Endocrine, vol. 4, no. 1, 1–10, February 1996 0969-711X/96/4:1-10/$7.50

Cell-Specific and Hormonal Regulation of the Rat Kidney Androgen-Regulated Protein (KAP) Gene

En-Mei Niu, Anne Crozat, and James F. Catterall

The Population Council, Center for Biomedical Research, New York, NY

In mouse kidney, the kidney androgen-regulated protein (KAP) gene is regulated in a sex-dependent manner by a complex tissue- and cell-specific multihormonal system. KAP is also found in mouse uterus during the period surrounding birth. We describe here for the first time the existence of KAP in a species other than mouse. The rat cDNA sequence was determined and the derived peptide sequence displayed only 53% identity with murine KAP, although the genomic organization of the genes was identical. Expression of rat KAP was restricted to kidney and uterus, but was constitutive in the latter and drastically induced at parturition. The renal expression of the rat KAP gene was sexually dimorphic and regulated by physiological levels of steroid hormones. The effects of castration, hypophysectomy, thyroidectomy, and castration plus thyroidectomy on KAP mRNA levels in both kidney and uterus were determined. Constitutive expression of the protein was strictly dependent on thyroid hormone in female kidneys where it was modulated by estrogens and other ovarian factor(s). In the uterus, KAP mRNA was mainly under estrogen control. In males, expression of the KAP gene was under the dual regulation of thyroid hormone and androgens. Its complex regulation suggests a carefully delineated role for KAP in the kidney and uterus, but its physiological function remains unknown.

Key Words: KAP; rat; kidney; uterus; steroid hormones; thyroid hormone.

Introduction

Kidney androgen-regulated protein (KAP) is the product of a relatively abundant mRNA expressed in the mouse kidney, in the proximal convoluted tubule epithelium of the cortex (S1 and S2 cells), and in the epithelial cells of the outer medullary segment (S3 cells) (Meseguer and Catterall, 1987). The mRNA is regulated by androgens in the cortex but is under different regulation in S3 cells (Meseguer and Catterall, 1987), where the gene requires both pituitary thyrotropin and estrogen for maximal expression (Meseguer and Catterall, 1992). In addition, KAP mRNA was identified in the mouse uterus where it is transcriptionally induced by steroid hormones during the period surrounding birth, at levels comparable to those attained in the kidney (Kasik andRice, 1993). Interestingly, KAP mRNA is not expressed in mouse uterus at any other time. This complex multihormonal, tissue-, and cell-specific control suggests a carefully delineated role for KAP in the kidney, as well as uterus; however, its function remains unknown. Homologues of KAP have not been described previously in species other than mouse. Studies of homologous proteins from several species often lead to identification of conserved structures that may indicate functional significance. For this purpose, we have cloned and sequenced the cDNA for the rat KAP, aligned the predicted amino acid sequences of both species, and compared their genomic organization. We also studied the distribution of KAP mRNA in several tissues and analyzed its specific regulation in the kidneys and uterus of rats subjected to different hormonal stimuli.

Received August 7, 1995; Revised October 5, 1995; Accepted October 19, 1995.

Author to whom all correspondence and reprint requests should be addressed: Dr. James F. Catterall, The Population Council, Center for Biomedical Research, 1230 York Avenue, Box 273, New York, NY 10021.

Results

Nucleotide Sequence of Rat KAP mRNA

A rat kidney cDNA library (Clonetech) was screened using the mouse KAP cDNA as a probe, and a 704 nucleotide cDNA clone was isolated. The nucleotide sequence of the cDNA insert is shown in Fig. 1A. Primer extension, using total rat kidney RNA as template, indicated that the cDNA corresponded to the full-length mRNA (Fig. 1B). The cDNA exhibited 78% sequence identity with mouse KAP mRNA (Fig. 1A), leading to the conclusion that the isolated clone was, indeed, the rat homologue of mouse KAP mRNA. The rat mRNA has a longer 5' noncoding segment than the mouse (154 nucleotides compared with 47 in the mouse, and a 3' noncoding region of 187 nucleotides [198 in mouse]) that includes an AATAAA polyadenylation signal, 17 bases 5' of the poly(A) site.

