CCAAT/Enhancer Binding Protein-Mediated Role of Thyroid Hormone in the Developmental Expression of the Kidney Androgen-Regulated Protein Gene in Proximal Convolutated Tubules

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The kidney androgen-regulated protein (KAP) gene is exclusively expressed in proximal tubules of mouse kidney and in the uterus of pregnant females before they give birth. It displays an exquisite and differential regulation of expression by steroid and thyroid hormones (THs) in different proximal tubule segments. Whereas the pars recta (PR cells) responds to thyroid and sexual hormones, the pars convoluta (PCT cells) represents a truly androgen-dependent compartment because expression occurs only in the presence of androgens and functional androgen receptors. Nevertheless, different hypothyroidism models have indicated that TH might also contribute to the androgenic response in PCT cells. In the present study, we aimed to determine the molec-

ular mechanisms that ultimately control KAP expression in these cells. Using several genetically deficient mouse models and different pharmacologic and hormonal treatments, we determined that thyroid and GH modulate CCAAT/enhancer binding protein α and β levels that, in turn, control KAP expression in PCT cells in a developmentally dependent manner. We demonstrated that these factors bind to sites in the proximal KAP promoter, thereby collaborating with androgens for full KAP expression. Finally, we propose that TH and GH, acting through CCAAT/enhancer binding protein, may constitute a general regulatory mechanism of androgen-dependent genes in mouse kidney. (*Molecular Endocrinology* 20: 389–404, 2006)

THE KIDNEY ANDROGEN-regulated protein (KAP) was originally identified by *in vitro* translation of male mouse kidney mRNA (1). It constitutes the most abundant (4% of total polyadenylated mRNA) and specific gene expressed in proximal renal tubule cells, as shown by SAGE (serial analysis of gene expression) (2), SADE (a sage adaptation for down-sized extracts) (3), and expression profiling of active genes (4). Cell specificity and the complex regulatory mechanisms involved in KAP mRNA expression (5), together with KAP mRNA relative abundance, point to a significant role for its encoded protein. Analyses of both nucleotide and peptide sequences failed to reveal significant homology with other genes, expressed sequence tag or proteins, or with known structural or functional domains. The absence of known functional domains greatly reduced experimental

approaches to elucidating KAP function, prompting us to perform a yeast two-hybrid assay that showed that KAP interacts with CsA binding protein CyPB. CsA administration reduces KAP steady-state levels in mouse kidney, and KAP overexpression reduces CsA toxicity in cultured proximal tubule cells. Thus, our data indicate a functional KAP, CyPB, and CsA-mediated toxicity relationship in kidney (6).

Our laboratory has been interested in understanding the regulation of KAP mRNA that displays a tissue- and cell-specific expression restricted to epithelial cells of proximal convoluted tubules in mouse kidney (7). This gene is subjected to strict and exquisite regulation by thyroid and sexual steroid hormones in different segments of proximal tubules. *In vivo* studies have shown that S3 cells (pars recta), which correspond to the outer stripe of the outer medulla, express the gene in the presence of thyroid hormone (TH/T₃) (8), and are also sensitive to androgen and estrogen action (9). Females and castrated males express the gene exclusively in S3 cells (7). Segments S2 and S1 (pars convoluta), which correspond to the cortical part of the kidney, express the gene only in the presence of androgens and a functional androgen receptor (AR); therefore, females, castrated males or AR-deficient mice of the *Tfm/Y* strain are negative for cortical expression of KAP mRNA (10).

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Abbreviations: ARE, Androgen receptor response element; CAT, chloramphenicol acetyl transferase reporter gene; KAP, kidney androgen-regulated protein; LUC, luciferase; ODC, ornithine decarboxylase; PCT, pars convoluta; Pit-1, pituitary transcription factor-1; TH, thyroid hormone; PR, pars recta; PRL, prolactin.

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Accordingly, androgen replacement in castrated males or treatment of females with testosterone induces expression in the cortical segments of the tubule (7). We also observed that male mice genetically deficient for TH (*hyt/hyt* strain) exhibit lower cortical KAP mRNA expression than wild-type control males and that exogenous T_3 injection prompts full expression recovery (8). Moreover, treatment of adult male control mice with potassium perchlorate ($KClO_4$), which reversibly competes for entry of iodine into the thyroid gland, resulting in severe pharmacological hypothyroidism, produces the same effect (11). These results, and the fact that simultaneous treatment of $KClO_4$ and TH restored KAP expression in the cortex, strongly suggested the involvement of TH in the androgenic control of KAP mRNA expression in cortical tubule segments. Presence of $KClO_4$ did not modify plasma testosterone levels or expression of the androgen receptor itself (11). More remarkably, animals born to mothers exposed to $KClO_4$ during pregnancy and after delivery were unable to express KAP mRNA in any compartment of the kidney, including the cortical convoluted segments, even at postnatal d 90 (11). Because T_3 is unable to induce cortical expression in castrated males (8), we concluded that androgens are necessary but not sufficient to induce KAP expression in the cortex and that presence of TH is also required.

In the present work, we aimed to gain further insight into the molecular mechanisms that control TH involvement in the developmental sexual dimorphic pattern of KAP expression in mouse kidney by identifying the molecular elements that collaborate with androgens in the cortical expression of the KAP gene. To this end, *in vivo* and *in vitro* studies were conducted using the proximal tubule-derived cell line PKSV-PCT, originally developed in Dr. Vandewalle's laboratory (12, 13) that, together with the PKSV-PR, are alone in supporting androgen-dependent expression of kidney-specific genes in an isolated cell system (14). For years, the lack of an appropriate cell line has hampered detailed characterization of the molecular elements mediating androgen-responsive gene expression in the kidney. Our studies proved the hormone-specific regulation of the KAP gene promoter in these cultured mouse renal proximal tubule-derived cells (15), by demonstrating that these cell lines constitute an excellent and valuable *ex vivo* model for analyzing the mechanisms and further characterizing the molecular elements controlling the intricate and complex regulation of KAP gene in mouse kidney.

RESULTS

Postnatal TH Requirement and GH Involvement in the Androgen-Dependent Expression of the KAP Gene in Kidney Cortex

Previous work showed that male mice exposed to $KClO_4$ from d 10 of gestation until being euthanized were not expressing the KAP gene, even at postnatal

d 90, and related this effect to the absence of TH (11). To assess whether TH played a role during prenatal development, after birth or in both time periods, four different groups of animals were studied by *in situ* hybridization using specific antisense cRNA probes for KAP mRNA. Results showed that male and female control water-treated mice (group 1) and animals treated with 1% $KClO_4$ for the entire experiment (group 4) expressed the gene in the expected manner, as described (7, 11) (Fig. 1A). Results obtained in groups 2 and 3 showed that only animals treated with 1% $KClO_4$ during the gestational period (group 2) were able to express the gene like those in the control water-treated group.

