Hormone-specific regulation of the kidney androgen-regulated gene promoter in cultured mouse renal proximal-tubule cells

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The kidney androgen-regulated protein (KAP) is specifically expressed and differentially regulated by androgens and tri-iodothyronine (T_3) in intact mouse early (PCT) and late (PR) proximal-tubule cells. Until now, detailed characterization of the molecular elements mediating androgen-responsive gene expression in the kidney has been hampered by the lack of appropriate cultured cell systems suitable for DNA transfection studies. In the present study we have analysed the hormone-dependent transactivation of the KAP gene promoter in immortalized differentiated PCT and PR proximal-tubule cells derived from L-PK/Tag1 transgenic mice. Transient transfection studies with different KAP promoter constructs indicated that a 224 bp-truncated fragment was sufficient to mediate cell-specific expression of the KAP promoter. Dihydrotestosterone (DHT) stimulated in an androgen-dependent manner the transactivation of KAP in PCT and PR cells, while mutation of a putative androgen-response element (ARE) sequence located at −39 bp from the transcription initiation site abolished the transactivation induced by DHT. Furthermore, insulin-like growth factor 1 (IGF-1), but not T_3, enhanced the androgen-dependent transactivation of KAP in cultured PCT cells. These results demonstrate that the short 224 bp fragment of the KAP promoter is sufficient to drive the proximal-tubule androgen-specific regulated expression of KAP and reveal synergistic interactions between IGF-1 and androgens for KAP regulation in PCT cells.

Key words: gene expression, KAP, promoter assays, steroid hormones.

INTRODUCTION

Androgens exert a variety of effects on many target tissues. Although they play an important role in the reproductive system [1], they also exert their effects on non-genital tissues. Among these, the kidney has been well characterized as an androgen-responsive tissue. It is profoundly affected by androgenic hormones, primarily in epithelial cells of proximal tubules [2]. Although a great deal of biochemical and genetic information on androgen-inducible mRNA expression in the mouse kidney has accumulated, detailed characterization of the molecular elements mediating androgen-responsive gene expression in the kidney has been hampered by the lack of appropriate cultured cell systems suitable for DNA transfection studies. For example, testosterone failed to modulate the endogenous expression of ornithine decarboxylase (ODC) and RP-2 genes in several cell lines of renal origin, including DB.2 [3] and MCT cells [4]. Asadi et al. [5] showed that dihydrotestosterone (DHT), the active metabolite of testosterone, stimulated a set of androgen-regulated genes in primary cultures of mouse proximal-tubule cells. However, even this primary cultured cell system was not effective enough for transient transfection assays required for the identification and characterization of elements controlling androgen-inducible transcription in mouse proximal tubule.

Using a strategy of targeted oncogenesis in transgenic mice, Cartier et al. [6] established two lines of proximal-tubule cells derived either from early proximal-convoluted-tubule (pars convoluta), PKSV-PCT cells, or the late straight portion of the proximal tubule (pars recta), PKSV-PR cells, microdissected from the kidneys of transgenic mice carrying the large T and little t simian-virus-40 (SV40) antigens placed under the control of the 5′ regulatory sequences of the L-type pyruvate kinase gene. Several studies have shown that these two proximal-tubule cell lines have conserved the main features of the parental cells from which they were derived [6–8]. We also showed that levels of the kidney androgen-regulated protein (KAP) and β-glucuronidase (β-gluc) transcripts, differentially expressed in two cultured PKSV-PCT and PKSV-PR cell lines, decreased when cells were shifted from a serum-supplemented to a steroid-free medium, whereas DHT induced a slight increase in β-gluc and a more marked increase in KAP transcripts in both cell lines [9].

The KAP product, initially identified as an abundant 20 kDa protein by *in vitro* translation of male mouse kidney mRNA [10], is encoded by a gene recently identified by means of serial analysis of gene expression (SAGE) [11], SAGE adaptation for down-sized extracts ('SADE') [12] and expression profile of active genes [13] as the most abundant and specific gene expressed in mouse kidney proximal tubules, since it represents about 4% of the total poly(A) species that are induced by androgens in mouse kidney [10–13] and it is highly restricted to the proximal-tubule epithelial cells and to the uterus of pregnant females prior to delivery [14]. Previous studies performed on intact mouse kidneys showed that KAP expression is subject to complex hormonal regulation according to the different segments (early or late) of

Abbreviations used: AR, androgen receptor; ARE, androgen-response element; CAT, chloramphenicol acetyltransferase; CsA, cyclosporin A; CyPB, the CsA-binding protein cyclophilin B; DHT, dihydrotestosterone; FCS, fetal-calf serum; gal, galactosidase; GH, growth hormone; β-gluc, β-glucuronidase; GR, glucocorticoid receptor; *HAGT*, human angiotensinogen gene; IGF-1, insulin-like growth factor 1; KAP, kidney androgen-regulated protein; LUC, luciferase; MMTV, murine-mammary-tumour virus; ODC, ornithine decarboxylase; PCT, intact mouse early proximal-tubule cells; PR, intact mouse late proximal-tubule cells; RT-PCR, reverse-transcription PCR; RXR, retinoid X receptor; SAGE, serial analysis of gene expression; SV40, simian virus 40; T3, tri-iodothyronine; THR, thyroid-hormone receptor.

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proximal tubules [15–20]. However, more detailed characterization of the mechanisms involved in such regulation has been hampered by the lack of a suitable *ex vivo* model of androgen-responsive proximal-tubule cells. Apart from its cell-specific expression, strict regulatory mechanisms and relative abundance of the *KAP* gene, little is known of the function of its encoded protein, which does not exhibit known structural or functional domains, or homologies with other sequences in the databases. Recently, some of us have shown that KAP interacts with the cyclosporin A (CsA)-binding protein cyclophilin B (CyPB), and that CsA reduces KAP steady-state levels in mouse kidney, whereas overexpression of KAP leads to a reduction in CsA toxicity in stably transfected proximal-tubule cells exhibiting tetracycline-controlled overexpression of KAP [14]. The results from that study provided the first demonstration of the existence of a functional link between KAP, CypB and CsA-mediated nephrotoxicity, thereby suggesting that KAP may play an important role in renal physiology and physiopathology.

In the present study, the molecular mechanisms involved in transcriptional control of the *KAP* gene in mouse kidney were analysed by transfection studies using the immortalized PKSV-PCT and PKSV-PR cell lines. The main results from the present study clearly show that these mouse proximal-tubule cells in culture can mimic the intact kidney in relation to the transcriptional activation of the androgen-regulated kidney-specific gene *KAP*. The *KAP* promoter is transcriptionally activated by androgen receptor-dependent mechanisms in these cells, through a putative androgen-response element (ARE) located at position −39 from the transcription initiation site [21]. We also analysed how tri-iodothyronine (T_3) and insulin-like growth factor 1 (IGF-1) could modulate the transcriptional capacity of the *KAP* promoter in PKSV-PCT cells. Finally, the effects of growth hormone (GH) on KAP mRNA expression in mouse kidney were tested using genetically deficient strains of mice.