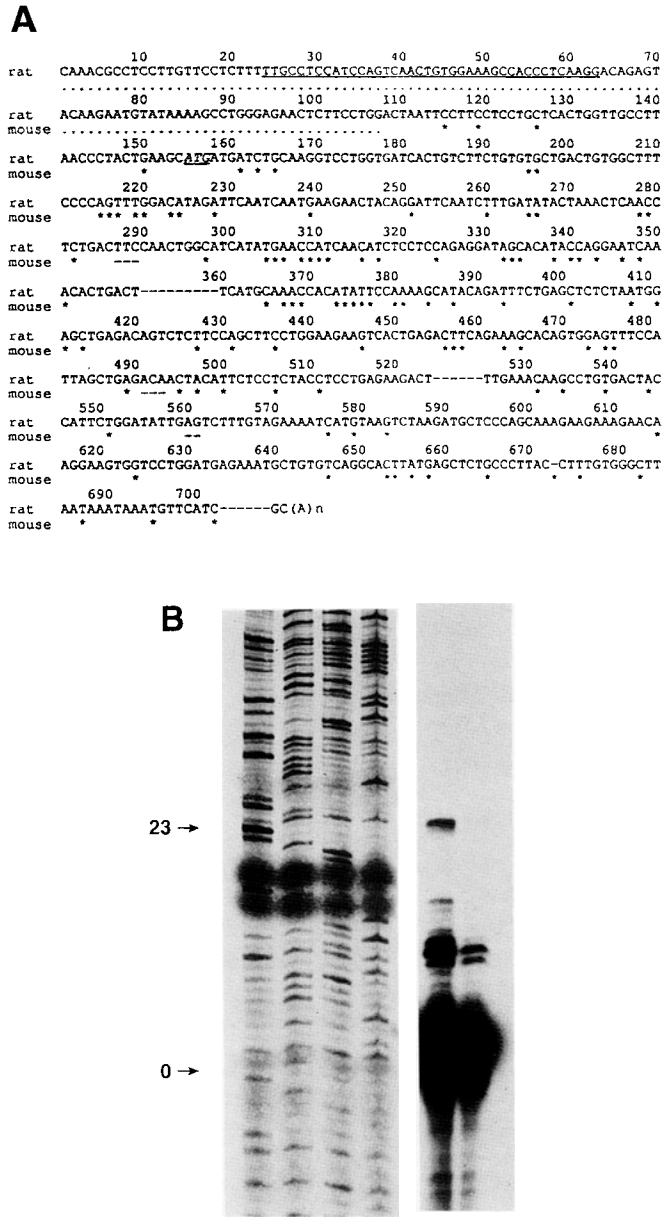

Fig. 1. Nucleotide sequence of the rat KAP cDNA. **(A)** Rat KAP cDNA and comparison with the mouse sequence. Sequences were compared using the "align" algorithm. Dashes represent gap insertions to optimize the alignment. The mouse mRNA transcription start site is at nucleotide 108 of the rat sequence. Dots underline the rat 5' UT sequence absent from the mouse mRNA. Asterisks indicate mismatches between the two sequences. The oligonucleotide used for primer extension is underlined. (A) refers to the poly(A) sequence. The start codon ATG is indicated in italics and underlined. **(B)** Determination of the transcription start site. Primer extension analysis was carried out on total RNA from rat kidney, using a 40 mer synthetic oligonucleotide as primer (underlined in panel A). A sequencing ladder utilized to determine the size of the reaction products is shown in the four lanes on the left side. Numbering indicates the position of the most 5' nucleotide of the primer ("0") and the size of the entire extension product, calculated from the 5' end of the primer ("23"). Additional bands can be seen below the putative transcription start site that represent truncated products of the reaction, conceivably owing to pauses of the polymerase. Over exposure of some part of the gel was necessary to carefully determine the location of the start site.

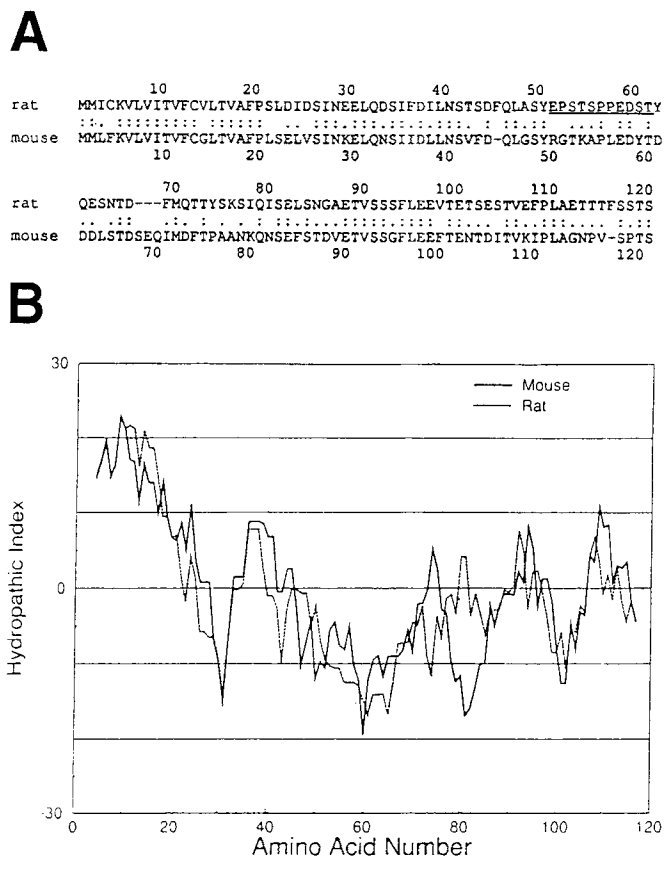

Fig. 2. Comparison of the amino acid sequences of rat and mouse KAP proteins. **(A)** Deduced amino acid sequences. The rat protein has 120 amino acids and the mouse 121. In each sequence "-" indicates a gap insertion character inserted to maximize the alignment of both sequences, ":" indicates two identical amino acids, and "." indicates a conservative change. The potential glycosylation site is indicated in bold characters and the PEST motif is underlined. The sequence of rat KAP displays 53% identity with the mouse sequence. **(B)** Hydropathy plots. Computer-generated plots of both proteins were produced by the method of Kyte and Doolittle, using a window size of 7 amino acids. Regions above the horizontal axis (0) are hydrophobic, whereas regions below the horizontal axis are hydrophilic.

Comparison Between Mouse and Rat KAP Amino Acid Sequences

The rat cDNA contains a predicted open reading frame of 360 nucleotides, beginning at nucleotide 155 and ending at nucleotide 517. Although, as in mouse mRNA, the rat nucleotide sequence surrounding the proximal ATG triplet (TGA AGC ATG ATG), does not follow the Kozak consensus rules (Kozak, 1984), two in-frame stop codons are present 5' to the first ATG, supporting the assignment of the initiation codon. The polypeptide of 120 amino acids specified by this sequence (Fig. 2A) has a calculated molecular weight of 13,183. In comparison, mouse KAP is 121 amino acids long and has a predicted molecular weight of 13,265 (Fig. 2A) (Meseguer et al., 1989). The two rodent proteins have only 53% identity, but this number rises to 80% when conservative amino acid substitutions are taken into

account. Analysis of the hydropathicity (Fig. 2B) shows that the distribution of hydrophobic and hydrophilic sections along the predicted protein sequences is well conserved between the two species, with the exception of a domain between amino acids 70 and 90 in which the plots are roughly reciprocal. In both species the N-terminus of the molecule is very hydrophobic, suggesting the presence of a signal peptide. This is further supported by the prediction for two potential signal sequence cleavage sites between amino acids 18/19 and 20/21 of the rat sequence (normalized probability of 0.89 and 1.0, respectively) (Von Heijne, 1986). In addition to the common features found between the mouse and the rat KAP proteins, the latter has a very strong "PEST" cluster of amino-acids (from residue 51 to 61), which is generally associated with the rapid turnover of a protein (Rogers et al., 1986), and a potential glycosylation site at residue 40. No significant homologies for either the mouse or the rat KAP sequences were found to other proteins of known sequence when compared to available protein sequence databases.