Average size and weight of mice in each group showed significant differences for animals exposed to 1% $KClO_4$ (Fig. 1A, including table). For those under permanent treatment (group 4), weight and size were 2.5- and 1.5-fold lower, respectively, than in control animals (group 1), which suggests that an associated GH deficiency-related event to the strong hypothyroidism caused by the goitrogen treatment. As for KAP expression, effects on weight and size were clearer in postnatal treated mice (group 3) than in those exposed to treatment before birth (group 2). These results indicated that, at least in part, GH could be involved in KAP cortical expression. From previous work, we knew that when TH deficiency occurs in the absence of a dwarf-associated phenotype, *i.e.* in the *hyt/hyt* mouse model, cortical expression is diminished but not abolished (8); therefore, we hypothesized that, the combined absence of GH and TH would likely result in lack of KAP expression. To demonstrate this hypothesis, we used two genetically deficient mouse models, GH and prolactin (PRL)-deficient *little* mice and pituitary transcription factor-1 (Pit-1)-deficient *C3H/HeJ dwarf* mice from The Jackson Laboratory (Bar Harbor, ME). The specific pituitary transcription factor Pit-1 deficiency led to absence of GH, PRL, and TSH in the *C3H/HeJ* mice, resulting in a combined hypothyroid and dwarf phenotype. Results from this experiment showed that deficiency of GH and PRL does not compromise androgen-dependent cortical KAP expression (*little*) as does combined absence of GH, PRL, and TSH (*Jackson dwarf*) (Fig. 1B).

Goitrogen-treated male mice were injected with T_3 , GH, or both at the same time, from postnatal d 7–21, to further verify the involvement of these hormones in the pharmacological model and to demonstrate that the results obtained were not indirectly related to putative nephrotoxic effects of $KClO_4$ in the kidney. *In situ* hybridization experiments showed that T_3 administration restored KAP expression in both kidney compartments (outer medulla and cortex). This effect was enhanced by coinjection of GH, although GH administration alone produced no effect (Fig. 1C).

Fig. 1. TH and GH Influence KAP mRNA Expression in Kidney Cortex

A, Effects of pre/postnatal TH deprivation on KAP gene expression. Frozen male and female kidney sections from animals exposed permanently to regular tap water (Group 1), those treated with KClO₄ 1% in drinking water from d 10 of gestation until birth (Group 2), those with KClO₄ 1% from birth until euthanized at postnatal d 40 (Group 3) or those exposed to KClO₄ 1% from d 10 of gestation until euthanized at postnatal d 40 (Group 4) were hybridized with strand-specific ³⁵S-KAP3 antisense cRNA. The magnification chosen for these images (×40) permitted visualization of the three major compartments of the kidney (c, cortex; o, outer medulla; i, inner medulla). At right, the table displays average weight and size of the animals belonging to each group and classified by sex. B, KAP gene expression in *lit/lit* and Jackson *dw/dw* mice. Frozen kidney sections from control (left panel), homozygous *lit/lit* (middle panel), and Jackson *dw/dw* (right panel) male mice were hybridized with strand-specific ³⁵S-KAP (KAP3) cRNA as previously described. The left panel corresponds to a kidney section from a normal male of the C57BL/6 *Lit/+* genetic background. Hybridization of frozen sections from a normal male of the C³H/HeJ *dw/+* strain yielded similar results (data not shown). C, KAP gene expression in male mice exposed to 1% KClO₄ and treated with T₃- and/or GH. Frozen kidney sections from C57BL/6 untreated control males (left upper panel), potassium perchlorate 1%-treated males from birth until euthanized at postnatal d 40 (middle upper panel), or similar mice treated with TH (right upper panel), GH (left lower panel), and TH plus GH (right lower panel) from postnatal d 7–21 were hybridized with strand-specific ³⁵S-KAP (KAP3) cRNA as previously described.

Fig. 2. Punctual Presence of TH Triggers KAP Expression in Cortex

A, Restoration of TH synthesis on goitrogen removal at different postnatal time points permits recovery of KAP gene expression in potassium perchlorate-treated male mice. Frozen kidney sections from C57BL/6 potassium perchlorate-treated males from birth until euthanized at postnatal d 40 (control; *upper left panel*) were hybridized with strand-specific ³⁵S-KAP (KAP3) cRNA, as previously described and compared with similar animals exposed to tap water from indicated time points (see figure) and further exposed to perchlorate in drinking water until euthanized. *In situ* hybridization of nontreated control males from birth until

Punctual Presence of T_3 Is Sufficient to Trigger KAP Expression

We next aimed to investigate at what time points in postnatal development TH was required to trigger KAP expression. To avoid stressing the animals with daily T_3 injections, a different strategy was used based on replacing 1% $KClO_4$ containing water by normal tap water during the time points studied. Before proceeding with this novel approach, we tested whether this treatment had equivalent effects to T_3 injection in animals exposed to 1% $KClO_4$ during the same period of time. Results on KAP expression when mice were euthanized (postnatal d 40) confirmed that both procedures had similar effects (data not shown); concomitantly, weights of animals under both treatments were practically identical: $13.31 \text{ g} \pm 0.1$ for those exposed to T_3 from postnatal d 7–21 and $13.98 \text{ g} \pm 0.1$ for those exposed to tap water from postnatal d 7–21. Control males had a weight of $19.7 \text{ g} \pm 0.1$ vs. $8 \text{ g} \pm 0.1$ of those permanently exposed to 1% $KClO_4$.

Once the procedure had been confirmed as effective, hypothyroid animals (potassium perchlorate-treated males) were exposed to regular tap water for different time periods (see Fig. 2A) and KAP mRNA expression was compared with that of age- and sex-matched animals exposed to tap water (non-treated control male) or potassium perchlorate (control) at 40 d postnatally. Results showed in Fig. 2A, indicated that the presence of T_3 around postnatal d 12 and beyond facilitated KAP expression and that 48 h exposure to tap water seemed sufficient to produce this effect. Female mice treated with tap water from birth to d 14 were negative for KAP expression in cortex at d 40 (Fig. 2B), whereas males treated in the same manner exhibited KAP expression in this compartment. These results indicated that only in males was the presence of TH after postnatal d 12 required to trigger KAP expression in cortex and that once this had occurred, the presence of TH was no longer required.

