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Identification and characterization of an androgen-responsive Kap promoter enhancer located in the intron II region of human angiotensinogen gene

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ABSTRACT

Transgenic expression of the human angiotensinogen (HAGT) gene directed by the mouse kidney androgen-regulated protein (*Kap*) gene promoter is proximal tubule cell-specific and androgen-regulated *in vivo*. The same *Kap* promoter fragment did not support similar regulation of other genes, but a transgene based on the original chimeric KAP-hAGT construct successfully directed NHE3 to kidney, suggesting that sequences within the HAGT gene fragment of the construct contributed to the regulation of its expression *in vivo*. In the present study, androgen-responsive regulatory sequences in the HAGT gene portions of the transgene were examined in transfected renal cells. A 1.4-kb enhancer between exons 2 and 3 was identified that increased the basal expression of *Kap* promoter 1.5- to 2-fold, its induction by dihydrotestosterone (DHT) 2- to 3-fold and its induction by dexamethasone (Dex) 4- to 5-fold. Sequence analysis revealed two potential hormone-responsive elements. Mutational assays and electrophoretic mobility shift assay showed one of these elements was androgen-specific. These findings may influence future strategies for the design of inducible, cell-specific transgenes.

1. Introduction

Steroid hormone receptors (SRs) are intracellular receptors that act upon binding of their ligand to regulate gene transcription. Upon activation by their cognate hormone, the SRs form homodimers that are able to bind to an imperfect palindromic DNA sequence, called the hormone response element (HRE). Investigation as to how SR regulates hormone-responsive promoters and enhancers, and mechanisms of steroid receptor specificity are ongoing in several experimental model systems [1–5]. HREs are found in various genes as hexameric palindromes or hexameric direct repeats (DR) with 3–5 bp spacers [6–9]. *In vitro*, when androgen receptor (AR) bound with random sequences preceding TGTTCT, AR selected a partial overlapping direct repeat of three hexamers [9]. Less is known about the selectivity for AR at HREs *in vivo*.

The kidney androgen-regulated protein gene (*Kap*) is tissue-specific and regulated by androgen [10]. The *Kap* promoter has been for the study of androgen-regulated gene expression [10] and also as an inducible gene-targeting tool [11–13]. The expression of a transgene consisting of the *Kap* promoter and the human angiotensinogen (HAGT) gene is proximal tubule cell-specific and androgen-regulated *in vivo* [14]. The degree of androgen induction of HAGT mRNA in these transgenic mice was 10–15 times higher than for endogenously expressed *Kap* [15]. As enhancers within introns or the 3′-untranslated region have been shown to increase the transcriptional efficiency and expression of chimeric transgenes in transgenic mice [11,16] and many attempts to use the *Kap* promoter to direct expression of intronless DNA have failed, the question arose of whether the HAGT gene contained an androgen-inducible enhancer. Enhancers in HAGT have been reported to direct cell type-specific expression of hAGT [17,18], however none have been associated with androgen response.

The aim of the current study was to locate portions of the hAGT gene that may act as enhancers in androgen-regulated expression in kidney cells and then identify its HREs *in vitro*. Experimental strategies used included overlap analysis to find the location of downstream enhancers, site-directed mutagenesis and DNA binding assay to evaluate putative response elements.

2. Materials and methods

2.1. Materials

OK (opossum kidney epithelial), was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). DMEM, and

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fetal bovine serum were obtained from Gibco. All oligonucleotides were synthesized at Shanghai Sangon (PR China). Transfection reagent Fugene 6 was purchased from Roche (USA). Luciferase reporter plasmids and luciferase substrate were obtained from Promega (USA). Dihydrotestosterone (DHT) and dexamethasone (Dex) were obtained from Sigma. All enzymes used for molecular cloning were from New England Biolabs (USA).