MATERIALS AND METHODS

Materials

Media for cell culture and LIPOFECTAMINE™ reagent were obtained from Life Technologies (Gaithersburg, MD, U.S.A.). Fetal-calf serum (FCS), trypsin, glutamine and essential amino acids were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Other supplements for cell culture, acetyl-CoA, sodium orthovanadate, steroid hormones and silica-gel TLC sheets were obtained from Sigma (St. Louis, MO, U.S.A.). Restriction and modifying enzymes were purchased from either Life Technologies, Promega (Madison, WI, U.S.A.), Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.), TaKara Shuzo Co. Ltd. (Shiga, Japan) or Roche Molecular Biochemicals (Mannheim, Germany). Oligonucleotides were synthesized by Tib Molbiol Syntheselabor (Berlin, Germany).

Plasmid constructs

To obtain the promoter fragment consisting of nts −224 to +1 in the *KAP* gene (relative to the start site of transcription), primers including these endpoints were used to amplify this region by PCR using a genomic subclone of the *KAP* gene. The 5′ primer (designated K67) had the sequence GCTCTAGAGGTACC**GGGTGGTGCACCTAGTCAG** (the KAP-specific portion is indicated here and below by the **bold** font). Recognition sites for the restriction enzymes *Xba*I and *Kpn*I were introduced into the 5′ end of the oligonucleotide. The 3′ primer (designated K54) had the sequence CCAGATCT**CCAAAAAGAGTCCTCCTGG**. The recognition site for *Bgl*II was introduced into the 5′ end of the oligonucleotide. An annealing temperature of 55 °C was used for this amplification. The *Kpn*I and *Bgl*II sites were used to insert the fragment into the same sites in the reporter. To obtain the promoter fragment consisting of nts −637 to +1, an upstream primer (designated K55) with the sequence CCATCGAT**GATCTTAGCCATTCTGACT** replaced K67. To obtain the longer promoter fragment extending to nt −1542, a genomic fragment was excised from a plasmid subclone using *Sac*I and *Xba*I and joined to the *Xba*I site from the KAP637 promoter fragment. All constructs were verified by extensive restriction mapping and partial DNA sequencing. All promoter lengths are available in both chloramphenicol acetyltransferase (CAT) and lucerifase (LUC) promoterless vectors. The pCAT-Basic and pGL3-Basic vectors were obtained from Promega Corp. (Madison, WI, U.S.A.).

The expression plasmid for β-galactosidase (β-gal) pCH110 (Amersham Pharmacia Biotech) and the *Renilla* LUC reporter plasmid pRL-TK (Promega Corp.) were used to normalize the transfection efficiencies of CAT and LUC assays respectively. Plasmid pSVAR0 containing an SV40 promoter that directs the transcription of the full-length human androgen receptor cDNA [22] or the human glucocorticoid receptor were kindly provided by Dr C. López-Otín (Departmento de Biologia Funcional, Universidad de Oviedo, Oviedo, Spain) and Dr M. Beato (Centre de Regulacio Genomica, Universitat Pompeu Fabra, Barcelona, Spain) respectively. Thyroid-hormone receptor (THR), consisting of the cDNA encoding rat $T_3R\alpha1$ cloned in pCDM8 and retinoid X receptor (RXR) expression vector, the human RXRα cDNA, cloned in plasmid pSG-5, were kindly provided by Dr J. Bernal and Dr A. Muñoz [Centro de Investigaciones Biomedicas, Consejo Superior De Investigaciones Científicas (CSIC), Madrid, Spain] [23]. The reporter control plasmid for androgen-dependent promoter activity, pCAT1430, which contains 1430 bp from the proximal promoter of the human pepsinogen C gene fused to the CAT gene, was also kindly provided by Dr C. López-Otín [22]. A LUC promoter control construct for androgen-dependent reporter activity was prepared by using the minimal promoter of the mouse *CYP4B1* gene, i.e., 98 bp fragment from the initiation of transcription (J. Isern, personal communication), fused to a consensus ARE sequence in the pGL3-LUC reporter gene (pARE98CYP-LUC). The glucocorticoid responsive murine-mammary-tumour-virus (MMTV) long terminal repeat fused to the *LUC* gene under the thymidine kinase promoter (MMTV-LUC) was kindly provided by Dr M. Beato. The SaltK reporter construct, which corresponds to a thyroid hormone-responsive element introduced into the reporter plasmid pBLCAT2 upstream of the heterologous HSV-tk promoter, was kindly given by Dr. Alberto Muñoz [24].

Cultured cells

The mouse renal proximal convoluted PKSV-PCT cells and late proximal-tubule PKSV-PR cells have been previously established and characterized [6–8]. In order to obtain consistent and reproducible inter- and intra-assay results, the cell lines were cloned by limiting dilution. Cloned cells were selected according to their differentiated dome-forming phenotype and maximal endogenous *KAP* gene expression, measured by semi-quantitative reverse-transcription (RT)-PCR assays. The cloned PKSV-PCT, referred to as PCT3, and cloned PKSV-PR, referred to as PR10 cells, were used in the present study. Cells were grown in the same modified medium used for the parental PKSV-PCT and PKSV-PR cells, as previously described [6,7]. CV1 cells were purchased from the American Type Culture Collection (Rockville, MD,

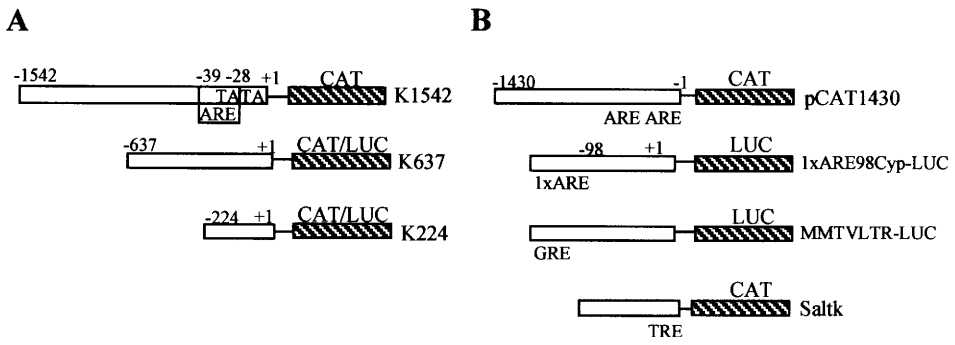

Figure 1 Set of reporter constructs used in the transfection assays

(**A**) K1542, K637 and K224 constructs containing various regions of the KAP promoter (−1542, −637 and −224 bp respectively) from the transcription initiation site, which is considered to be the +1 position, fused to the CAT or LUC reporter genes. (**B**) The pCAT1430 construct containing the 1430 bp of the human pepsinogen C gene proximal promoter fused to the CAT gene and the minimal promoter of the mouse CYP4B1 gene fused to the LUC gene were used as control for androgen inducibility in transfection assays. The glucocorticoid responsive MMTV long terminal repeat fused to the *LUC* gene under the thymidine kinase promoter (MMTV-LUC) was used as control for glucocorticoid inducibility. SaltK was used as a control for thyroid-hormone action.