Expression of CCAAT/Enhancer Binding Protein (c/EBP) α and β Genes under Different Hormonal Conditions and Correlation with KAP Expression in Mouse Kidney

Because TH was not able to induce KAP mRNA in the cortex of castrated males (8), and because KAP proximal promoter does not contain canonical TH receptor binding sites, we proposed an indirect action of TH in

KAP expression. Among different possibilities, we first explored whether other transcription factors under TH control might be collaborating with androgens on KAP regulation. Inspection of the KAP proximal promoter indicated different putative transcription factor binding sites, including several for the c/EBP family members, at positions –457, –429, –110, and –66, from the transcription initiation site (16). Because it had been described that TH was regulating c/EBP α and β gene expression (17) and also because of the significance of these transcription factors in development, we investigated their expression levels in mouse kidney, under different hormonal conditions and correlated them with those of KAP mRNA.

c/EBP α and β levels in kidneys of male mice exposed to 1% $KClO_4$ or castration were assessed by Western blot assays. Results clearly showed c/EBP α and β expression to be residual in 1% $KClO_4$ -treated mice and levels of both factors to be reduced in castrated males (Fig. 3A). Semiquantitative RT-PCR in the same kidney samples revealed an almost undetectable expression of KAP mRNA in the presence of 1% $KClO_4$ and an obvious decrease in castrated males (Fig. 3B), in agreement with previous *in situ* hybridization results. Changes in KAP mRNA expression correlated well with c/EBP α and β protein levels, thereby indicating a possible functional relationship. To further test this hypothesis, c/EBP α and β protein levels were determined in kidneys of potassium perchlorate-treated males (controls), animals additionally given TH from postnatal d 7–21 (7–21 d T_3) and controls (non-treated control males). Western blot assays indicated that c/EBP α and β were recovered in the TH-treated males (Fig. 3C) and that KAP mRNA was also expressed under these conditions (Fig. 3D). In summary, the clear correlation observed among hormonal status of the animals, c/EBP α and β levels and KAP mRNA expression strongly suggests involvement of c/EBPs in KAP gene expression.

Effects of c/EBP α and β on KAP Promoter Transcription in PCT3 Cells

PCT3 cells, derived from the parental PKSV-PCT, have already shown their ability to support androgenic regulation of reporter gene constructs containing the kidney-specific KAP promoter in transient transfection assays (15). To further investigate the concept that TH-dependent expression of KAP mRNA in cortical segments of proximal tubules might relate to the presence of c/EBP α and β , transient transfection assays using the –638 KAP proximal promoter fragment

euthanized has also been included (*rightmost panel*) (c, cortex; o, outer medulla; i, inner medulla). B, Differential KAP cortical expression in potassium perchlorate-treated male and female mice, after postnatal d 14. Frozen kidney sections from C57BL/6 potassium perchlorate-treated male and female mice from birth until euthanized at postnatal d 40 (*left upper and lower panels*, respectively) were hybridized with strand-specific ^{35}S -KAP3 cRNA probes, as previously described, and compared with similar animals exposed to tap water from birth until postnatal d 14, and to potassium perchlorate from d 15 until euthanized at d 40 (*right upper and lower panels*).

Fig. 3. c/EBP Levels Correlate with KAP mRNA Expression in Cortex

A, Effects of postnatal hypothyroidism and castration on c/EBP α and c/EBP β protein levels in mouse kidney. Western blot assays were performed with nuclear (N) and cytosolic (C) extracts from kidneys of untreated control C57BL/6 male mice (control male), mice treated with 1% KClO₄ in drinking water from birth until euthanized at postnatal d 40 (KClO₄ 1% male) and castrated mice (castrated male). Antibodies against c/EBP α (p42) and c/EBP β (p35/p38) were used as described in *Material and Methods*. Specificity of the signals obtained with these antibodies was previously tested by incubation of each antibody with a 5-fold excess of their specific peptide (sc-61 P for c/EBP α and sc-150 P for β , both from Santa Cruz Biotechnologies) (not shown). Blots presented in the figure were performed with antibodies blocked with nonspecific peptides (M-20 for mouse gp130). Protein loading was assessed by gel staining with Coomassie brilliant blue and indicated on the figure as loading control for c/EBP α and β blots, respectively. **B**, Endogenous KAP mRNA expression levels in postnatal hypothyroid and castrated male mice. Endogenous KAP mRNA levels were measured by semiquantitative RT-PCR technique (see *Materials and Methods* for details) in extracts obtained from the contralateral kidney of the same animals used for c/EBP protein detection (see text of Fig. 3A). The KAP/Cyp A ratio is expressed as fold increase over basal level, as previously described (15). **C**, c/EBP α and β protein levels in hypothyroid male mice treated postnatally with TH. The experimental procedure was the same as described in Fig. 3A. c/EBP α and β levels were determined in kidneys of 1% potassium perchlorate-treated males from birth until euthanized (40 d postnatal) (control), in similar animals treated from postnatal d 7–21 with daily doses of TH (1.2 μ g) (7–21 T₃-treated males) and both were compared with naive male mice of the same age (nontreated control male). **D**, KAP mRNA levels on hypothyroid male mice treated postnatally with TH. *In situ* hybridization in contralateral kidneys of the same animals used for c/EBP protein detection (see text of panel C). Technical procedures were the same as those described in Fig. 1A. c, Cortex; o, outer medulla; i, inner medulla.

fused to chloramphenicol acetyl transferase reporter gene (CAT) were performed (Fig. 4A). Experiments were carried out in PCT3 cells under different hormonal treatments and in the presence or not of co-transfected expression vectors for c/EBP α and β cDNAs.

Results indicated that c/EBP α or c/EBP β alone (lanes 7 and 8, respectively) exhibited the same transactivation capacity as DHT in the presence of a transfected functional androgen receptor (lane 3) on the –638 KAP promoter (~3-fold) (Fig. 4A). When androgens and androgen receptor were included together with c/EBP α , β or both, activity was around 5- to 6-fold higher (lanes 4–6, respectively). These results

indicated a role for c/EBP α and β as well as an additive effect of androgens and these transcription factors on KAP gene transactivation.