2.2. Plasmid construction

First, pGl3-Kap1542 was constructed by inserting *Kap* promoter fragments −1542/−1 into SacI and HindIII sites of pGL3-Basic as previously described [14]. Sequence analysis showed single restriction site in hAGT for BglII, BamHI and XhoI site and double restriction sites for SalI and KpnI. A diagram of the restriction fragments from hAGT that were inserted as enhancer fragments is shown in Fig. 1A. Because BglII leaves ends compatible with those left by BamHI and XhoI leaves ends compatible with those left by SalI, subclones *Bgl-Sal* (7.9 kb), *Bgl-Bam* (2.7 kb), *Bam-Sal* (5.2 kb), *Sal-Sal* (1.7 kb), *Bam-Xho* and *Xho-Sal* were constructed by inserting corresponding fragments into a BamHI and (or) SalI cloning site following the poly A region of pGl3-Kap, that was inserted upstream of the promoter fragments. Subclone *Bgl-Sal-Sal* (7.9 kb) containing the whole hAGT genomic region in *Kap-HAGT* transgenic construct was constructed by inserting 1.7 kb SalI fragment into the SalI site of subclone *Bgl-Sal* and selecting clones with correct orientation of the SalI fragment after sequence analysis. The 1.4 kb KpnI fragment was inserted into the KpnI site of the polylinker in pGL3-Kap1542 to make subclone *Kpn*|*Kap*1542.

The positive control vector for glucocorticoid and androgen response was constructed by insertion of the luciferase reporter (excised from pGL3-Basic with NheI and XbaI) into the NheI site of pMSG-CAT (Pharmacia, Piscataway, NJ, USA).

pET-rARDBD1 was constructed by insertion of a polymerase chain reaction DNA fragment with primer ARDBD1 (GGGGATCCCCCATCGACTATTACTTC) and ARDBD2 (GCGAATTCTTCTCCTTCTTCCTGTAG), encoding the DNA binding domain of rat AR, from amino acids 530–627, into the BamHI and EcoRI site of and pET28a (Novagen). The pET-ARDBD1 construct generated a 97-amino acid protein with a His tag on the amino terminus.

2.3. Site-directed mutagenesis

The QuikChange® II kit (Stratagene, La Jolla, CA, USA) was used for site-directed mutagenesis. For the alteration of potential AREs in the KpnI fragment of HAGT, the primers 5′-GTCTGCCCAGTGGTTGCTATGTTCCTGAGCATGGG, 5′-GGGACAAGAGTTATATGGGAAGTTTCTCGATTTCTGCTCGATTTTGCTGTGAA, 5′-ACAAGAGTTATATGGGAACTTTCTCTGTTTCTGCTCGATTTTGCTGTGAAC and the exact complements of each were used as pairs. All constructions were confirmed by DNA sequence analysis.

2.4. Cell culture and transient transfection assay

Opossum kidney epithelial cells (OK cells) were grown in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics (penicillin/streptomycin). Transfection assays on OK cells were done using Fugene® 6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) under the manufacturers directions. In detail, cells were seeded in 48-well tissue culture plates at a density of 2.5×10^4 cells in phenol red-free DMEM supplemented with 10% charcoal/dextran-treated FBS. 24 h later, when cells reached about 50–60% confluence, FuGene was resuspended (5 min at room temperature) in serum-free medium and allowed to complex with DNA constructs at the ratio of 6:1 (reagent in microliters to DNA in micrograms) for 15 min at room temperature. The plasmid/FuGene mixture was then overlayed on the cells in a final volume of 0.4 ml of fresh charcoal-stripped complete medium. Each assay well contained 5 ng *Renilla* luciferase vector, phRL-TK (Promega) as the internal control, and 25 ng pSG5rAR as the source of androgen receptor (AR) or 25 ng pSG5-hGR as a source of glucocoticoid receptor (GR). Each experiment included pGL3-Basic as the negative control, and 50 ng/well pMSG-Luc as a positive control for androgen and glucocoticoid induction. The experimental promoter constructs were transfected in amounts equimolar to each other and to the pGL3-Basic control. After incubation for 24 h at 37 °C, medium was replaced and cells were treated with/without 15 nM dihydrotesterone, 15 nM dexamethasone or ethanol vehicle for