U.S.A.) and N2A cells were kindly provided by Dr Juan Bernal (Centro de Investigaciones Biomedicas, CSIC, Madrid, Spain). Cells were grown and maintained in Dulbecco's modified Eagle's medium containing 1000 mg/l glucose, supplemented with penicillin/streptomycin (1 %), non-essential amino acids (1 %), sodium pyruvate (1 %) and FCS (10 %) at 37 °C and under air/CO_2 (19:1).

Transient transfection studies

For each transfection experiment using the CAT reporter plasmid, cells were seeded at 2.5×10^5 cells in a 60 mm-diameter dish and transfected 18 h later with 2 μg of the appropriate CAT construct, 0.5 μg of expression vectors containing the nuclear receptors and 2 μg of the internal control (pCH110) using the LIPOFECTAMINE® reagent following the manufacturer's indications. Plasmid pCH110 contains the structural gene for β-gal under control of SV40 early promoter and was used as an internal control of transfection efficiency. For transfection assays using the LUC reporter vector, 1.5×10^5 cells were seeded in 35 mm-diameter dishes and transfected with 1 μg of the appropriate LUC reporter vector, 0.5 μg of the expression vector containing the nuclear receptor and 20 ng of the internal control (pRL-TK) following the same procedure. Briefly, the DNA/liposome mixture was added to cell-culture dishes containing an appropriate volume of OptiMEM® I Reduced Serum Medium (Life Technologies). Complete fresh medium was added after 5 h incubation at 37 °C. At 18 h after DNA addition, cells were washed with PBS and replenished with medium containing steroid-free and thyroid-hormone-free serum by means of charcoal and Dowex resin treatment. Where required, complete medium supplemented with 1 μM DHT and/or 10^{-6} M T_3, 100 ng/ml IGF-I or 100 μM sodium orthovanadate were added to the cultures for 48 h before harvesting.

CAT and LUC assays

For CAT assays, cells were harvested (40 mM Tris/HCl, pH 7.4, 150 mM NaCl and 1 mM EDTA) and lysed by successive freeze–thaw cycles. Cell lysates were centrifuged for 5 min at 16000 ***g*** at 4 °C to remove cell debris. Cell lysates were heat-inactivated at 65 °C for 10 min. Upon β-gal normalization, CAT assays were performed using appropriate amounts of each lysate with 70 μg of acetyl-CoA and 0.025 μCi of [^{14}C]chloramphenicol (56 mCi/mmol) at a final volume of 130 μl and incubated 2–4 h at 37 °C. Reaction mixtures were extracted with ethyl acetate, dried, and ^{14}C-acetylated products separated by TLC. CAT activity was visualized by Fujifilm BAS-1800 Bio-imaging Analyzer and quantified with a Fujifilm Image Reader and the MacBAS program. LUC assays were performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and quantified using a Luminoskan RS reader (Labsystems, Helsinki, Finland). Stimulation of CAT and LUC activities was expressed as fold increase over activities of non-induced transfected cells with the same reporter plasmids (set to 1) and were based on at least three independent experiments.

Site-directed mutagenesis of ARE

The K224LmARE and the K637LmARE contain a mutated ARE located at −39 nt in the fragments of −224 and −637 nt of the KAP promoter (K refers to KAP, 224 and 637 are the sizes of the truncated KAP promoters tested, L means Luc and mARE means mutated ARE). The wild-type ARE is 5′-GGTACAGGATGTATAAA-3′ and the mutated ARE lacks the underlined nucleotides (5′-GGATGTATAAA-3′).

K224L and K637L were used as the DNA mutagenesis template to anneal with the mutagenic primers (upper primer: 5′-CCTTCAGGGAGAGGGATGTATAAAAGCC-3′; lower primer: 5′-GGCTTTTATACATCCCTCTCCCTGAAGG-3′). The mutant strands were synthesized with Pfu Turbo® DNA polymerase and used to transform Epicurian Coli XL1-Blue Supercompetent cells by using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.). Plasmid DNAs were isolated from the selection plates and mutants were identified by sequencing.

RNA extraction and semi-quantitative RT-PCR

Confluent PCT3 and PR10 cells were dissolved in 4 M guanidine monothiocyanate/25 mM trisodium citrate/0.5 % sarcosyl/0.1 M 2-mercaptoethanol and extracted sequentially with phenol, chloroform/3-methylbutan-1ol ('isoamyl alcohol') (49:1, v/v) and 0.2 M sodium acetate [25]. Total RNA was precipitated, ethanol-washed, dried, and resuspended in diethyl pyrocarbonate-treated water. Total RNA (0.5 μg) was used in each RT-PCR reaction using the SuperScript® One-Step® RT-