In Vitro Translation

The full-length rat cDNA was subcloned into pGEM3Z and transcribed in a cell-free system using the T7 RNA polymerase. When the mRNA was translated in a reticulocyte lysate in the presence of ^{3}H-Leucine, a single translation product with a molecular weight of about 22,500 was observed (Fig. 3, lane 1), which depended strictly on the presence of an in vitro transcript (data not shown). When microsomal membranes were added cotranslationally, two new products of mol wt 21,000 and 24,500, respectively, were identified (Fig. 3, lane 2). We speculate that these products represent glycosylated (24,500) and unglycosylated (21,000) KAP species with a cleaved amino-terminal signal sequence. Removal of the signal sequence without glycosylation of the mature KAP peptide results in a shorter product, whereas for the slower mobility chain, the presence of the added carbohydrate moiety more than offsets the increased mobility caused by removal of a signal sequence. Because glycosylation and signal peptide cleavage are cotranslational modifications occurring exclusively in the lumen of the endoplasmic reticulum (Jackson and Blobel, 1977; Lingappa et al., 1978), the processed forms of the rat KAP peptide probably represent products integrated into or translocated across the microsomal membrane. To characterize their orientation with respect to the membrane, rat KAP synthesized in the presence of membranes was subjected to proteolysis with proteinase K, either under conditions that maintained the microsomal membrane bilayer intact or in the presence of Triton X-100, which solubilized the bilayer. When the products synthesized in the presence of microsomal membranes were subjected to proteinase K digestion, only the slower product of mol wt 24,5000 was detected (Fig. 3, lane 3). No labeled product was detected in the presence of detergent (Fig. 3, lane 4). Thus, at least one peptide was localized to the vesicle lumen and hence protected from protease degradation in the absence of detergent. In contrast, after synthesis in the absence of microsomes, the translated product of mol wt 22,500 was protease sensitive regardless of the presence of TX-100 (data not shown), indicating that protection from digestion was provided by the membranes.

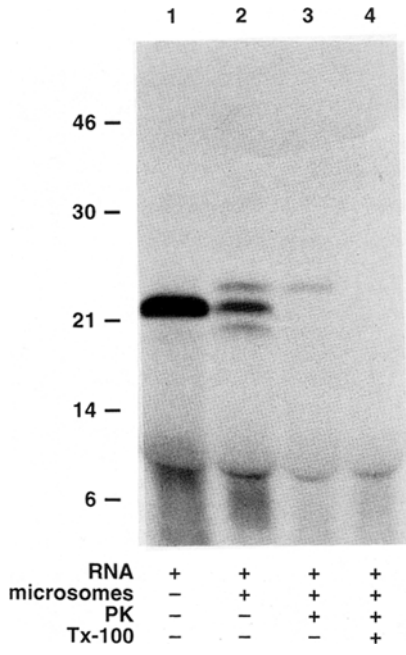

Fig. 3. In vitro translation of the rat KAP cDNA. Transcription-linked translation was performed in a reticulocyte lysate cell-free system containing ^{3}H-Leucine, in the absence (lane 1) or presence (lanes 2, 3, and 4) of dog pancreas microsomal membranes. Translation products were treated with proteinase K (lanes 3 and 4) in the absence (lane 3) or presence (lane 4) of detergent, resolved by SDS-PAGE (12%) and detected by fluorography. Mol-wt markers (10^3) are indicated on the left.

Genomic Organization of the Rat KAP Gene

The rat KAP cDNA was used as a probe to screen a rat genomic library and five independent clones were isolated. Restriction endonuclease analysis of the genomic fragments show that all five clones were identical and contained an insert of about 5 kbp encoding the entire gene. Sequencing of the exon-intron junctions and of the intervening sequences showed that the overall organization of the rat KAP gene is very similar to that of the mouse (Niu et al., 1991). Figure 4A shows the comparison of the genomic organization in both species where KAP has four exons and three intervening sequences (IVS), each located at the same relative positions. Exon 1 is longer in the rat than in the mouse gene, owing to the use of an alternate transcription initiation site, 108 bp upstream of the start site of the mouse gene as demonstrated by primer extension (Fig. 1B). Exons 2, 3, and 4 are 171 bp, 63 bp, and 243 bp vs 177 bp, 63 bp, and 251 bp in the rat and mouse, respec-