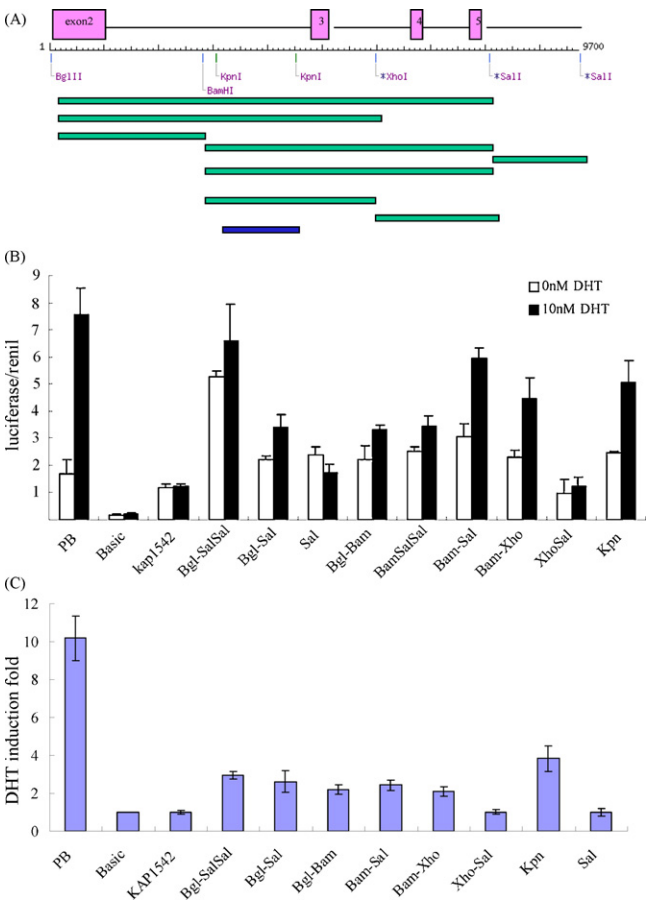

Fig. 1. Transient transfection assay of the androgen-responsible enhancer effect of hAGT gene. (A) Map of overlap constructs. The HAGT reporter includes exons 2–5, as well as intervening sequences between the included exons and some 3′-untranslated sequence. The overlapping fragments were made using available restriction sites, and the lines indicate the portions of the structural gene represented. Kap promoter fragments were inserted into the multiple cloning sites preceding the luciferase reporter (LUC). The HAGT fragments were inserted into a BamHI and SalI cloning site following the poly A region, except for the KpnI fragment, that was inserted upstream of the promoter fragments. (B) Transfection. Kap1542 plasmids containing various fragments of the HAGT gene were co-transfected with androgen receptor expression vector into OK cells in equimolar amount to the negative control, pGL3-Basic. pPB-Luc was used as androgen-inducible positive control. pRL-TK was used as the internal control. Each construct was represented by six wells of a 48-well assay plate, three of which were exposed to 15 nM DHT. Cells were harvested 48 h after transfection. Activities of firefly and Renilla luciferases were measured sequentially from the same aliquots using the Dual-Luciferase Reporter Assay System (Promega). A single experiment that is representative of at least three experiments was shown. The inductions by DHT were: PB, 20-fold, Kap1542, 1.2-fold, Bgl-Sal-Sal, 2.4-fold, Bgl-Bam, 2.5-fold, Bam-Sal, 1.7-fold, Bam-Xho, 1.6-fold, Xho-Sal, 1.1-fold, Kpn, 3.2-fold, and Sal, 1.2-fold. (C) Average induction fold by DHT. In each experiment, the induction fold of every sample was divided by that of the negative control, pGL3-Basic. Average data from five independent transfection experiments were shown (±S.E.).

24–30 h. Each transfection assay included six wells for each DNA constructs (three with and three without steroid) and was carried out at least three times on separate days.