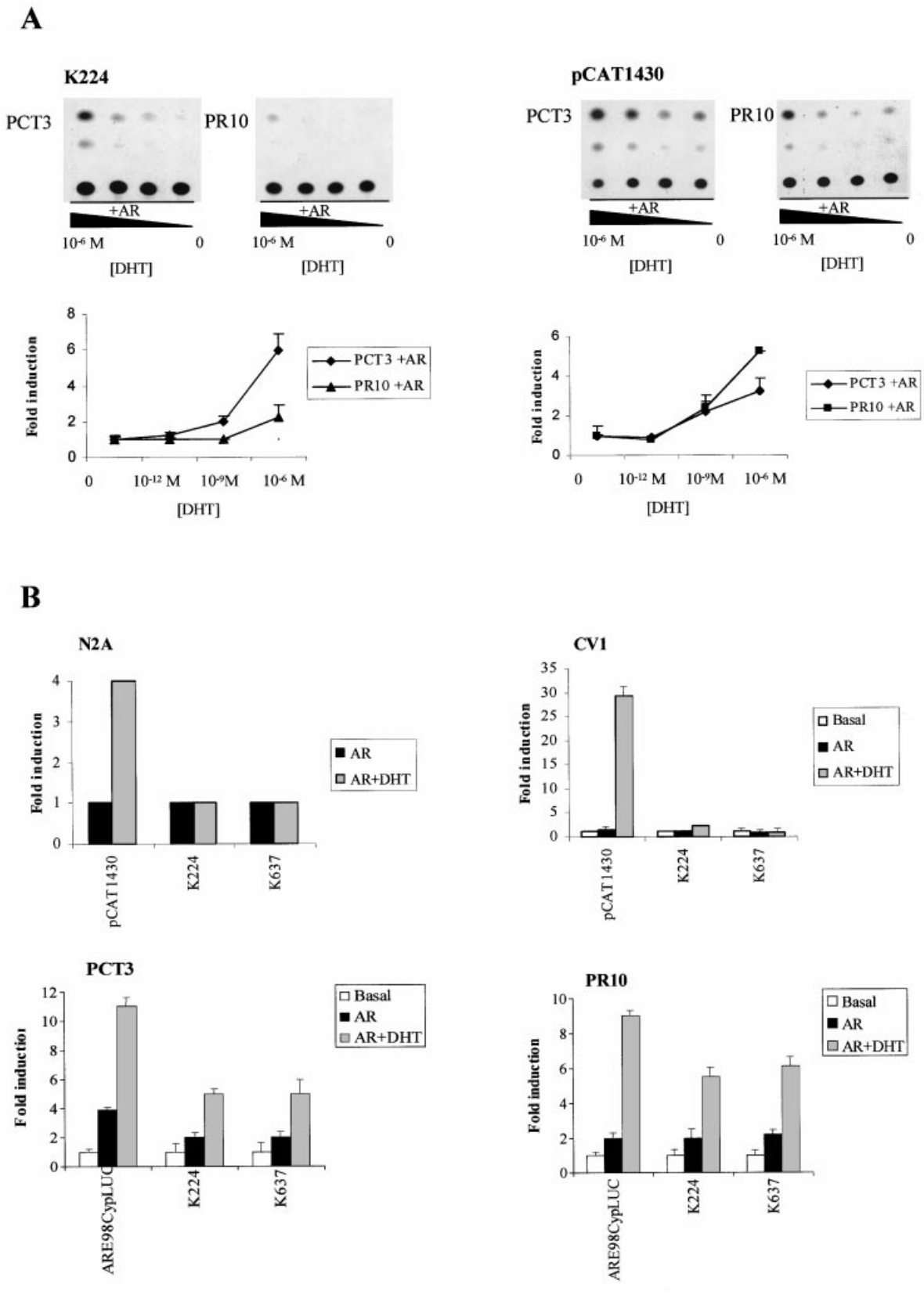

Figure 2 Dose-dependent androgen response of the K224 and pCAT1430 promoter constructs

(**A**) Cultured mouse PCT3 and PR10 cloned cells were transfected with 2 μg of the K224 or the pCAT1430 control plasmid, in the presence of 0.5 μg of co-transfected human AR expression vector pSVAR0 and treated for 48 h with vehicle or with the indicated doses of DHT, in steroid-free medium. Autoradiograms of CAT assays measured in homogenates of transfected cells are shown in the upper part of the Figure. CAT activities were adjusted according to the β-gal activity produced by 2 μg of transfected pCH110 plasmid. CAT/β-gal relative activities are shown as fold induction

PCR System (Life Technologies) under conditions of linearity with respect to input RNA and the number of cycles (25 cycles). The sequences of the primers used for KAP were 5′-GCATGATGCTTTTCAAGG-3′ and 5′-TCAGGAAGTAGGGGAGACTGG-3′. Those for mouse CypA cDNA, used as an internal control for RNA integrity and quantity, were 5′-ATGGTCAACCCCACCGTG-3′ and 5′-CAGATGGGGTAGGGACG-3′. Amplification products were separated on 2%-(w/v)-agarose gel and ethidium bromide-stained products measured using the Bio-Rad Gel Doc 1000 and the Molecular Analyst 1.40 program. For relative quantification of KAP mRNA in the cells, decreasing amounts of KAP cDNA (from 1 ng of pGEM-T-KAP to 0.0016 ng) and CypA cDNA (from 10 ng of pGEM-T-Cyp A to 0.0097 ng) were amplified by one-step RT-PCR performed on a PCT-100® Programable Thermal Controller (MJ Research, Waltham, MA, U.S.A.). The amplification signals were utilized to construct standard curves for each cDNA tested. KAP and CypA values from each sample tested were interpolated on their respective standard curves and the relative mKAP over mCypA (KAP/CypA) ratios were calculated.

RESULTS

Androgen-dependent transactivation of the KAP promoter in mouse renal proximal-tubule PCT3 and PR10 cells

The ability of mouse proximal convoluted PCT3 and PR10 cells to support androgenic regulation of reporter-gene constructs containing the kidney-specific KAP promoter was analysed by transient transfection assays. A construct containing 1542 bp from the 5′-flanking region of the *KAP* gene (K1542), which had proved to be sufficient to drive expression of a heterologous reporter gene in a tissue-specific, cell-specific and androgen-regulated fashion in transgenic mice [26], and two truncated fragments of 637 bp (K637) and 224 bp (K224) were each inserted upstream of the *CAT* and *LUC* reporter genes. We also included 1430 bp of the proximal promoter of the non-kidney-specific human pepsinogen C gene fused to the CAT gene (pCAT1430) [22] and the pARE98CYP-LUC reporter plasmids for androgen inducibility, and the MMTV-LUC reporter plasmids as control for glucocorticoid inducibility in PCT3 cells (Figure 1).

We first tested the optimal concentration of DHT (from the range 1 pM–1 μM) necessary for maximal androgen induction of pCAT1430 control and K224 promoter constructs in PCT3 and PR10 cells (Figure 2A). Relative CAT/β-gal activity shows that maximal efficiency occurs at the 1 μM DHT dose for both constructs in both cultured cell types. This concentration of DHT was previously reported to be the most effective in the induction of domes formed by PKSV-PCT and PKSV-PR cells, corresponding to localized regions of the monolayer lifted from the Petri dishes, which are good indicators of vectorialized ionic transport processes and differentiation state [9]. This concentration of DHT was also reported to be the most effective for maximal androgen-dependent transactivation of the pCAT1430 promoter in human breast-cancer cells [22], which was also used as a control in the present study. We then assayed the activities of different KAP promoter constructs in the cultured proximal tubule PCT3 and PR10 cells as compared with those from KAP-transfected neuroblastoma N2A and green-monkey kidney-derived CV-1 cells. The induction of the relative CAT/β-gal activity or LUC/*Renilla* LUC activity was tested in the four cell lines, by using different KAP-CAT or KAP-LUC constructs and the pCAT1430 and pARE98CYP-LUC constructs which were used as controls of the androgen-driven expression. The results were expressed as fold induction which corresponded to the CAT/β-gal or LUC/*Renilla* LUC activity ratios for each construct normalized to basal values measured in the absence of co-transfected androgen receptor (AR) and in steroid-depleted medium. None of the KAP constructs tested exhibited induced CAT activity in AR-co-transfected and DHT-treated N2A cells, whereas the pCAT1430 control plasmid exhibited a 4-fold induction (Figure 2B, left upper panel). Similarly, AR-transfected CV-1 cells showed a 30-fold induction of the pCAT1430 control construct in the presence of 1 μM DHT versus a 2-fold increase for the K224 promoter, and a complete lack of induction for the K637 construct (Figure 2B, right upper panel).