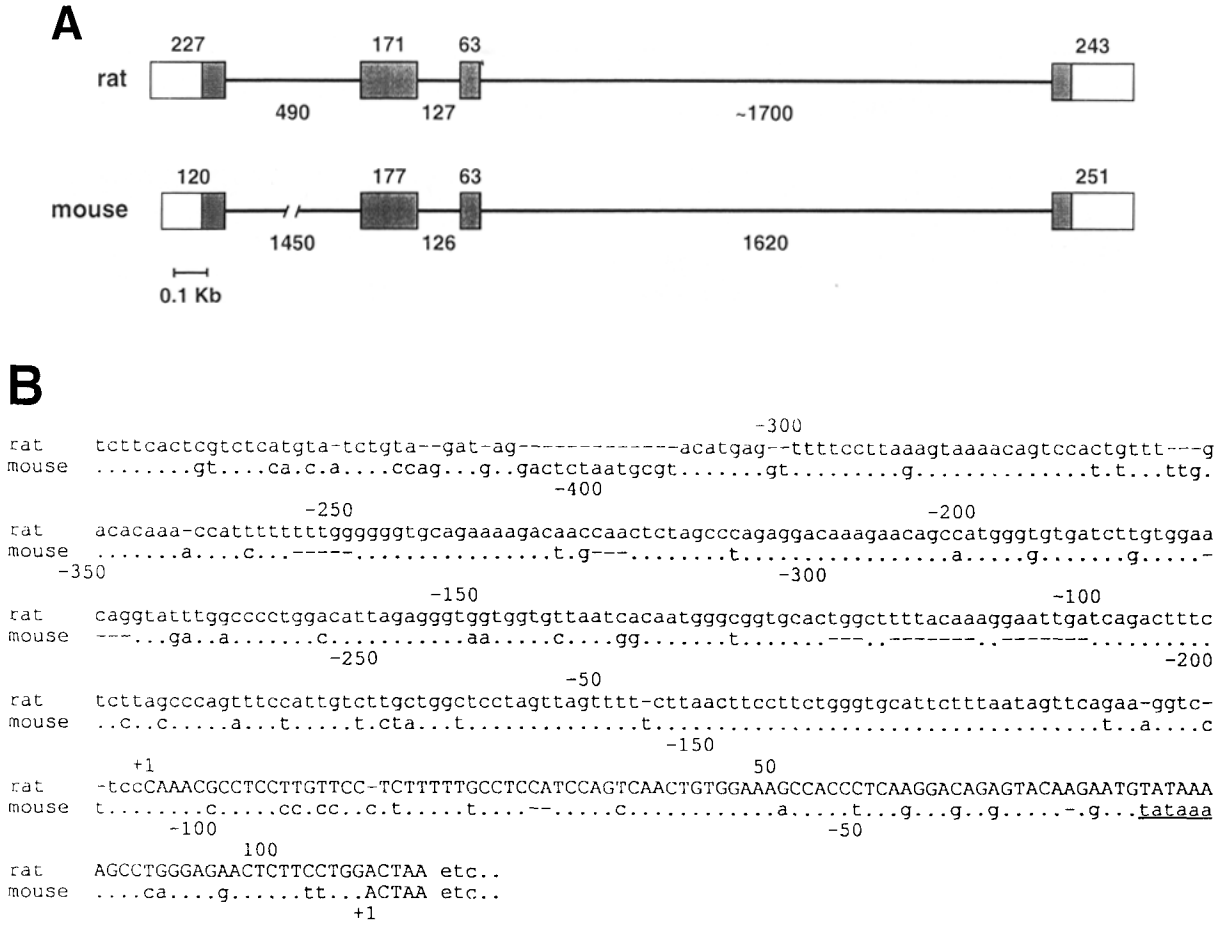

Fig. 4. Comparison of the rat and mouse KAP gene organizations. **(A)** Genomic organization of rat and mouse KAP. The genomic organization and sequence of the mouse KAP gene was described previously (Niu et al., 1991). The 4 exons of the KAP genes are depicted by boxes, with the closed and open boxes referring to the protein-coding region and untranslated sequences, respectively. The lengths of the exons are shown above the boxes and those of the introns below the solid lines. **(B)** Proximal promoters of the rat and mouse KAP genes. Sequence analysis shows 75% identity between the mouse promoter and the corresponding sequences of the rat gene. The coding sequence is indicated in capital letters. Numbering starts at the cap site. Dots indicate identical nucleotides between rat and mouse sequences. The mouse TATAA box is underlined.

tively. Although exons 2 and 4 have slightly different sizes in the two species, the relative positions of the exon/intron junctions are conserved (Fig. 4A). In addition, the first intron in the rat is significantly shorter (490 instead of 1450 bp) than its homologue in the mouse gene. Other intervening sequences have approximatively the same sizes (127 vs 126 for IVS2 and about 1700 bp vs 1620 for IVS3 in the rat and mouse, respectively), resulting in an overall length for the rat gene of about 3000 nucleotides (3807 for the mouse). 347 basepairs of rat proximal 5'-flanking DNA sequence that we have sequenced and the first 107 bp of 5'-untranslated region of rat mRNA have 75% identity with the mouse promoter region (Fig. 4B).

Tissue-Specific Expression of KAP mRNA in the Rat

Northern blot analysis of total RNA from various rat tissues reveals the presence of a single KAP transcript of approx 800 nucleotides, which was detectable only in the kidney and uterus, but not in any other tissue tested (male brain, heart, muscle, bladder, prostate, epididymis, seminal vesicle, testis, small intestine, liver, and female ovaries; data not shown). The relative abundance of the mRNA was two- to threefold higher in male than in female kidneys. This result is consistent with the sexual dimorphism of KAP expression in the renal tissue already described in the mouse (Toole et al., 1979). The relatively high basal level of expression observed in female kidneys is unusual among androgen-regulated genes (Catterall et al., 1986) and was also reported in the mouse (Meseguer et al., 1989), where it has been attributed to an estrogen-dependent expression of the gene in the epithelial cells of the S3 segment of the tubules (Meseguer and Catterall, 1990). Expression of KAP in uterus has also been reported in the mouse (Kasik and Rice, 1993), but occurs at levels comparable to the kidney only in pregnant animals during the period surrounding birth. KAP mRNA is not detectable in the mouse uterus at any other time. In contrast, rat KAP mRNA is present in the uterus of nonpregnant animals at about the same level found in the female kidney.

Hormonal Regulation of Kidney KAP mRNA Levels

Expression of KAP mRNA in both male and female rat kidneys was analyzed by Northern blot following either castration, hypophysectomy, thyroidectomy, or thyroidectomy plus castration, and compared to the levels of expression in control animals. Pools of total RNA from three animals of the same group (lanes 4, 9, and 10) or total RNA from a prototype animal (lanes 1–3, 5–8) were used in Fig. 5 to show a representative profile of the levels of KAP and glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) mRNA under these conditions (panel A), and the relative levels of KAP mRNA measured by phosphor imaging and normalized to the corresponding G3PDH signal (panel B). Castration of male rats decreased KAP mRNA concentration in the kidney to about 30% of intact values (lane 2 vs 1) to a level of expression slightly lower than those observed in female kidneys (lane 6). Ovariectomy, on the other hand, dramatically increased KAP mRNA expression in the female tissue (lane 7 vs 6), implying that some ovarian factors may, in fact, inhibit the expression of KAP either directly or indirectly.