2.5. Luciferase reporter assay

Cells were harvested for assay 48 h after transfection. The medium was removed, the wells were washed once with PBS, and 50 μl of passive lysis buffer (Promega) was added to each well. The lysates were incubated at room temperature for 2 h. Aliquots of 20 μl cleared lysate were assayed for Firefly and Renilla luciferase activities in a luminometer (Luminoskan Ascent, Thermo Electron Corporation, Helsinki, Finland) using the reagents and directions provided in the Dual-Luciferase Reporter assay System (Promega). The results shown in the figures are a single experiment that is representative of at least three experiments, and plotted using Microsoft Excel software. The inductions given in the text of the Section 3 are the average of three experiments.

2.6. Expression and purification of ARDBD

The *Escherichia coli* strain BL21(DE3) was transformed with pET-rARDBD1. 100 ml cells were grown to OD_{600} = 0.4–0.6 and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h. After harvesting, cells were resuspended in 5 ml of Buffer A (20 mM Tris–HCl, pH 7.9, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The supernatant was incubated with 1.5 ml of Ni-NTA-agarose (Qiagen) resin in batch, rocked gently for 30 min at 4 °C, and then loaded on to a small Bio-Rad Econo-column. After successively washing the resin with 15 ml of Buffer A and 15 ml of Buffer A containing 20 mM imidazole, ARDBD was eluted with Buffer A containing 150 mM imidazole. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Peak fractions were combined. The final preparation contained 100 μg/ml ARDBD.

2.7. Electrophoretic mobility shift assay (EMSA)

Mobility shift experiments were performed with 5 μg of purified ARDBD/GRDBD and 5′ end DIG-labeled double-stranded oligonucleotides containing the desired HRE sequences. Typically, the binding reactions were carried out in 20 μl containing 10 mM Tris–HCl (pH 7.5), 65 mM NaCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 10% glycerol, 1 mg/ml bovine serum albumin, and 25 μg/ml poly(dI-dC) as a carrier for 30 min at 4 °C. In competition experiments, a 100-fold molar excess of the cold competitor was preincubated with purified ARDBD/GRDBD for 30 min at 4 °C before the labeled DNA fragment were added. The complexes were resolved on nondenaturing 6% polyacrylamide gels in 0.5× TBE buffer for 1 h at 14 V/cm, and viewed following ECL detection.

3. Results

3.1. Androgen response and enhancer effect of hAGT gene

In vivo data from transgenic mice suggested that a 1542 bp *Kap* promoter fragment (*Kap*1542) alone was sufficient to drive the PTC-specific, androgen-regulated hAGT expression, but not several other genes, whereas the chimeric *Kap*1542-HAGT could successfully drive the PTC-specific, androgen-regulated human renin and rat NHE3 expression. Therefore *Kap* promoter sequences seemed necessary but not sufficient for such expression and additional regulatory elements within the hAGT genomic sequence might contribute to tissue-specific expression. Therefore, the relationship between the *Kap* promoter and hAGT reporter gene was studied.

To analyze 1542 bp *Kap* promoter activity *in vitro*, the fragment (nt −1542 to +1 for *Kap*) was cloned into a luciferase reporter plasmid (see Section 2). The reporter activity was assayed by transient transfection in OK cells, Cos-1 cells, NIH3T3 cells and LNCaP cells. The AR was co-transfected into all lines except for LNCaP, which has an endogenous AR. Luciferase activity from the *Kap* promoter construct was induced 1.2-fold in the presence of 10^{-6} M 5α-DHT only in OK cells. Under the same conditions, the mouse mammary tumor virus (MMTV)-Luc construct (positive control) showed 8- to 10-fold steroid-induced transcription in all cell lines, and pGL3-basic (negative control), showed no induction in the presence of androgens (data not shown). Therefore it seemed that OK cells, but not other cell lines, provided factors necessary for *Kap* promoter activity, so OK cells were chosen for additional experiments.

The hAGT reporter used in the transgenic mouse construct included exons 2–5, intronic regions, and some 3′-flanking sequence. This 9.7 kb sequence was inserted into the enhancer site of the *Kap*1542, and referred to as *Bgl-Sal-Sal* (Fig. 1a). The chimeric construct was tested in OK cells. *Bgl-Sal-Sal* had a 1.5- to 3-fold higher luciferase expression than that of *Kap*1542 without the HAGT and was induced an additional 2- to 3-fold by DHT, whereas *Kap*1542 was not or slightly induced (Fig. 1b). From this result, we inferred that the HAGT sequence included androgen-responsive sequences, although the level of induction in transfection assay was much lower than that for the transgene *in vivo*.