Site-Directed Mutagenesis of Putative c/EBP Binding Sites in the KAP Promoter

Site-directed mutagenesis was performed on the 224K and 638K KAP constructs fused to the luciferase (LUC) reporter gene to further investigate whether the putative c/EBP binding sites identified in the KAP promoter were functionally relevant. The K224 construct contains two putative sites, from –66 to –56 and from –110 to –101 from the transcriptional initiation site,

Fig. 4. c/EBP Affects KAP Promoter Transactivation in PCT Cells

A, Effects of androgens and c/EBP α and β on the transcriptional activity of the K638 promoter construct in PCT3 cells. Cultured mouse PCT3 cells were transfected with 2 μ g of the K638 construct, in the presence of 0.5 μ g of cotransfected human androgen receptor (AR) expression vector pSVAR0 (lanes 2–6) and treated for 48 h with vehicle (lanes 1, 2, 7, and 8) or with 10^{-6} M DHT (lanes 3–6) in steroid-free medium. A total of 0.5 μ g of pMSV-c/EBP α and pMSV-c/EBP β expression vectors, which contain the entire open reading frame of rat c/EBP α and mouse c/EBP β (p35 LAP), was also cotransfected to the same cells, either alone (lanes 7 and 8) or in different combinations that include cotransfection of AR and presence of DHT (lanes 4–6). 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 -galactosidase activity produced by 2 μ g of transfected pCH110 plasmid. Bars indicate the mean ($\pm$ SE) of fold induction of reporter activities over control unstimulated, nonreceptor or c/EBPs-transfected cells (=1) for a minimum of three independent experiments. B, Outline of the proximal KAP promoter. ARE and the four putative C/EBP binding sequences positions on proximal KAP promoter are shown.

which were individually deleted and assayed by transient transfection in PCT3 cells. The same was done within the K638 construct where sites –66 to –56, –110 to –101, –429 to –420 and –457 to –448 were

individually deleted and tested (henceforth referred to as: –66, –110, –429 and –457) (Fig. 5A). Although results from these experiments indicated no significance for any of the individually mutated sites (Fig. 5B), a second set of experiments revealed that the simultaneous mutation of the –457 and –429 sites in the K638 construct produced a strong decrease in their transcriptional activity, which dropped approximately 35- to 40-fold, compared with that of the K638 control construct (Fig. 5C). c/EBP α or β cotransfection did not affect the transcriptional capacity of the K638 double-mutated construct (Fig. 5C). Triple mutated constructs, including the other two sites at positions –110 or –66, did not produce additional effects to those obtained with the double mutated construct (Fig. 5C).

From the *in vivo* experiments, we concluded that TH controls androgen-dependent cortical KAP gene expression through modulation of factors that might collaborate with androgens in the presence of a functional AR. To determine whether the sites at positions –457 and –429 were affected by the absence of steroid hormones in the culture media and/or the absence of the androgen receptor response element (ARE), previously defined at position –39 on the KAP promoter (15), transient transfection assays in complete or steroid hormone-free media were performed. Results (Fig. 5D, upper left panel) showed that mutation of the ARE further decreased the relative activity of the –457 and –429 mut K638 construct that went from 40-fold to 100- to 150-fold. In the absence of steroid hormones (Fig. 5D, right side of the upper panel), the effects were comparable to mutation of the ARE; no significant differences were observed between the K638(Δ 457–448, Δ 429–420) or the K638(Δ 457–448, Δ 429–420, Δ 39–35) construct, indicating that androgens, acting through this androgen receptor binding site, together with factors binding to the –457 and –429 sites collaborated on the transcriptional activity of the KAP promoter.

The transcriptional activity of the pGL3 basic vector was influenced neither by the presence of transfected c/EBP expression vectors nor by the culture conditions used (Fig. 5D, lower panels), which proved that the effects observed were KAP promoter dependent.

c/EBP α and β Bind to the –457 Box

All evidence found in the above-described experiments, both in kidney and in PCT3 cells, pointed to c/EBP α and β as the putative transcription factors able to control KAP gene expression in the presence of androgens.

To reinforce this concept, gel retardation assays were performed with oligonucleotides corresponding to the 457 and 429 boxes, in the presence of nuclear extracts obtained from kidneys or PCT3 cells. Results (Fig. 6A) showed that there were nuclear factors in PCT3 cells able to bind to these two boxes and, also,

Fig. 5. Functional Analysis of Putative c/EBP Mutated Sites on the KAP Promoter

A, Set of the different mutated versions of the LUC K638 and K224 KAP promoter reporter constructs. Nucleotides deleted from native sequences on the K224 and K638 promoter constructs were the following: positions 66–56 [K638/224 (Δ66–56)]: ACTGTGGA; 110–101 [K638/224 (Δ110–101)]: CTTCCCAAC; 429–420 [K638/224 (Δ429–420)]: CTCCAGCAAT; and 457–448 [K638 (Δ457–448)]: CTTTTCGAAT. B, Functional assays of single deletion of the putative c/EBP binding sites on the KAP promoter. Functional activities of 1 μ g K224 or K638 (wild type) were tested on PCT3 cells and compared with 1 μ g of each mutated construct. LUC activities were adjusted according to the *Renilla* LUC activity produced by 20 ng of transfected pRL-TK

to a consensus wild-type (WT) c/EBP box, but not to a mutant one (MUT). To determine the specificity of these retarded band, competition experiments including 30-fold excess of cold 457, 429, c/EBP WT, and c/EBP MUT oligonucleotides were performed. Results (Fig. 6B) showed that the retarded band on 457 box is competed out by cold WT c/EBP or by the same 457 sequence, but not by the c/EBP MUT. Because box 429 was only competed by its own sequence, our results suggested that the factor bound to the 457 sequence, but not that one bound to the 429 sequence, may belong to the c/EBP family. Next, we aimed to identify the nature of this factor by performing supershift assays with antibodies against c/EBP α and β . As expected, the retarded band for the 429 box was unaffected by the presence of the antibodies. Anti-c/EBP β , but not anti-c/EBP α , produced a supershift of the 457 and c/EBP WT boxes (Fig. 6C). The lack of c/EBP α supershift could not be attributed to absence of this isoform because its presence in the PCT3 nuclear extract was determined by Western blot analysis (not shown). Similar experiments performed with male mouse kidney nuclear extracts produced retarded bands for the 457 box that were competed by c/EBPWT but not by c/EBPMUT (Fig. 6D). When using these extracts, supershifts occurred with both anti-c/EBP β and anti-c/EBP α antibodies (Fig. 6E) indicating that, contrary to what occurs in PCT3 cells, both c/EBP factors are able to bind to the 457 box in mouse kidney extracts. Note that kidney extracts contain c/EBPs from all their different cell types. Whether this difference in binding ability to the 457 box is due to a different cellular origin of the active c/EBP α in whole kidney (*i.e.* not necessarily coming from cortex cells) or reflects an abnormal behavior of c/EBP α of the PCT cells is not known. The nature of the factor that binds to the 429 box and the mechanisms that operate to make c/EBP α incompetent to bind to the 457 box in PCT3 cells remain to be determined.

DISCUSSION

Although earlier *in situ* hybridization assays in mouse kidney had clearly demonstrated that the presence of androgens and functional androgen receptors were instrumental and apparently sufficient for KAP gene expression in cortical S1 and S2 segments of proximal tubules (10), later studies revealed the important contribution of TH to achieve a full androgen-dependent cortical response in males (8).