Similar experiments using PCT3 and PR10 cells showed an 8–10-fold induction of relative LUC activity for the pARE98CYP-LUC control promoter construct and significant (5–6-fold) LUC activity for the K224 and K637 constructs in both cell lines (Figure 2B, lower panels). In cells not treated with DHT, the 2-fold increment observed for the K224 construct was similar to the maximal activity achieved with this construct in CV-1 cells in the presence of androgens. The results obtained with the K1542 construct in PCT3 cells indicated that the longer KAP promoter fragment was ineffective at inducing the androgenic response (results not shown). As controls, the parent vectors lacking the KAP sequence were unaffected by the presence of AR and DHT (results not shown).

To further determine whether the GGTACAggaTGTATA putative ARE sequence found in the KAP proximal promoter at position −39, which had proved to bind AR prepared from recombinant baculovirus-infected Sf9 (*Spodoptera frugiperda* 9) insect cells (results not shown), was functionally active, mutated ARE K224 and ARE K637 constructs lacking the GGTACA underlined sequence were transfected in PCT3 and PR10 cells. The androgen-inducibility of the reporter gene was then compared with those obtained with the intact K224 and K637 constructs. Ablation of this ARE sequence in the KAP promoter abolished androgen-inducibility for both constructs in both cell lines, at the level observed for the K224 construct in PCT3 cells (shown in Figure 3). Thus these results indicated that the predicted ARE in the KAP promoter is functional.

Effects of thyroid hormone on the transactivation capacity of the KAP promoter in PCT3 cells

Previous *in situ* hybridization studies performed in congenitally hypothyroid *hyt/hyt* male mice had shown that (i) T_3 was unable to induce any response in cortical-tubule segments in females and castrated males, i.e., in the absence of androgens, and that (ii) androgen-dependent expression of the endogenous *KAP* gene in intact PCT was enhanced in the presence of T_3 [18].

To determine whether the androgen-responsive PCT3 cells were able to mimic the effects exerted by T_3 alone or in combination with androgens in the intact mouse kidney, KAP promoter constructs were tested under different hormonal conditions. To

over control level, i.e. in the absence of androgens and of a co-transfected AR. (**B**) Androgen-dependent activity of the two KAP promoter constructs in different cell lines. A 2 μg portion of the K224 and K637 KAP constructs fused to CAT or LUC reporter genes was transfected in N2A, CV-1, PCT3 and PR10 cells in the presence of 0.5 μg of co-transfected AR expression vector pSVAR0 and 1 μM DHT for 48 h. CAT (in N2A and CV-1) and LUC (in PCT3 and PR10) activities were measured as indicated in Figure 2(A) and compared with that of control pCAT1430 and pARE98CYP-LUC constructs respectively. Bars indicate the means ± S.E.M. of fold induction of reporter activities over control unstimulated, non-receptor transfected cells (= 1) for at least three independent experiments.

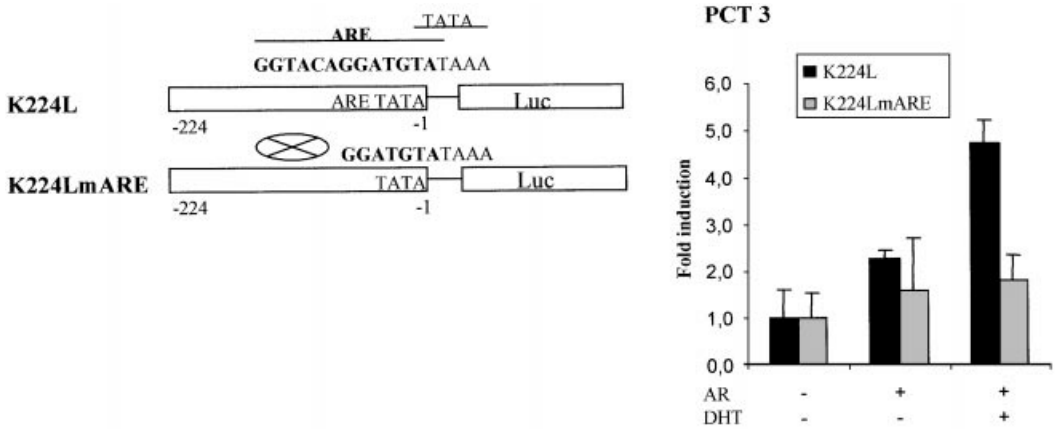

Figure 3 Mutation strategy of the putative ARE element and functional assay of the mutated K224 construct

The nucleotides deleted from the native sequence are shown in the left part of the Figure. Functional activities of 1 μg of K224L (wild-type) and 1 μg of K224LmARE (mutated) constructs were tested on PCT3 cells transfected with 0.5 μg of the AR expression vector pSVAR0 and treated for 48 h with vehicle alone or with 1 μM DHT in PCT_3 cells. LUC activities were adjusted according to the *Renilla* LUC activity produced by 20 ng of transfected pRL-TK in the same sample. Fold induction over basal level of relative LUC activity for both constructs was compared and results shown in the right panel of the Figure. Bars indicate the means $\pm$ S.E.M. of fold induction of LUC activity over control unstimulated, non-receptor transfected cells (= 1) for at least three independent experiments.

assay the effects of T_3 alone, THR and RXR were co-transfected with KAP reporter constructs and the relative CAT activity was measured in the presence or absence of T_3. None of the three KAP constructs was induced by T_3, but the thyroid hormone-responsive control SaltK plasmid proved to be activated in a T_3-dependent manner (Figure 4A). The most significant induction (2.5-fold) was obtained with the K637 construct in the presence of co-transfected receptors and in the absence of T_3. These results indicated that, as *in vivo*, T_3 by itself has no effect on the transactivation capacity of the proximal KAP promoter in cultured PCT3 cells.

To test whether T_3 could modulate androgen-dependent responses in this cell system, CAT assays using the K224 and K637 constructs were measured in a variety of situations (Figure 4B). Results demonstrated that the androgen-dependent response attained by the K224 promoter in PCT3 cells (5.4-fold) significantly decreased by 2.5-fold when cells were co-transfected with RXR and THR. Addition of T_3 did not significantly further diminish CAT activity (Figure 4B). Results with the K637 construct performed under similar experimental conditions demonstrated a complete lack of effect on the androgen-dependent activation of this construct.

Since the co-operative effect observed by androgens and T_3 on cortical PCT from the mouse kidney was not observed on the KAP promoter constructs in cultured PCT3 cells, we analysed the levels of endogenous KAP gene expression in transfected PCT3 cells. Semi-quantitative one-step RT-PCR analysis using specific primers of mouse KAP cDNA was performed to determine endogenous KAP mRNA levels. Consistent with the relative CAT levels found in transfection assays, KAP mRNA expression was enhanced by AR transfection and DHT treatment and was not induced in T_3-treated THR/RXR-transfected cells. Similarly, the presence of THR and RXR and T_3 clearly blunted the androgen-dependent response (Figure 5).

Stimulation of androgen-regulated KAP transactivation by protein phosphorylation in PCT3 cells

The effect of 100 μM sodium orthovanadate, a phosphatase inhibitor, on the androgen-dependent transactivation capacity of the K224 promoter was tested in PCT3 cells (Figure 6A). Phosphatase inhibition caused a 2.1-fold increment in relative CAT activity over the values exhibited by AR and DHT alone. The inhibitor showed no effect unless it acted in concert with AR and DHT simultaneously.