We previously showed that steroid hormones alone are not fully responsible for KAP mRNA expression in mouse kidney and demonstrated the involvement of pituitary hormones by the absence of KAP mRNA in kidneys of hypophysectomized mice (Meseguer and Catterall, 1990). Comparable results were observed in male rats in which KAP mRNA was not detectable after hypophysectomy (lane 3), also suggesting the requirement of pituitary hormone(s) for KAP gene expression in the rat kidney, possibly via regulation of androgen production or directly on the gene transcription. The picture seems more complex for the female since expression of KAP was still detectable after hypophysectomy (lane 8) and expressed at the levels of intact animals as if gonadal factors alone were sufficient to support KAP expression. However, in view of the absence of KAP expression after thyroidectomy (lane 9), the more likely explanation for a residual expression in hypophysectomized females is incomplete surgery.

By analogy to the mouse model, and assuming that the pituitary hormone involved in KAP gene regulation was also TSH in the rat, we examined the levels of KAP mRNA in rats of both sexes after thyroidectomy. Following surgery, animals were treated for 4 wk with methimazole, a drug that suppresses iodination of thyroglobulin, to inhibit any residual production of thyroid hormones. KAP mRNA was virtually undetectable under these conditions in female (lane 9), indicating that basal KAP gene expression is under thyroid hormone control in this sex. Additional ovariectomy did not restore KAP gene expression in thyroidectomized females (lane 10), suggesting that the increased expression observed in ovariectomized rats could be owing to an inhibitory effect of estrogens and/or other ovarian factor(s) on TH induction. In thyroidectomized males, the level of KAP mRNA was significantly decreased but was still expressed at 26% of the level observed in intact males (lane 4 vs lane 1). Effects of androgens on the residual expression of the KAP gene in thyroidectomized male rats is shown in lane 5 where it is further decreased to almost undetectable levels after thyroidectomy plus castration (lane 5).

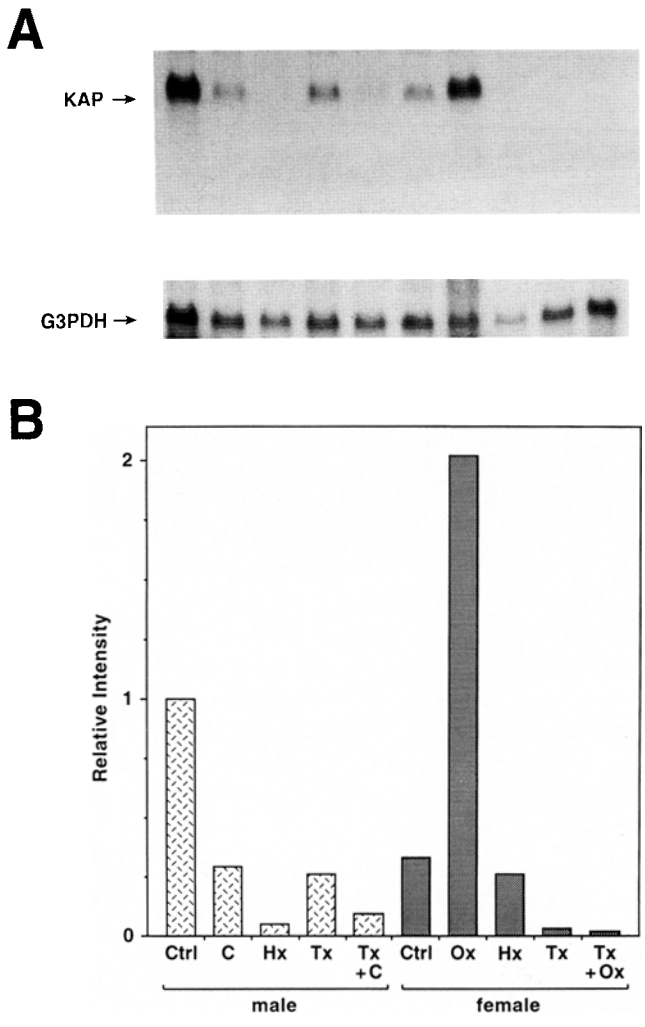

Fig. 5. Hormonal regulation of rat KAP mRNA in kidney. Adult Sprague-Dawley rats were surgically and chemically treated as described in Materials and Methods. **(A)** Representative Northern blot. Total RNA was extracted from renal tissues and subjected to Northern blot analysis (10 μg per lane), using ^{32}P-labeled rat KAP cDNA and human G3PDH probes. Exposure time was 17 h for KAP mRNA and 2 d for G3PDH. 1, control (Ctl) male; 2, castrated (C) male; 3, hypophysectomized (Hx) male; 4, thyroidectomized (Tx) male; 5, castrated plus thyroidectomized (Tx+C) male; 6, control (C) female; 7, ovariectomized (Ox) female; 8, hypophysectomized (Hx) female; 9, thyroidectomized (Tx) female; 10, ovariectomized plus thyroidectomized (Tx+Ox) female. **(B)** Relative levels of KAP mRNA. KAP and G3PDH mRNA levels were measured by phosphorimaging and KAP mRNA values were normalized relative to G3PDH signals. The value of 1 was given to male control kidney.