Although TH involvement was established, striking differences arose in KAP mRNA expression levels between congenital hypothyroid *hyt/hyt* adult males (8) and adult mice exposed to potassium perchlorate from d 10 of gestation until euthanized at postnatal d 90 (11). The *hyt* mice present primary hypothyroidism related to the hyporesponsiveness of the thyroid gland to TSH, which results in very low TH serum levels and abnormally high TSH levels (18). Perchlorate inhibits the transport of iodine into the thyroid in a competitive and reversible manner, which results in a decrease of TH production (T_4 and T_3) and an increase in TSH production (19). In the past, we had determined T_4 and T_3 concentrations in kidneys of hypothyroid mice (8, 11). Adult animals treated for 1 wk with $KClO_4$ gave values that ranged from 0.258–0.524 ng of T_3 and from 0.734–1.119 ng of T_4 /g tissue. Although very difficult because of their small size, congenital perchlorate-treated mice were also analyzed and values of T_3 and T_4 in kidney tissues ranged from 0.306–0.742 ng of T_3 and from 0.643–2.260 ng of T_4 /g of tissue. Animals of the *hyt/hyt* strain had values ranging from 0.548–0.974 ng of T_3 and from 0.857–2.85 ng of T_4 /g of tissue. We concluded that $KClO_4$ treatment was effective to render the animals hypothyroid, with TH values being even lower than those in the *hyt/hyt* mice. Untreated control mice ranged from 1.3–2.4 or higher ng of T_3 /g of tissue.

in the same sample. Fold induction over basal level of relative LUC activity for both constructs (K224, K638) was compared; results are shown in panel B. Bars indicate the mean ($\pm$ SE) of fold induction of LUC activity over control nonmutated constructs (=1), for a minimum of three independent experiments. C, Functional assays of double and triple deletion of the putative c/EBP binding sites on the KAP promoter. Functional activities of 1 μ g of K638 mutated constructs in two sites: K638 (457–448, 429–420) or in three sites: K638 (457–448, 429–420, 110–101) and K638 (457–448, 429–420, 66–56) were tested in PCT3 cells and compared with 1 μ g of K638 (wild type) following the same procedures as described in Fig. 5A. For each mutated construct, an additional situation, which corresponds to cotransfection of 0.5 μ g of pMSV-c/EBP α and PMSV-c/EBP β expression vectors, was included. Bars indicate the mean of fold induction of LUC activity over control nonmutated constructs (=1), for a minimum of three independent experiments. Error bars are not visible because values were too small to be depicted at this scale. D, Effects of steroid hormone depletion and/or ARE mutation on transcriptional activity of the K638 (Δ 457–448, Δ 429–420) construct. The K638 (Δ 457–448, Δ 429–420) construct was additionally deleted at the identified functional androgen receptor site (ARE) at position –39 from the transcription start site on the KAP promoter (15). The mutated ARE lacks the *underlined* sequence: 5'-GGTACAGGATGTATAAA-3' from positions –39 to –34. Functional activities of 1 μ g of K638 mutated constructs K638 (Δ 457–448, Δ 429–420) and K638 (Δ 457–448, Δ 429–420, Δ 39–34) were assayed in PCT3 cells and compared with 1 μ g of K638 (wild type), following the same procedure as described in Fig. 5A. The experiment was conducted in complete medium that includes presence of steroid hormones (*left upper panel*) or in steroid hormone-depleted media (*right upper panel*). For each mutated construct, an additional situation corresponding to cotransfection of 0.5 μ g of pMSV-c/EBP α and PMSV-c/EBP β expression vectors was included. As a control, 1 μ g of pGL3 basic vector was transfected or cotransfected with 0.5 μ g of pMSV-c/EBP α and PMSV-c/EBP β expression vectors, in complete (*left lower panel*) or steroid-free media (*right lower panel*). Bars indicate the mean of fold induction of LUC activity over control nonmutated constructs (=1), for a minimum of three independent experiments. Error bars are not visible because values were too small to be depicted at this scale.

Fig. 6. Box 457 Binds c/EBP

A, EMSA of boxes 429 and 457. Five micrograms of nuclear extracts from PCT3 cells were incubated with 32 P-labeled oligonucleotide duplex containing sequences corresponding to box 457 (lane 8) and box 429 (lane 6) from the KAP proximal promoter and analyzed by gel shift assay. 32 P-labeled c/EBP consensus WT (lane 2) or c/EBP MUT sequences (lane 4), from Santa Cruz Biotechnologies, were used as positive and negative binding controls, respectively. Lanes 1, 3, 5, and 7 are controls for free DNA probe. The sequences for each probe are described in *Materials and Methods*. B, Competitive EMSA of boxes 429 and 457. Specific protein binding by 32 P-labeled oligonucleotide duplex probes c/EBP WT (lanes 1–5), box 429 (lanes 7–10) and box 457 (lanes 12–15), incubated with 5 μ g of nuclear extracts from PCT3 cells, was competed with 30-fold molar excess of cold oligonucleotides, corresponding to c/EBP WT (lanes 2, 8, and 13), c/EBP MUT (lanes 4, 9, and 14), box 429 (lanes 5 and 10) and box 457 (lanes 3 and 15) sequences, described in Fig. 6A. Band patterns with no competitor oligonucleotide added are also shown in each case (lanes 1, 7, and 12). Lanes 6 and 11 are controls for free DNA probe. C, Supershift assays of complexes bound in boxes 429 and 457. The composition of complexes formed in the 429 and 457 boxes (lanes 6 and 10) was further analyzed by the incorporation of anti-c/EBP α (lanes 7 and 11) and c/EBP β (lanes 8 and 12) antibodies to the binding reaction and compared with the c/EBP WT sequence (lanes 2–4). The supershifted complexes (lanes 4 and 12) are indicated by a *thick arrow* and nonshifted complexes by a *thin arrow*. Lanes 1, 5, and 9 are controls for free DNA probe. D, Competitive EMSA of the 457 box using mouse kidney nuclear extracts. Specific protein binding by 32 P-labeled oligonucleotide duplex probe 457, incubated with 25 μ g of nuclear extracts from adult male mouse kidney (lane 2), was competed with the indicated-fold molar excess of cold c/EBP WT (lanes 3–6) and c/EBP MUT (lanes 7–10) oligonucleotides. Lane 1 represents a control for free DNA probe. The position of the complexes is indicated by an *arrow* on the *left*. E, Supershift assays of complexes bound in box 457 with kidney extracts. The composition of complexes formed in the 457 box (lane 2) in the presence of mouse kidney extracts was further analyzed by the incorporation of anti-c/EBP α (lane 3) and c/EBP β (lane 4) antibodies to the binding reaction. The supershifted complexes (lanes 3 and 4) are indicated by *thick arrows* on the *right*; a *thin arrow* marks the position of unshifted complexes. Lane 1 represents a control for free DNA probe.