Growth factors, particularly GH, have been shown to modify the response to androgens in mouse kidney, with IGF-1 acting as intermediate effector [27,28]. Since IGF-1 has been shown to participate in phosphorylation processes, we tested whether its presence could have an effect on AR interaction with the KAP promoter. The K224 construct was transiently transfected in PCT3 cells grown in IGF-1-free medium or in 25–200 ng/ml IGF-1-supplemented medium. Experiments performed in the presence of co-transfected AR and 1 μM DHT showed that 100 ng/ml IGF-1 increased 2.3-fold the transactivation capacity of the KAP224 promoter (Figure 6B). By contrast, no change in transactivation activity was detected in the absence of AR (Figure 6B). This increment in the AR-mediated response induced by IGF-1 was similar to that obtained when 100 μM orthovanadate was included in the culture medium.

DISCUSSION

The results of the present study showed that immortalized mouse renal proximal-tubule cells derived from L-PK/Tag1 transgenic mice represented valuable *ex vivo* cell systems for analysing hormone-specific regulation of the KAP gene promoter. All previous cell-transfection studies using the 1542 bp KAP promoter fragment or other promoter truncations did not permit characterization of the specific sequences involved in the regulation of the KAP gene. All KAP promoter fragments used were unable to direct reporter gene expression in a number of cell types in which androgen regulation of control constructs was evident. Since this was a common situation for other androgen-regulated kidney-specific genes, understanding of the molecular mechanisms underlying androgenic regulation of gene expression in kidney has been limited. Although production of transgenic mice, such as those produced by Ding et al. [26], to analyse the proximal promoter of the *KAP* gene constitutes an invaluable model to

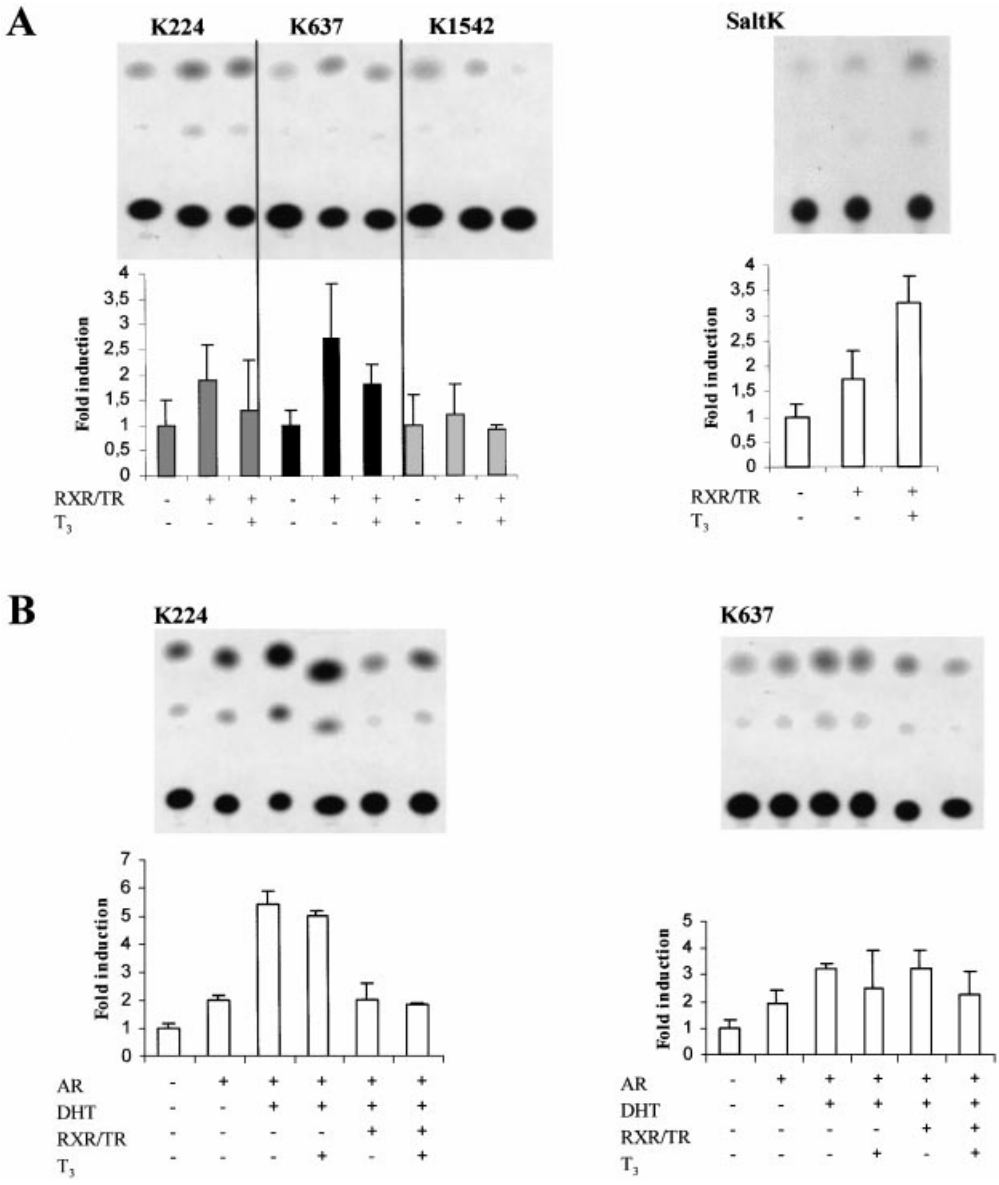

Figure 4 (A) Effects of thyroid hormone on the transactivation ability of KAP promoter constructs and (B) effects of thyroid hormone on the androgen-dependent transactivation of K224 and K637 promoter constructs

(**A**) The PCT3 cells were co-transfected with 2 μg of K224, K637 or K1542 constructs and 0.5 μg of RXRα and 0.5 μg of thyroid receptor α. Cells were grown for 48 h in the absence or presence of 1 μM T_3. In the right panel, similar cultures were transfected with SaltK, which is a thyroid-hormone-inducible reporter plasmid and which was used as a positive control for T_3 treatment. CAT activities were adjusted according to the β-gal activity produced by 2 μg of pCH110 transfected in the same sample and were expressed as fold increase over basal level. (**B**) PCT3 cells were co-transfected with either 2 μg of K224 or K637 in the absence or presence of 0.5 μg of human AR, 0.5 μg RXRα and 0.5 μg of TRα. Cells were grown for 48 h with and without 1 μM DHT and/or 1 μM T_3. CAT activities were measured as described previously.

study the transactivating capacity of the promoter *in vivo*, this approach remains limited, since each analysis of a given mutation would require a new strain of transgenic mice.