Regulation of the KAP mRNA Levels in Rat Uterus

In addition to kidney, KAP is also expressed in rodent uterus (unpublished results, Kasik and Rice, 1993; Runic et al., 1996). Uterine hormonal regulation of rat KAP mRNA was studied by Northern blot analysis, following either ovariectomy, hypophysectomy, thyroidectomy, or thyroidectomy plus castration. Pools of total RNA from two or three animals of the same group (lanes 4 and 5) or total RNA from a prototype animal (lanes 1–3, 6–8) were used to obtain the typical Northern blot presented in Fig. 6, which shows that, after ovariectomy, levels of uterine KAP mRNA fall to 30% of those observed in intact females (lane 2 vs 1), as opposed to the kidney where ovariectomy resulted in an increase of KAP mRNA, implying a positive control by estrogens on KAP regulation in the uterus. The remaining uterine KAP gene expression observed after ovariectomy suggested that KAP could also be under multihormonal regulation in this tissue since hypophysectomy further decreased the expression of KAP to about 10% of the control animals (lane 3). Thyroidectomy alone does not have any effect on KAP expression (lane 4), indicating that uterine KAP expression is not under TH control. The additional decrease observed in lane 3, compared to ovariectomy, could then be owing to other sources of estrogens that were suppressed after hypophysectomy or to an influence of TH on estrogen effects since ovariectomy plus thyroidectomy shows a significant additional reduction of mRNA levels when compared to castration alone (lane 5 vs lane 2).

To obtain data concerning the expression of uterine KAP mRNA under different conditions, we collected rat uteri at 10 and 20 d of pregnancy. KAP RNA disappears at d 10 (lane 7) but displays a 3.7-fold increase over the nonpregnant control at the end of gestation (lane 8), suggesting that estrogens, and not progesterone, regulate levels of KAP gene expression in uterus. However, when intact female rats were treated for 8 d with a pharmacological dose of 17 β-estradiol (βE_2), no significant effect was observed on KAP mRNA levels (compare lanes 6 and 1), implying that KAP may be stimulated by a transient acute peak of estrogen as observed at the end of pregnancy, but is unaffected by chronic treatment, perhaps owing to desensitization.

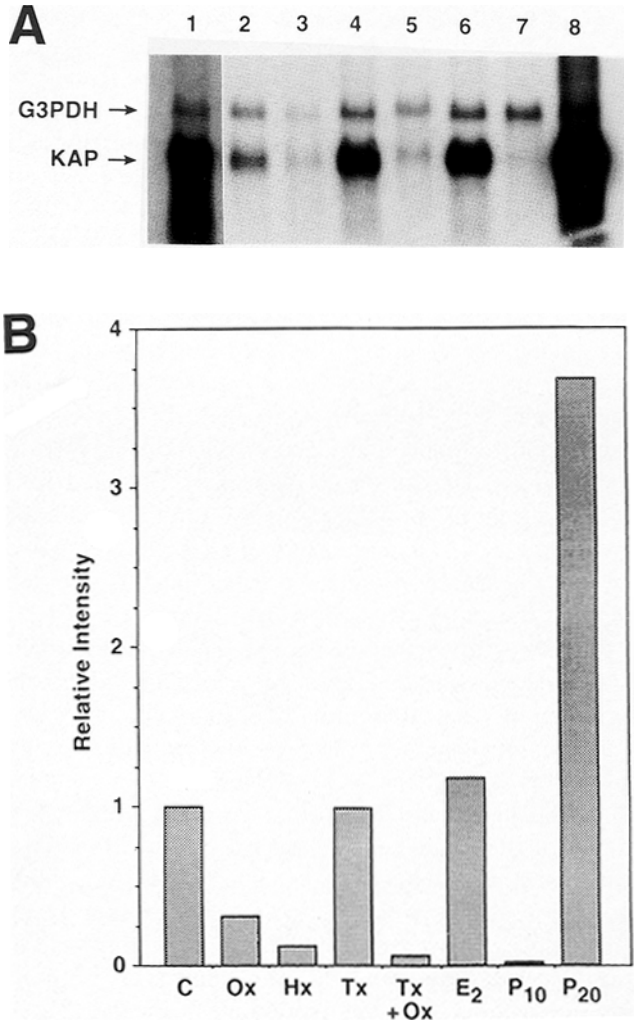

Fig. 6. Hormonal regulation of rat KAP mRNA in uterus. Adult female Sprague-Dawley rats were surgically and chemically treated as described in Materials and Methods. **(A)** Representative Northern blot. Total RNA was extracted from uteri and subjected to Northern blot analysis (10 μg per lane), using ^{32}P-labeled rat KAP cDNA and human G3PDH probes. Exposure time was 60 h except lane 1 which was exposed for 10 d. 1, control (C) uterus; 2, ovariectomized (Ox); 3, hypophysectomized (Hx); 4, thyroidectomized (Tx); 5, ovariectomized plus thyroidectomized (Ox+Tx); 6, Estrogen treated (E2); 7, 10 d pregnant (P10); 8, 20 d pregnant (P20). **(B)** Relative levels of KAP mRNA. KAP and G3PDH mRNA levels were measured by phosphorimaging and KAP mRNA values were normalized relative to G3PDH signals. The value of 1 was given to female control uterus.

Discussion

Although the biological function of KAP is unknown, KAP gene expression has been a useful marker of androgen action in mouse kidney. We and others have shown that the name of the protein is a misnomer since the gene is regulated not only by androgens, but also by estrogens and thyroid hormones (Meseguer and Catterall, 1990; Sol et al., 1994), and KAP was recently found in uterus of mouse during the period surrounding birth (Kasik and Rice, 1993). In mouse kidney, KAP gene is expressed in two different cellular locations, in the epithelial cells of the outer medullary S3 segment of the proximal tubules, where the levels of KAP expression seem to be primarily regulated by estrogens (Meseguer et al., 1989) and thyroid hormones (Sol et al., 1994), and in similar cells of the cortical S1 and S2 segments in the proximal tubule, where the primary response to androgens occurs (Meseguer and Catterall, 1987; Meseguer et al., 1989).

We cloned the cDNA for the rat homologue of mouse KAP, and showed that they have 78% identity in their nucleotide sequences and encode proteins with 80% homology.