Because we already knew that the KClO_4 treatment was effective to render the animals hypothyroid, and animals in this study presented the same dwarf phenotype as before, hormone levels were not measured.

Although both models apparently showed the same defect, homozygous mice for the recessive *hyt* mutation exhibited lower cortical expression than controls, whereas perchlorate-treated animals showed complete absence of KAP mRNA from the early stages of development (11). In addition, in potassium perchlorate-treated adult males, cortical expression did not disappear but only decreased (11). In light of these data, our first hypothesis to explain the differences between models was that exposure to maternal TH, in obligated heterozygous *hyt*/+ mothers, was responsible for priming and enabling KAP cortical response in adult *hyt/hyt* males, whereas mice born of mothers exposed to goitrogen during gestation became unable to express KAP in adult life. Although the damage caused by lack of maternal and fetal TH in the developing brain had been reported (see Ref. 20 for review), the results of this work do not support the concept that prenatal TH deprivation is responsible for KAP expression prevention, but rather demonstrate that its postnatal presence makes expression possible.

Another distinctive feature between both hypothyroidism models was the significant growth failure associated with perchlorate treatment, which has also been described in childhood hypothyroidism (21). *Hyt* mice, previously used in our laboratory (8), present slightly lower weight than normal littermates, approximately 20 g, whereas animals under postnatal perchlorate treatment are about half the weight, i.e. approximately 10 g. TH involvement in the regulation of the rat GH promoter (22), its interaction with retinoic acid receptors (23) and the participation of cell-specific transcription factors such as Pit-1/GH factor-1 (24) to fully accomplish GH expression have been reported.

This observation prompted us to hypothesize that, although absence of TH diminishes KAP cortical expression, combined TH and GH deficiency abolishes it. *In situ* hybridization analysis performed in the GH-deficient *lit/lit* mice (25) and Jackson *dwarf* mice, deficient in TSH, PRL, and GH due to an inactivating mutation in the anterior pituitary-specific transcription factor Pit-1 (26), revealed that GH and TH combined deficiency does compromise KAP gene expression in cortical segments in this genetically deficient mouse model. These results provided further evidence of the *in vivo* interactions between GH and T_3 for the regulation of KAP gene and, also, that lack of KAP mRNA was not related to putative undesirable perchlorate side effects.

This concept was further challenged by administration of T_3 , GH or both hormones simultaneously to perchlorate-treated males on d 7–21. When mice were euthanized, on postnatal d 40, it became clear that T_3 replacement overcomes the inhibitory effects of perchlorate and that this effect is even more prominent in

the presence of GH. These results, together with our previous data (11), strongly support the idea that TH deprivation without consequences on severe growth retardation, i.e. in *hyt* mice and in adult perchlorate-treated mice, reduces but does not abolish KAP expression, whereas absence of TH associated with severe growth retardation, i.e. Jackson/dwarf mice and mice treated postnatally with perchlorate results in absolute absence of KAP mRNA. Transient transfection assays from our laboratory had demonstrated that the KAP promoter enhances its response to androgens by 2.5-fold, in the presence of IGF-I (15). These data, together with the *in vivo* results presented in this work, support the concept that GH can modulate androgen-dependent KAP cortical expression in mouse kidney.

Although poorly studied in mice, the participation of TH and GH in gonadal development and puberal maturation has been described in rats (27–31). According to those studies, the lack of expression of sexual dimorphic kidney markers such as KAP could, in part, be related to insufficient androgen levels; nevertheless, our data indicate that other factors must be involved because discrete presence of T_3 for 48 h anytime after postnatal d 10, promotes cortical expression of the gene, when analyzed at postnatal d 40. This time, which is too short to reprogram gonadal development in males, might be sufficient to induce one or more T_3 /GH-dependent factors required to collaborate with androgens in KAP expression.

From the *in vivo* data presented here, we propose that postnatal expression of KAP mRNA in proximal convoluted tubules requires the presence of androgens and, also, the contribution of developmentally regulated factors triggered essentially by T_3 . Taking into account the punctual need of T_3 , we also hypothesize that, after TH priming and in the presence of some androgen levels, the one or more factors involved in KAP expression must be either autoregulated or under the control of hormones or stimuli other than T_3 .

The transcription factors CCAAT/enhancer-binding proteins (c/EBPs) are members of the bZIP (basic region leucine zipper) class of DNA-binding proteins. They play an important role in cellular differentiation and development (32–36) and fulfill many of the requirements of the hypothesized factor(s) involved in the regulation of KAP expression in kidney cortex. Congenital hypothyroidism causes a significant decrease in expression of both c/EBP α and β isoforms at early stages of postnatal liver development (17), and T_3 positively regulates c/EBP α gene expression through a functional TH response element found in c/EBP α proximal promoter (37). c/EBP β has also been reported to contribute to regulating c-fos expression mediated by GH (38). It has recently been reported that GH promotes relocation of c/EBP β to heterochromatin, in association with the activation of MAPK signaling, introducing a new level of transcriptional regulation mediated by this hormone. GH-mediated

phosphorylation and nuclear redistribution of c/EBP β may be coordinated to achieve spatial-temporal control of gene expression (39).

Correlation of c/EBP α and β levels in nuclear and cytoplasmic kidney extracts of perchlorate-treated males with KAP mRNA expression, in contralateral kidneys of the same animals, showed that, in the absence of both forms of c/EBP, there is no expression of KAP. Moreover, T₃ injection in perchlorate-treated males recovers levels of both c/EBPs and restores KAP expression. Direct evidence of c/EBPs involvement in KAP transactivation was obtained by performing transient transfection assays of the KAP proximal promoter (K638) fused to the CAT reporter gene in PCT3 cells. This promoter fragment (Fig. 4A) was selected for transfection because it contains four putative c/EBP binding sites at positions –66, –110, –429, and –457. Our results showed that transfection of c/EBP α or β (Fig. 4B, lanes 7 and 9) exerts the same effect on transcriptional activity as transfection of the AR in the presence of DHT (Fig. 4B, lane 3) and also that simultaneous cotransfection of α and/or β c/EBPs and AR, in the presence of androgens, (Fig. 4B, lanes 4–6) produces an additive effect on K638 transcriptional activity. Altogether, these results support the concept that the effects of T₃ on the androgenic response of KAP observed in kidney are, at least in part, mediated by c/EBP α and β .

Site-directed mutagenesis was performed at specific sites to elucidate which of the putative c/EBP binding sites on the promoter were functionally significant (Fig. 5A). Results (Fig. 4B) indicated that mutation of individual sites had no effect on promoter activity, whereas simultaneous mutation of boxes –429 and –457 decreased the transcriptional activity of the promoter approximately 40-fold (Fig. 4C), thereby indicating that these sites are functionally significant when they work in a cooperative fashion.