3.2. Identification of an androgen-responsive enhancer region in the HAGT reporter

Two liver cell-type dependent enhancer elements have been reported in the downstream region of the HAGT gene [17,18]. Therefore, chimeric constructs consisting of the mouse *Kap* promoter and hAGT restriction fragments that either included or excluded known enhancers were subcloned and assayed by transient transfection in OK cells (Fig. 1b). The cleavage of the hAGT gene by SalI yielded the subclones *Bgl-Sal* (7.9 kb) and *Sal-Sal* (1.7 kb) (Fig. 1a). Luciferase reporter activity from the *Sal-Sal*, which contained the previously identified core enhancer d61, was about 2-fold higher than the basal activity of *Kap*1542, but was not induced by DHT. However, induction by DHT of luciferase activity of the *Bgl-Sal* was slightly higher than that of *Bgl-Sal-Sal*. The subclone *Xho-Sal* included the previously identified core enhancer B2-5 but was not induced by androgen and did not increase the basic promoter activity of *Kap1542*. The subclone *Bam-Xho* had a similar androgen response to *Bam-Sal* (Fig. 1b). We concluded that neither B2-5 nor d61 were androgen-responsive enhancers of Kap1542.

A smaller KpnI fragment (+2987 to +4171), located within *Bam-Xho* (Fig. 1a), increased the basal activity and androgen induced activity of the *Kap*147 promoter by 2- to 3-fold (Fig. 1b). This induction was higher than for the other constructs containing portions of hAGT, therefore the KpnI fragment was most likely to contain an androgen-response element.

3.3. Steroid specificity of the hAGT KpnI fragment

We tested the responses of the construct containing hAGT KpnI fragment to both androgen and glucorticoid in OK cells. To measure androgen response, the luciferase construct was co-transfected with pSG5-mAR and treated with 5α-DHT (Fig. 2a). To measure glucocorticoid response, the hAGT construct was co-transfected with pSG5-hGR and treated with dexamethasone (Fig. 2b). The positive control in each case was pMSG-luc, which is inducible by either androgen or glucorticoid. As shown in Fig. 2, expression of *KpnKap*1542 was induced 2.4-fold by DHT and expression of *KpnKap* 1542 was induced 4.5-fold by DEX. The inductions by DEX