The KAP protein has a potential signal peptide that is cleaved during in vitro processing (Fig. 3), suggesting that in vivo it could be either secreted or targeted to the ER or the Golgi apparatus. We do not know if the low translocation efficiency observed in vitro (Fig. 3, lane 1 vs 2) has any relevance with regard to the rate of translocation in vivo; however, at least for mouse KAP, two bands were detected in the kidney by Western blotting, which presumably correspond to the precursor and processed forms of mouse KAP, suggesting a slow translocation rate in vivo as well. The calculated molecular weight of the rat mature protein is mol wt 10,973; however, when translated in vitro, the translation product shows an abnormal migration at mol wt 24,500 after signal peptide cleavage and glycosylation (Fig. 3). Such abnormal migration was also observed for mouse KAP (data not shown). Surprisingly, in vitro, the cleaved but not yet glycosylated form of rat KAP, which was expected to be protected in the presence of microsomes, disappears after proteinase K digestion (Fig. 3, lane 4). In practice, it is rarely possible to achieve 100% protection of translocated peptides, owing to damage or rupture of vesicles during handling; at this time, we have no other explanation for the apparent selective sensitivity of this intermediate form of KAP. Most proteins that transit through the ER are either membrane-associated or assigned for secretion. Secretion of KAP was not detected in mouse urine nor in the media of stably transfected cell lines (data not shown). On the other hand, hydropathicity plots do not show any potential transmembrane domain that could earmark KAP as an integral membrane peptide. However, other types of membrane association could be foreseen (involving protein–protein interactions) that cannot be predicted from the primary amino acid sequence. Alternatively, KAP could be a resident of the ER or Golgi apparatus, although no recognizable retention signal was identified in the predicted amino acid sequence. Further analysis will be performed to distinguish between these possibilities.

In addition to the common features that rat KAP shares with its mouse homolog, it has specific characteristics, including a potential glycosylation site at amino acid 40 and a strong PEST consensus sequence between amino acids 51 and 61 (Fig. 2A). Glycosylation of rat (Fig. 3) but not mouse (data not shown) KAP occurs in vitro, supporting the hypothesis that glycosylation occurs at residue 40 of the native rat sequence. At this position, the mouse sequence encodes for Asn Ser Val instead of Asn Ser Thr. It is unlikely that glycosylation plays a role in the function of the rat protein, as it has not been conserved across species. More interesting is the repartition of homologies along the mature protein sequence, as they are not homogeneously distributed but, to some extent, seem to fall into two "domains" of higher homology (Fig. 2A). From amino acids 26–41 and 81–100, the degree of identity between the two molecules reaches 75 and 70%, respectively. The similarities between these regions are 100 and 90% when conservative amino acid changes are included. The overall sequence identity between the mature peptides of the two species is 48% and the similarity is 79%, both of which are relatively low for such closely related species. These conserved patterns may indicate functional domains of the protein and could help us in the determination of a biological role for KAP.

The genomic organization of KAP is remarkably well conserved between rat and mouse; the high degree of identity also observed in the proximal promoters (Fig. 4B) extends to intervening sequences (data not shown). The rat gene, however, uses an alternative transcription start site that is located 108 bp upstream of the start site in the mouse gene. Interestingly, only one transcript can be detected on Northern blots of rat tissues (Figs. 5 and 6), even though the proximal TATAAA box used in the mouse gene is conserved in the rat 5' untranslated region, indicating that no transcript is made from this more proximal transcription start site in the rat.

Sexual dimorphism is not always identical among rodents. For instance, there is sexual dimorphism of renal ornithine decarboxylase activity in mice but not in rats (Pajunen et al., 1982; Hoang and Bergeron, 1987). The present study clearly confirms a restricted tissue-specific expression of rat KAP mRNA to kidneys and uterus and its regulation by physiologic levels of steroid hormones as we already reported in mice (Meseguer and Catterall, 1987). In addition to the kidney and late pregnant uterus, rat KAP is also expressed in uterus of nonpregnant animals at levels comparable to those observed in kidney. This is not the case in mouse, where uterine expression is detectable only during the last days of pregnancy (Kasik and Rice, 1993).

Removal of the gonads, the most important organs involved in steroidogenesis of sex hormones, did not completely eliminate expression of the renal KAP gene in male (Fig. 5, lane 2), and even induced a significant increase of expression in female (Fig. 5, lane 7), implying that part of KAP expression is steroid independent in this target tissue. We also observed a steroid-independent expression of KAP mRNA in S3 cells of mouse kidney (Meseguer and Catterall, 1990), and looked for a possible effect of pituitary hormones, showing that TSH determined the constitutive expression of the gene (Meseguer and Catterall, 1992). Using congenital hypothyroid mice (hyt/hyt) we have demonstrated that thyroid hormones and not TSH were responsible for KAP expression in S3 cells and that the androgen-dependent cortical response in the male was partially impaired in hypothyroid mice, suggesting cooperation between steroid and thyroid hormones (Sol et al., 1994). Thyroid hormones are pleiotropic hormones that affect the metabolism of almost all body tissues, and have been implicated in many multihormonal regulatory systems where they interact, in combination with other hormones, to control specific cellular responses. More

specifically, they have been implicated in normal maturation of rat testis (Francavilla et al., 1991; Cooke et al., 1992; Hardy et al., 1993) and their loss in hypothyroidism is associated with severe impairment of body and testicular growth (Francavilla et al., 1991). Thyroid hormones are involved in the development and modulation of sexual dimorphism of several tissues in rodents (Buzzell etal., 1989; Ram and Waxman, 1990; Black et al., 1992) and other organisms (Catz et al., 1992). At a molecular level, thyroid hormone (TH) is involved in many multihormonal systems, such as the regulation of α_{2u}-globulin synthesis and mRNA levels in the rat liver, which are modulated by thyroid hormone, androgen, growth hormone, and glucocorticoid, and repressed by estrogen (Kurtz et al., 1976; Roy et al., 1976, 1977). Thyroid hormone receptors have been identified in a variety of rat tissues, and in particular in kidneys where the receptor concentration was among the highest of all tissues examined (Oppenheimer et al., 1974). In addition, kidney and liver contain the highest concentration of mRNA for the type I iodothyronine 5'-deiodinase (5'DI), which converts T4 to T3, the active form of thyroid hormone. These two tissues are considered the most important quantitatively in the peripheral production of T3 from T4 (Berry et al., 1991). 5'DI mRNA is expressed only in the cortex and outer medulla, but not the inner medulla and papilla, and is restricted to the tubular cells of the proximal S3 segment (Lee et al., 1991).