Deprivation of steroid hormones and/or mutation of the ARE at 39 bp from the transcriptional initiation site was performed to further investigate whether factors binding to these functional sites could cooperate with the androgenic response. The 100- to 150-fold decrease in promoter activity obtained in either situation clearly demonstrated a functional relationship between transcription factors bound to the –457 and –429 boxes and the AR.

Competitive shift and supershift assays performed to verify that the –457 and –429 sites were binding-specific nuclear factors and identify their nature demonstrated that c/EBP α and β bind to the –457 box but not to the –429. The nature of the factor bound to box 429 remains to be identified. Overall, the data presented here, together with previous contributions from our laboratory indicate that expression of the KAP gene in epithelial cells of proximal convoluted tubules depends not only on androgens and functional androgen receptors, but also on transcription factors that bind at boxes –457 and –429 in the proximal pro-

moter. c/EBP α and β can bind to the –457 box, activate transcription and, together with a third party, bind to box –429 and trigger expression of the gene when androgen levels are sufficient in males. After birth, the presence of TH and GH is required for sexual maturity and expression of c/EBP α and β . Once these factors have been expressed and probably because they can be autoregulated (40, 41), TH becomes dispensable and cortical KAP expression occurs in the presence of androgens alone.

Study of the KAP gene has raised the theory that androgens are necessary, but not sufficient, to initiate an androgenic response in kidney and that other developmentally regulated factors, ultimately controlled by T₃, are involved. Studies with the ornithine decarboxylase (ODC) gene demonstrate that the lack of renal androgenic response during the postnatal period cannot be related only to the low levels of plasma testosterone at that time, but rather to immaturity of renal mechanisms to respond to androgens, which follow a maturational process in which testosterone could be only one of the factors implicated (42). Induction of renal ODC by androgens in adult mice requires activation of the transduction cascade mediated by catecholamines binding to α 1-adrenergic receptors (43). Reports describe the existence of a critical period for T₃ to play a role in development of renal α 1-adrenergic receptors (44); the influence of TH in the development of β adrenergic control of ODC in rat kidney and heart (45) and evidence that c/EBP α is required for transcription of β -adrenergic receptors in adipose tissue (46) suggest that c/EBPs under T₃ control might not only be involved in the androgenic control of KAP gene expression, but rather constitute a more general phenomenon for transcriptional control of androgen-regulated genes in kidney.

MATERIALS AND METHODS

Materials

Media for cell culture and LIPOFECTAMINE reagent were obtained from Life Technologies, Inc. (Gaithersburg, MD). Fetal calf serum, trypsin, glutamine, and essential amino acids were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Other supplements for cell culture, acetyl coenzyme A, steroid hormones, THs, and thin-layer chromatography plates were obtained from Sigma (St. Louis, MO). Human GH was a generous gift from Kabi-Pharmacia (Uppsala, Sweden). Restriction and modifying enzymes were purchased from either Life Technologies, Inc., Promega (Madison, WI), Amersham Pharmacia Biotech (Amersham, Buckinghamshire, UK), TaKara Shuzo Co. Ltd. (Shiga, Japan), or Roche Molecular Biochemicals (Mannheim, Germany). Oligonucleotides were synthesized by TIB MOLBIOL-SynththeseLabor (Berlin, Germany). All other reagents were obtained as previously reported (6, 8, 11).

Animals

Jackson *dwarf* (strain C³H/HeJ-*dw*¹/*dw*¹), *little* mice (strain C57BL/6J-*lit*/*lit*) and their genetically matched normal controls (strain C³H/HeJ *dw*¹/*+* and C57BL/6J-*lit*/*+*) were obtained at 4–8 wk from The Jackson Laboratory. C57BL/6 control mice were obtained at 6 wk of age from Charles River España (Barcelona, Spain).

Mice were housed in the animal facilities in covered cages at 22 C with 12 h light and darkness cycles. Pelleted food and tap water were supplied *ad libitum*.

At the time of the experiment, animals were euthanized by cervical dislocation. Kidneys were removed and snap-frozen immediately in liquid nitrogen in the presence of 2-methylbutane. All animal experimental procedures were conducted in accordance with Institutional standards which fulfill the requirements established by the Spanish Government and the European Community (Real Decreto 223/1988: B.O.E. no. 67, 3/18/88) and B.O.E. no. 256, 10/25/90).

Hormone Treatment

T₃ hormone was resuspended in 10 mM KOH and 1.2 µg/animal/d were administered sc human GH was reconstituted with Na₂CO₃ 25 mM (pH 9.4) in 0.9% NaCl and 18 µg/animal/d were injected, sc. Pharmacologically induced congenital hypothyroidism was achieved through addition of 1% KClO₄ to drinking water of pregnant dams at d 10 of gestation. From birth until being euthanized, animals were exposed to goitrogen, even when other treatments were simultaneously performed. The corresponding sham treatments were effected in all experimental situations. Hypothyroidism in newborn mice was induced using the same drugs at the same concentration until the animals were euthanized. When required, KClO₄-treated water was replaced by normal tap water.

Probe Synthesis and *in Situ* Hybridization Histochemistry

³⁵S-Labeled transcripts from pKAP3 or pKAP4 cDNA plasmids, which correspond to antisense and sense cRNA, respectively, were prepared as previously described (14). The preparation of renal sections, hybridization protocol and autoradiographic analysis were all performed as reported (8, 11). Hybridized sections were examined under a light microscopy. Positive silver grain staining (appearing in black) determined the site of KAP mRNA synthesis (using KAP3 probe) in proximal tubule cells. All sections hybridized with the mRNA KAP4-specific strand sense probe exhibited no detectable silver grain staining (data not shown).

The magnification chosen for the images shown in the figures (×40) permitted visualization of the three major compartments of the kidney (cortex, c; outer medulla, o; inner medulla, i) and determination of the spatial location of KAP mRNA. Kidney sections from same figure were each analyzed in a single experiment. Consequently, slides were exposed to the same conditions throughout the entire procedure, and KAP mRNA expression levels on different slides were comparable. Photomicrographs shown in the figures were selected from similar results obtained in different experiments using animals from different litters.

RNA Extraction and Semiquantitative RT-PCR

Total RNA was extracted from kidneys of animals using the guanidinium thiocyanate method (47). Different amounts of total RNA were used in each RT-PCR using the SuperScript One-Step RT-PCR System (Life Technologies, Inc.) under

conditions previously reported. Primer sequences for KAP and CypA internal control genes have already been described (15).