Analysis of the androgenic control of β-gluc and KAP mRNA expression in mouse PKSV-PCT and PKSV-PR proximal-tubule-derived cells demonstrated that these cells retained the morphological and biochemical features of the parent cells from which they were derived [6–8] and were suitable for analysis of the pleiotropic action of androgens on proximal-tubule cells, since endogenous KAP and β-gluc mRNA levels were modulated by androgens [9]. We used the K1542 fragment of the KAP proximal promoter, known to be able to drive expression of the human angiotensinogen gene (*HAGT*) in transgenic mice [26]. These animals exhibited levels of human HAGT mRNA expression similar to those of the KAP endogenous gene in male mice and in androgen-treated females. Since females do not express the gene in S3 late-proximal-tubule cells, it has been concluded that the K1542 contains the *cis*-acting elements necessary for tissue-, cell- and androgen-dependent expression of KAP in PCT cells. By contrast, this promoter fragment did not contain the *cis*-acting elements necessary for androgen-independent regulated expression of the KAP gene in late (S3) proximal-tubule cells [26].

Previous *in-situ*-hybridization studies demonstrated that KAP mRNA is expressed, but differentially regulated, in two distinct

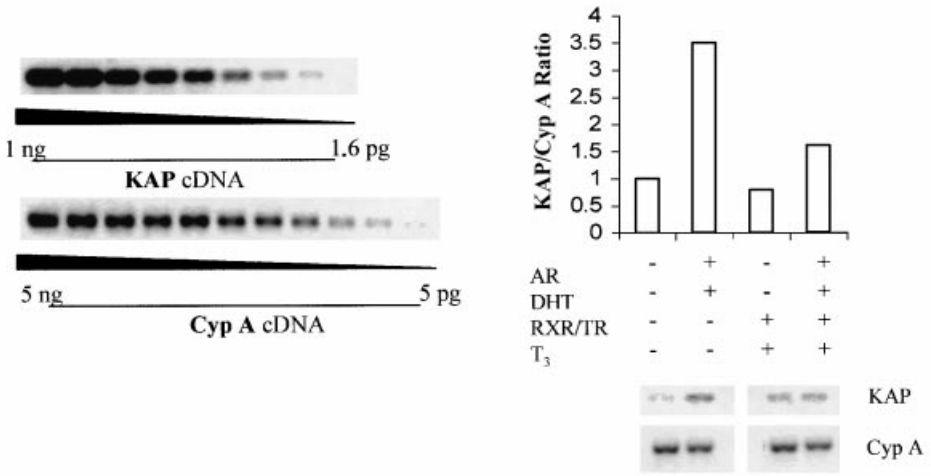

Figure 5 Endogenous KAP mRNA expression levels in transfected PCT3 cells

Endogenous KAP mRNA levels were measured by a semi-quantitative RT-PCR technique (see the Materials and methods section for details) in cells transfected with different combinations of human AR, RXRα and TRα expression vectors in the presence or absence of hormones. The ratio KAP/Cyp A is expressed as fold increase over basal level, as previously described.

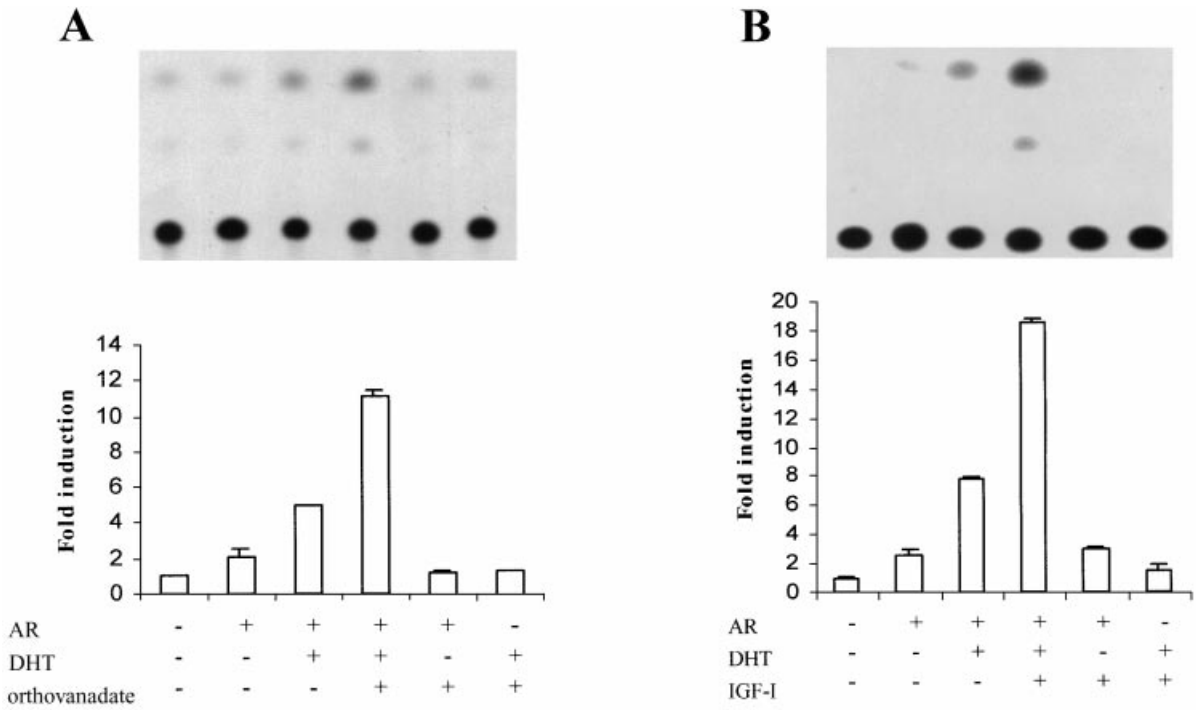

Figure 6 (A) Effects of phosphatase inhibition on the androgen-dependent transactivation capacity of the K224 construct in PCT3 cells and (B) effect of IGF-1 on the androgen-dependent response of the K224 promoter in PCT3 cells

(**A**) PCT3 cells transfected with 2 μg of the K224 construct and 0.5 μg of human AR were incubated with 1 μM DHT alone or in combination with 100 μM sodium orthovanadate for 48 h. CAT activities were adjusted according to the β-gal activity produced by 2 μg of pCH110 transfected in the same samples and ratios were expressed as fold increase over basal level. (**B**) This assay is similar to the one described in Figure 6(A), except that cells were treated with 100 ng/ml IGF-I for 48 h. CAT activities were measured as described above.

areas of the kidney [15]. Expression of the gene in the cortical proximal tubules (S1 and S2) occurs in normal male mice or testosterone-treated females, which bear a functional AR [16,17]. Moreover, in females and castrated males, or in Tfm/Y mice lacking functional ARs, KAP mRNA is exclusively located in the late S3 segments of the proximal tubules [17]. These results indicated, thus, that cells from the proximal straight tubule (S3) express the *KAP* gene in an androgen-independent fashion, whereas expression of KAP regulated by androgens depends on functional AR in the proximal convoluted tubule (S2) cells. Experiments performed in congenitally hypothyroid *hyt*/*hyt* mice demonstrated a complete absence of *KAP* gene expression in S3

cells of the kidneys of mutant females and a diminished expression in the renal cortex, more pronounced in the S3 compartment, from male mice. These results indicated that the expression in S3 cells was triggered by thyroid hormone, and also that physiological doses of androgens can also induce KAP mRNA expression in these cells. Furthermore, while castration completely abolishes KAP mRNA expression in *hyt/hyt* male mice, substitutive administration of pharmacological doses of DHT to castrated males was sufficient, in the absence of thyroid hormone, to restore normal levels of KAP expression in both convoluted (S1 and S2) and straight (S3) proximal-tubule cells [18]. Altogether, these results have provided a line of evidence that the KAP gene is up-regulated by thyroid hormone as well as by androgens in S3 proximal-tubule cells.