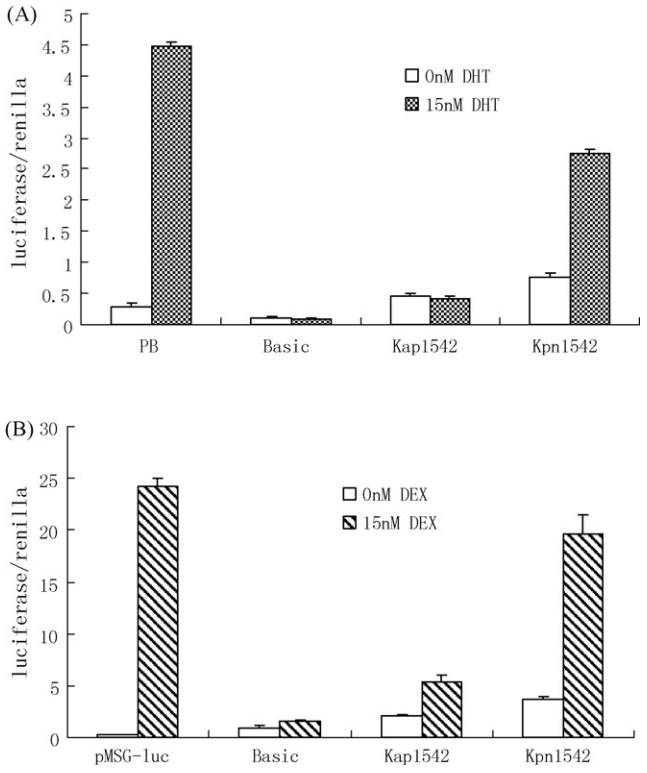

Fig. 2. Steroid specific of hAGT KpnI fragment. Constructs were co-transfected with pSG5-hGR into OK cells and assayed in the absence and presence of 15 nM dexamethasone (DEX) or 15 nM DHT. (A) The inductions by DHT were, Kap1542, 1-fold, KpnKap1542, 2.4-fold and (B) the inductions by DEX were, Kap1542, 2-fold, KpnKap1542, 4.5-fold.

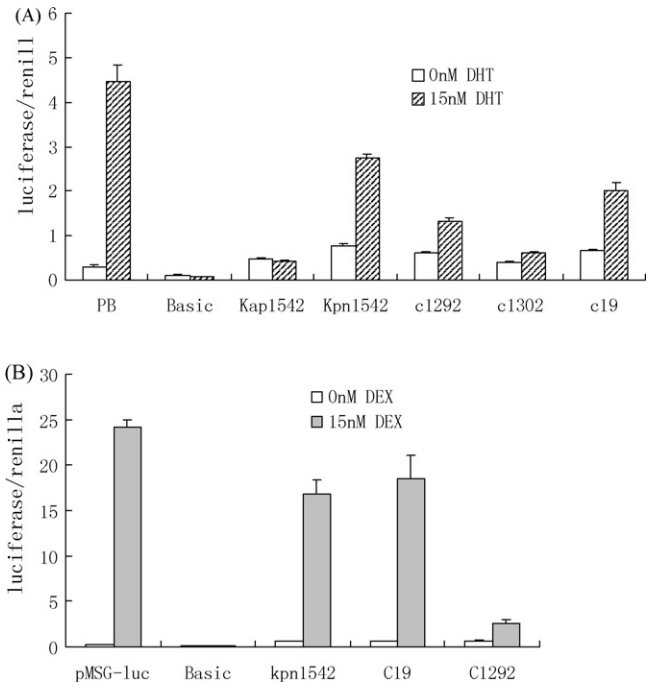

Fig. 3. Mutational analysis of ARE-like/GRE-like sequences in the HAGT Kpn I enhancer fragment. There were 2 possible androgen-responsive elements in the HAGT Kpn fragment (A, B) and three kinds of mutation (C19, C1292, C1302). (A) Induction by DHT. The unaltered construct was induced 3.6-fold by DHT. C19 contained a point mutation of a DR3 near the upstream KpnI site, this was induced 3.1-fold. C1292 and C1302 contain point mutations in a DR3DR3 near the downstream KpnI site, these mutations resulted in lowered induction in response to DHT, 2.2-fold and 1.53-fold and (B) induction by DEX. The unaltered construct was induced 27.9-fold by DEX. C19 contained a point mutations of a DR3 near the upstream KpnI site, this was induced 31.3-fold. C1292 contain point mutations in a DR3DR3 near the downstream KpnI site, this mutation resulted in lowered induction in response to DEX, 3.96-fold.

were greater than by DHT. Therefore the hAGT KpnI fragment did not appear to be selective for androgen.

3.4. Analysis of sequences involved in the steroid response in HAGT KpnI

Initially, we looked for potential AREs/GREs consisting of closely spaced hexamers resembling the core motif TGTTCT forming indirect or direct repeats. Finding none, the criteria were lowered to include partial direct repeats including any hexamer following the pattern A/TGTnCA/T. We found two possible AREs/GREs: a DR3, a direct repeat of hexamers separated by three nts close to the 5′ KpnI site, with the sequence A**G**TG**C**TtgcT**G**TG**C**T (designated A); and another DR3 nearer to the 3′ KpnI site with the sequence G**G**AA**C**TttcT**G**TA**C**T (designated B) (Table 1).