Preparation of Protein Extracts and Western Blotting

Kidney tissues were homogenized in buffer A containing 20 mM Tris-HCl (pH 6.8), 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 1% Triton X-100, 2 mM phenylmethanesulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM Na₃VO₄. Homogenates were centrifuged for 15 min at 13,000 rpm. Supernatant (cytosol and membrane fraction) was heated at 70 C for 5 min, centrifuged for 20 min at 14,000 rpm and supernatant (cytosol) frozen at –80 C. The pellet from the original kidney homogenates (including nuclear fraction) was resuspended in buffer B containing 25 mM Tris-HCl (pH 7.5), 1 mM EGTA, 2 mM EDTA, 1% SDS, and 2 mM phenylmethanesulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM Na₃VO₄. The suspension was sonicated until clarified, centrifuged for 15 min at 13,000 rpm and the supernatant (nuclear fraction) saved at –80 C. All steps were performed at 4 C. Protein concentration was determined by the micromethod of Bio-Rad (Hercules, CA) using BSA as a standard.

Protein extraction from cell cultures was performed using the same protocol except that, after washing of plates with PBS, cells were scraped with a rubber policeman and homogenized with buffer A on a rocking platform for 60 min at 4 C. The remaining procedures were the same as for kidney extracts.

Fifty micrograms of nuclear and cytosolic proteins from mouse kidney or cultured cells were size-fractionated by denaturing 12% SDS-PAGE. Proteins were electroblotted onto polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH) in transfer buffer (50 mM Tris-HCl, 40 mM glycine, 20% methanol) and blots blocked overnight at 4 C in 4% BSA in TBST [Tris-HCl 20 mM (pH 7.6), NaCl 130 mM and Tween 20 at 0.1%]. Primary polyclonal rabbit antibodies anti-c/EBPα (14AA) and anti-c/EBPβ (C-19) from Santa Cruz Biotechnologies (Santa Cruz, CA) were diluted at 0.2 mg/ml in blocking buffer. Washes were performed following the membrane manufacturer's instructions and secondary antibody (horseradish peroxidase-conjugated goat antirabbit; Dako A/S, Glostrup, Denmark) was diluted 1:5000 and incubated for 1 h at room temperature. After washing, bands were detected using the ECL+ chemiluminescence detection method (Amersham Pharmacia Biotech) and exposed to Hyperfilm. To assess specificity of the antibodies, several assays were performed using specific blocking peptides (5-fold excess) (sc-61) for c/EBPα, (sc-150 P) for c/EBPβ and non-specific (sc-565P) for gp-130, from Santa Cruz Biotechnologies.

Plasmid Constructs

The strategy for obtaining the promoter fragments consisting of nucleotides (nt) –224 to +1 and –638 to +1 in the KAP gene (relative to the transcription start site) has already been described (15). Constructs were verified by extensive restriction mapping and partial DNA sequencing. All promoter lengths are available in both CAT and LUC promoterless vectors. The pCAT-Basic and pGL3-Basic vectors were obtained from Promega Corp. (Madison, WI).

The expression plasmid for β-galactosidase pCH110 (Amersham Pharmacia Biotech) and the *Renilla* LUC reporter plasmid pRL-TK (Promega Corp.) were used to normalize transfection efficiencies of CAT and LUC assays, respectively. Plasmid pSVAR0 containing a simian virus 40 promoter that directs transcription of the full-length human androgen receptor cDNA was kindly provided by Dr. C. López-Otín (Universidad de Oviedo, Spain) (48). TH receptor, consisting

of cDNA-encoding rat T3R α 1 cloned in pCDM8, retinoid X receptor expression vector, human retinoid X receptor α cDNA cloned in plasmid pSG-5 and the SaltK reporter construct, which corresponds to a TH-responsive element introduced into the reporter plasmid pBLCAT2 upstream of the heterologous Herpes simplex virus-thymidine kinase promoter, were kindly provided by Dr. A. Muñoz (Instituto de Investigaciones Biomédicas Alberto Sols, Centro Superior de Investigaciones Científicas, Spain) (49). pMSV-c/EBP α and pMSV-c/EBP β are expression vectors that contain the entire open reading frame of rat c/EBP α and c/EBP β (p35 LAP), respectively, driven by the murine sarcoma virus promoter (50). They were kindly provided by Dr. M. Giral (Universitat de Barcelona).

Transient Transfection Studies

The cloned PKSV-PCT, referred to as PCT3 cells, has previously been described (15). The culture conditions have also been reported (12, 13). Transient transfection experiments were performed as previously reported (15).

CAT and LUC Assays

These assays were performed as previously reported (15). Stimulation of CAT and LUC activities were expressed as fold increase over activities of noninduced transfected cells with the same reporter plasmids set to 1 and based on at least three independent experiments.

Site-Directed Mutagenesis of Putative c/EBP Binding Sites

K224 (110–101), K224 (66–56), K638 (457–448), K638 (429–420), K638 (110–101), and K638 (66–56) contain deleted putative c/EBP binding sites of the KAP promoter. K224 and K638 were used as the DNA mutagenesis template to anneal with the mutagenic primers. Additional ARE mutation at position –39 was performed as described (15).

Mutant strands were synthesized with *Pfu Turbo* DNA polymerase and used to transform Epicurian Coli XL1-Blue Supercompetent cells using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Ca). Plasmid DNAs were isolated from the selection plates and mutants were identified by sequencing.

EMSA Experiments

Nuclear extracts from PCT3 cells were prepared as described by Dignam *et al.* (51) without dialysis. Nuclear extracts from C57BL/6 mice kidney were prepared as described by Landschulz *et al.* (52) starting from 3.5 g of tissue and without dialysis. For EMSA experiments, the ³²P-labeled DNA fragments (C/EBP WT: TGCAGATTGCGCAATCTGCAA, C/EBP MUT: TGCAGAGACTAGTCTCTGCAA, 429:CTTCACTGTTCTCCAGCAATCTGCCAGGAT, 457:GATAGTTCTGCTTTTGC-AATGAGCAGTTCT) were incubated with 25 μ g of kidney nuclear protein or 5 μ g of cellular nuclear protein in a binding buffer [20 mM Tris-HCl (pH 7.4), 75 mM KCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM DTT] in the presence of 100 ng of poly(deoxyinosine-deoxycytosine) and 50 ng of acetylated BSA for 30 min at 37 C in a final volume of 20 μ l. Oligonucleotide competition experiments were performed under the same conditions by previous mixing of all the reagents and increasing amounts of corresponding cold oligonucleotides before nuclear protein addition. Supershift assays were performed by incubating the complexes for a further 10 min more at 37 C with anti-C/EBP α and anti-C/EBP β before

loading. The samples were electrophoresed on 5% acrylamide 0.5 $\times$ TBE gels, dried, and developed with intensifier screen at –80 C.

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