Transient transfection analyses of KAP reporter constructs including the K1542 and two truncated forms (K637 and K224) from the transcription initiation site, have demonstrated for the first time the ability of S2 and S3-derived PCT and PR cells to promote androgen-dependent transactivation of the short K224 promoter fragment, in a ligand- and AR-dependent manner. Although the DHT concentration required for maximal induction of the KAP reporter constructs is higher than those reported *in vivo*, we do not believe that this effect is due to a pure pharmacological effect, since the well-recognized androgen-dependent promoter of the human pepsinogen C gene [22] used in our experiments as a control, responded in the same manner as the KAP constructs. Besides, androgen induction of the KAP promoter was only effective in the PCT3 and PR10 cell lines, since the N2A and CV-1 cells were unable to support its activation when compared with control reporter constructs. Mutagenesis of the putative ARE at position −39 bp from the transcription start site in the K224 and K637 constructs, known to bind AR directly by electrophoretic-mobility-shift assay, abolishes the androgen-dependent transactivation of the reporter gene in both PCT3 and PR10 cell lines. Thus the present findings suggest that androgens control *KAP* gene expression in both PCT cells and PR cells, by initiating gene transcription through this DNA element. Because it cannot be excluded that such DNA element binds other transcription factors, such as other members of the nuclear receptor superfamily, we have tested the ability of the glucocorticoid receptor to induce expression of the K224L and the ARE-mutated K224LmARE promoter constructs in PCT3 cells, in the presence of 10 nM dexamethasone. Wild-type and mutated ARE K224 constructs did not respond to the presence of glucocorticoid receptor (GR) and glucocorticoids, while the MMTV-LUC glucocorticoid responsive control construct exhibited a 4-fold increase with respect to the basal expression obtained in unstimulated PCT3 cells (results not shown). These results are consistent with earlier *in vivo* data showing that glucocorticoids are unable to restore KAP expression in castrated male mice (A. Meseguer and J. F. Catterall, unpublished work). Since GR and AR seem to bind to the same basic response element in the DNA, we favour the notion that specific co-activators of the AR present in these cells may participate in this androgen-dependent specific response. Because there are very few examples of tissue-specific coactivators of steroid receptors [29,30], cultured mouse renal PCT and PR cells should represent valuable cell systems for the future identification of cell-specific AR co-activators and/or co-repressors involved in the control of androgen-dependent gene expression in mouse kidney.

The diminished or even undetectable transcriptional activity observed in cultured PCT cells with the K1542 construct does not correlate with the results obtained in transgenic mice, where the 1542 bp fragment KAP promoter confers androgen-dependent expression on the heterologous *HAGT* reporter gene. This construct contains the functional ARE motif at position −39 bp, but has no transactivation capacity. It cannot be ruled out that these differences are due to the presence of putative negative regulatory sequences in the longer promoter fragment that can become exposed in transient transfection assays, when using non-nucleosomal DNA.

Thyroid hormones control the cell-specific expression of the *KAP* gene in intact late S3 proximal-tubule cells and co-operate with androgens in promoting cortical *KAP* gene expression [18,20]. We next aimed to analyse the effects of T_3 on *KAP* gene expression in cultured proximal-tubule cells to further determine their ability to mimic the events occurring in mouse kidney. Because the regulatory sequences involved in thyroid-hormone-dependent transactivation in S3 cells are apparently not included in the 1542-bp promoter fragment, we did not attempt to observe any regulatory effect of T_3 on the KAP constructs in cultured PR cells. On the other hand, the question arises as to whether T_3 could modify the androgenic responses of the K224 and K637 constructs in cultured PCT cells. Initially, we tested the three KAP-CAT constructs in the presence of THR and RXR in T_3-depleted or T_3-supplemented media and demonstrated that none of the three KAP constructs responded to the hormone. Similar experiments performed in AR-transfected and DHT-treated cells showed that co-transfection of THR and RXR alone or in combination with T_3 inhibited rather than stimulated the androgenic effect provided by the K224 promoter. Since these results do not fully correlate with those observed in the kidney [18,20], we hypothesize that the effects of thyroid hormone on cortical androgen activation may be indirect through the action of another hormone or factor. It can also be argued that other promoter regions may participate in the co-operative effect between androgens and T_3 or, alternatively, that fine tuning of hormonal interactions for *KAP* gene expression in the kidney requires a strict molar equilibrium among hormone receptors, co-regulators and other transcription factors, a situation which still remains difficult to establish in an *in vitro* isolated cultured cell system.

The positive effects of IGF-1 on the androgen-dependent transcriptional activity of K224 promoter in cultured PCT cells, together with earlier *in vivo* results [18,20] strongly suggest that, at least in part, thyroid hormone may induce KAP expression in kidney cortex by controlling the expression of GH and, in turn, the expression of IGF-1. This rationale is based on results observed in congenitally thyroid hormone-deficient *hyt/hyt* mice still expressing KAP mRNA in the cortex [18] and on pharmacologically induced congenital hypothyroid mice, totally devoid of KAP mRNA expression in the cortex [20]. Although plasma and kidney T_3 and thyroxine ('T_4') levels demonstrated profound hypothyroidism in both mouse models, the goitrogen-treated mice presented an associated GH deficiency, as was evident by their reduced body size and weight [20]. The dwarf phenotype exhibited by the goitrogen-treated mice suggested that not only thyroid hormone, but also GH, might be involved in androgen control of KAP expression. *In situ* hybridization analysis performed in the GH-deficient *lit/lit* mice [31] and Jackson *dwarf* mice, deficient in thyrotropin, prolactin and GH due to an inactivating mutation in the anterior pituitary-specific transcription factor Pit-1 [32], revealed that the GH and thyroid hormone combined deficiency does compromise *KAP* gene expression in cortical segments in this genetically deficient mouse model (results not shown). These results provided further evidence of the *in vivo* interactions between GH and T_3 for the regulation of *KAP* gene expression in mouse kidney cortex.

In summary, we have demonstrated that mouse PCT and PR cell lines provide excellent and valuable *ex vivo* models for analysing the mechanisms and further characterizing the molecular

elements controlling the intricate and complex regulation of the *KAP* gene in the mouse kidney.

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