Agtrap gene transcription in normal culture.
We analyzed the region from −381 to +72 based on the results showing maximum promoter activity. The results of luciferase assay using deletion mutants revealed the minimally required proximal promoter region from −150 to +72 that contains the X-box, E-box, and GC-box consensus motifs is able to direct substantial transcription of the Agtrap gene. Among these binding motifs, we confirmed that the E-box specifically binds Usf1 and Usf2 by employing EMSA, streptavidin-biotin complex assay, and ChIP. Such E-box-Usf1/Usf2 binding is functionally important in activating Agtrap expression for the following reasons: 1) mutation of the E-box to prevent Usf1/Usf2 binding reduces Agtrap promoter activity (Fig. 2); 2) transfection of siRNA for Usf1 increases and Usf2 decreases endogenous Agtrap mRNA and protein expression (Fig. 5), and 3) the decrease in Agtrap mRNA expression in the affected UUO kidney is accompanied by changes in Usf1 and Usf2 mRNA (Fig. 6). Taken together, these data indicate that Usf1 and Usf2 negatively and positively regulate Agtrap gene transcription, respectively. Because Usf1 and Usf2 bind to DNA with the same E-box sequence specificity, they most likely regulate Agtrap gene expression in a competitive manner.

Recently, the E-boxes in the promoter regions of renin and angiotensinogen were shown to be direct targets of Usf1 and Usf2 and suggested to be involved in the pathogenesis of both hypertension and renal injury (33, 56–58). In this study, it is
shown that Agtrap, an emerging modulator of the renin-angiotensin system, is another target gene of Usf1 and Usf2, and that Agtrap gene expression is activated through the binding of Usf2 and inhibited through the binding of Usf1 to the same canonical E-box sequence in the Agtrap proximal promoter region. Both Usf1 and Usf2 are reportedly activators of gene transcription via homodimerization or heterodimerization, with similar trans-activating capacities (59, 60), and they have also been proposed to function as repressors of a number of target genes (61). However, the results of this study show that Usf1 and Usf2 exert opposing regulatory effects on the expression of the same gene. Consistent with this notion, similarly opposing effects of Usf1 and Usf2 on the E-box of plasminogen activator inhibitor-1 gene, a key regulator of the fibrinolytic system, have been reported (62, 63). With respect to an interaction between Usf and other transcription factors, a previous study reported a contrasting functional and physical interaction between Usf and Sp1, a GC-box binding transcription factor, in the transcriptional regulation of the deoxycytidine kinase gene in liver-derived HepG2 cells (64). In the regulation of the deoxycytidine kinase promoter, the combination of Usf1 and Sp1 exhibited additive trans-activation at lower concentrations of Sp1, although Sp1 was inhibitory at higher levels, whereas trans-activation by Usf2 and Sp1 was synergistic in HepG2 cells (64). In this study, although the E-box and GC-box were found to be adjacent in the Agtrap promoter, the results of luciferase assay showed a positive and independent stimulatory effect of these binding motifs in kidney-derived mDCT cells (Fig. 2), possibly because of a difference in the network of transcription factors in the liver and kidney. However, it is still possible that a functional interplay of Usf1 and Usf2 with putative transcription factors other than Sp1 is involved in the opposing regulatory effect exerted by Usf1 and Usf2 on Agtrap gene expression (Fig. 5). Further studies are needed to elucidate the molecular mechanisms, including kinase cascades, such as PI3K (28), which are involved in the differential regulatory functional effect of Usf1 and Usf2 on Agtrap gene expression. Studies are also needed to examine the possible role of E-box modulation by methylation at the core CpG in the Usf1/Usf2 recognition site (5’-CACpGTG-3’) in the regulation of the Agtrap promoter (64).

Cardiovascular and renal diseases are closely related to circadian rhythms, which are under the control of an internal biological clock mechanism. The binding of the transcription factors BMAL1 and CLOCK to multiple extra- and intragenic E-boxes is reported to play an important role in the circadian
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rhythm-related regulation of certain genes in peripheral, cardiovascular, and renal tissues (65–67). However, the present results do not indicate any significant interaction of BMAL1 with the E-box of the mouse Agtrap promoter (Fig. 4). This may be because the BMAL1-CLOCK heterodimer binds to multiple E-boxes of target genes despite there being a single E-box in the mouse Agtrap proximal promoter (66). The human AGTRAP promoter contains two adjacently located E-box motifs (Fig. 2). However, we did not obtain any evidence to indicate the interaction of BMAL1 with these two adjacently located E-box motifs in the AGTRAP promoter, at least in human kidney-derived cells (Fig. 7). Further studies are needed to examine the potential interaction of the BMAL1-CLOCK heterodimer with the adjacent two E-box motifs in the AGTRAP promoter in other cells or tissues such as fat or liver, so as to exert cell type- or tissue-specific function. However, the results of the promoter assay and ChIP analysis clearly indicate the functional interactions of USF1/USF2 and the adjacently located two E-box motifs in the AGTRAP promoter. We also demonstrated functional E-box-USF1/USF2 binding in the human AGTRAP promoter, thereby suggesting that a strategy of modulating the E-box-USF1/USF2 binding may have novel therapeutic potential.

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