To assess the function of the putative ARE/GREs, we used site-directed mutagenesis to eliminate guanine or cytosine contact sites. The alteration of site A, to the sequence A**G**TG**G**TtgcT**A**TG**T**T (C19) (Table 1), eliminated any occurrence of nGnnCn. This alteration had little effect on androgen induction but no effect on glucocorticoid induction (Fig. 3). Site B was altered in either of two ways: C1292 with sequence GGAAGTttcTCGATT resulted in alteration of both hexamer half-sites, and G1303 with sequence GGAACTttcTCTGTT resulted in alteration of the right hexamer only (Table 1). Inductions of C1292 and G1303 by androgen were decreased from 3.6-fold to 2.2- and 1.5-fold, respectively (Fig. 3A). Inductions of C1292 by glucocorticoid were one-tenth those of the unaltered sequence (Fig. 3B). These data showed that site B was involved in both AR and GR response.

Table 1
Original and mutant DNA sequences of two possible steroid response elements in HAGT KpnI.

Original	HAGT(*Kpn*) 20–34 A	AGTGCTtgcTGTGCT	DR3
Altered	C19g	AGTG**G**TtgcT**A**TG**T**T	
Original	HAGT(*Kpn*) 1291–1317 B	GGAACTttcTGTACTttcTGCTCGA	DR3DR3
Altered	C1292	GGAA**G**TttcT**C**TA**T**TttcTGCTCGA	
Altered	C1302	GGAACTttcT**C**TGTTttcTGCTCGA	

Column 1 states whether this is the original sequence or the altered sequence. Column 2 refers to the location of the original sequence or the designation of the plasmid containing the altered sequence. Column 3 shows the putative androgen-response element, with the hexamer in upper case and the spacer in lower case. Column 4 refers to the kind of repeat – direct repeat (DR) or inverted repeat (IR) – and number of bases in the spacer.

3.5. Electrophoretic mobility shift assay (EMSA) of ARE/GRE in hAGT KpnI

To study the sequence-specific binding of AR and GR to site A and site B of hAGT *Kpn* fragment, we used 24-mer oligonucleotide probes corresponding to site A (HAGTA) and site B (HAGTB), in EMSA with ARDBD and GRDBD proteins. As shown in Fig. 4, HAGTA bound only to the ARDBD but not GRDBD, while HAGTB bound to both ARDBD and GRDBD. This was consistent with the result from site-directed mutagenesis that site A was a potential androgen-specific HRE and site B was not an androgen-specific HRE.

We used a set of unlabeled competitor oligonucleotides representing standard HAGTA, HAGTB, and variants in which the A/TGTnCA/T motif had been mutated (C19g, C1292). Sequence-specific complexes between HAGTA and purified ARDBD were completely suppressed by an excess of unlabeled competitor HAGTA, but not by mutant competitor C19g (Fig. 4a). Complex

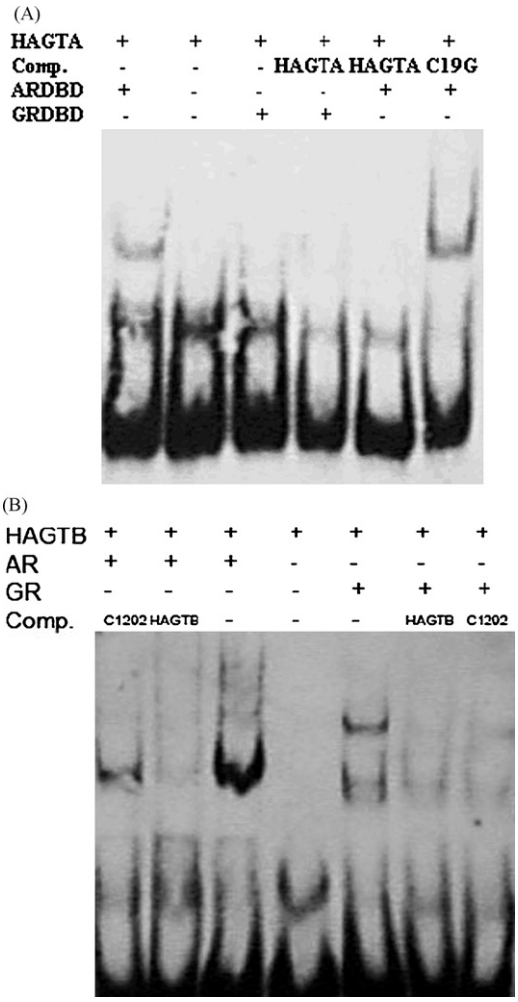

Fig. 4. Electrophoretic mobility shift assay (EMSA) for interactions of the hAGT Kpn fragments with purified recombinant ARDBD and GRDBD. Specific complexes are supressed by 50 ng of unlabeled hAGT Kpn HRE(s) (standard HAGTA, HAGTB) and unlabeled mutant hAGT Kpn HRE(s) (C19g, C1292) competitor. (A) HAGTA and (B) HAGTB.

formations between HAGTB and purified ARDBD or GRDBD were completely suppressed by an excess of unlabeled competitor HAGTB, and partly by mutant competitor C1292 (Fig. 4b).

4. Discussion

In vitro, the receptors for glucocorticoids, mineralocorticoids, progestins, and androgens recognize the same DNA consensus sequence, which consists of an inverted repeat of two 6-bp half-sites, separated by a three-nucleotide (nt) spacer 5′-GGTACAnnnTGTYCT-3′. The requirements for androgen receptor-specific recognition *in vivo* are less clear cut. The palindromic pattern sometimes applies to androgen-response elements found *in vivo*, such as in the prostatein gene [6], but direct repeats of hexamers form the androgen-response elements in the genes for probasin [5], sex-limited protein [7], and secretory component [8]. The spacing between hexamers can be variable, as in the Pem homeobox gene, the ARE of which is a direct repeat of hexamers separated by five nts [19].

In our previous study, a human gene fragment, hAGT, conferred androgen-inducibility to a tissue-specific promoter, *Kap*, in transgenic mice [14] and in the current study, the same fragment conferred androgen-inducibility to a *Kap*-luciferase reporter construct *in vitro*. Therefore, we used sequence homology algorithms to search for potential AR and GR contact sites in the hAGT KpnI fragment. When looking for potential AREs/GREs with TGTTCT hexamers that formed indirect or direct repeats, we found none. When the criteria were lowered to the hexamer A/TGTnCA/T and two AREs/GREs were located. Site-specific mutagenesis and EMSA in the DR3DR3 motif showed that site A was selective for androgen receptor, but either androgen receptor or glucocorticoid receptor could interact with site B.

The hAGT KpnI fragment did not overlap with other portions of *HAGT* shown to have enhancer activity for liver-specific expression. The enhancer elements identified elsewhere in HAGT are known as B2-5 and d61 [17,18]. When HAGT fragments including either B2-5 or d61 were tested in luciferase reporter constructs under the same conditions as the KpnI fragment, neither conferred androgen response to the *Kap* promoter.

The *HAGT* promoter directed expression of its own mRNA in liver, kidney, heart, adrenal gland, ovary, brain and adipose tissue of HuAoGen transgenic mice, furthermore, the HAGT expression in the kidney and adrenals of HuAoGen mice was regulated by androgen [20]. Substitution of the *Kap* promoter for the HAGT promoter restricted HAGT transgene expression to the proximal tubules and to the epididymis [14], another site of endogenous *Kap* expression. The expression of the *Kap-HAGT* transgene is normally undetectable in the kidney of female mice, can be dramatically induced in response to testosterone treatment [14]. We identified an androgen-response element in the *Kap* promoter [21], and in the current study showed that sequences from the HAGT contributed to hormonal response. This is the first report to suggest a basis for the androgen or glucocorticoid response in the downstream portion of this gene.

In summary, we found that an androgen-regulated downstream enhancer with two HREs is localized to a 1.4 kb sequence within intron II of the hAGT gene, one of which is specific to androgen. Future experiments will address the question of whether the hybrid HAGT KpnI enhancer-Kap promoter can be used to specifically target other genes to the proximal tubule cells and how is it regulated in vivo.

Acknowledgements

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