In male rat kidneys, hypophysectomy caused an almost complete inhibition of KAP mRNA synthesis (Fig. 5, lane 3). Castration or thyroidectomy allowed us to dissociate the effects of testosterone and TH, showing that both hormones contribute equally to KAP gene expression (Fig. 5, lanes 2 and 4). Thyroidectomy plus castration virtually abolished KAP gene expression, indicating that testosterone and TH are the only hormones involved in KAP gene regulation of the male rat kidney. In females, basal renal expression is under the exclusive regulation of TH. Indeed, thyroidectomy totally abolished KAP mRNA production (Fig. 5, lane 9), supporting our finding in *hyt/hyt* mice, where the expression of KAP mRNA in S3 cells was shown to be exclusively under thyroid hormone control (Sol et al., 1994). In addition, ovariectomy induced renal KAP gene expression to levels much higher than observed in intact females or males as if an inhibitor of TH action was removed. This hypothesis was further substantiated by the fact that, in ovariectomized plus thyroidectomized females, no KAP gene expression could be detected. The same observations did not stand in the uterus, where thyroidectomy had no effect on KAP gene expression. In this tissue, the main effector appeared to be estrogen, since ovariectomy decreased mRNA level to about 30% of the intact control. However, TH may have a permissive effect on KAP gene expression, since thyroidectomy plus castration results in further inhibition of KAP expression to nearly undetectable levels. During pregnancy, uterine KAP mRNA also responded dramatically to estrogens, both at its peak prior to delivery and in mid-pregnancy, where estrogens are low and KAP expression was undetectable. Surprisingly, a pharmacological dose of estrogen administered for 1 wk did not significantly increase KAP mRNA in nonpregnant rats, suggesting that, in the uterus, continuous estrogen stimulation may produce a desensitization of the KAP gene. Alternatively, cofactors essential for KAP gene activation may be induced at the end of pregnancy.

Our results clearly establish the multihormonal and tissue-specific regulation of KAP gene expression in rats, supporting a generalization of our observations in mice, and enforcing its relevance as a model for studying multihormonal regulation of gene transcription. The specific responsive cells in the rat kidney and uterus of both species are still to be established. In addition, the implications of KAP regulation during pregnancy on parturition remain to be investigated, and may provide important clues to the enigma surrounding its function.

Materials and Methods

Materials

Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN) or New England Biolabs (Beverly, MA). [γ-^{32}P]ATP, ^{32}P-dCTP, and ^{3}H-Leucine were purchased from Amersham (Arlington Heights, IL). Synthetic oligonucleotides were prepared using a Pharmacia (Piscataway, NJ) Gene Assembler automated synthesizer.

DNA labeling

Double-stranded cDNAs for library screening and Northern blot analysis were ^{32}P-labeled using the Pharmacia oligolabeling kit, according to the manufacturer's protocol.

Library Screening

The rat KAP cDNA was cloned from a commercially available cDNA library, prepared from adult male rat kidney (Clonetech, 5'-stretch, cat. no. RL1031a), using a ^{32}P-labeled probe, spanning the entire coding sequence of the mouse KAP cDNA (nucleotides 44 to 409) (Meseguer et al., 1989). Rat genomic clones were isolated from a commercial genomic library (Clonetech, cat. no. RL1005b) using the full ^{32}P-labeled rat cDNA as a probe. Both screenings were performed at 42°C in the presence of 50% formamide. The last washes were done at 42°C in 1X SSC. Phage DNA, prepared from minilysates (Wizard Lambda Preps, Promega, Madison, WI), was utilized for direct sequencing, using the fmol DNA sequencing system (Promega), according to the manufacturer's instructions. Selected clones were subcloned into pGEM3 (Promega) for further analysis.

Primer Extension

Primer extension was performed as described (Niu etal., 1991), using total rat kidney RNA as template.

In Vitro Transcription and Translation

Rat KAP mRNA was synthesized in vitro from a linearized cDNA template cloned in pGEM3, using the T7 Cap-Scribe transcription kit (Boehringer Mannheim). One μg of transcription product was used for cell-free translation using theTranslation Kit, Type I (Boehringer Mannheim) and ^{3}H-Leucine, in the presence or absence of 2 μL of canine pancreatic microsomes (Boehringer Mannheim), essentially as described (Zimmermann and Zimmermann, 1994). Briefly, reactions were carried for 1 h at 30°C and terminated by transfer to 0°C. Posttranslational sequestration assays were performed for 1 h on ice in the presence of 80 m*M* sucrose. When indicated, proteinase K (PK) at 50 μg/mL, Triton X-100 (TX-100) at 0.5%, or both, were added to the reaction. Samples were resolved on 15% SDS-PAGE, fixed, and visualized by fluorography.

Animals and Treatments

Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) at 8 to 10 wk of age. When indicated, surgery (castration, hypophysectomy, or thyroidectomy) was performed by the supplier 8 d before shipment. Thyroidectomized animals were given 0.025% methimazole and 1% calcium gluconate in drinking water for 4 wk. Calcium gluconate was given to maintain calcium balance and methimazole was given to block triiodothyronine (T3) production by residual thyroid tissue.

For estrogen treatment, adult female rats were injected daily with 5 μg β-estradiol (βE_2) for 6 d and were killed on d 7. Each subcutaneous injection consisted of 200 μL of βE_2 in cotton seed oil (25 μg/mL).

All studies involving the use of animals were conducted in accordance with the NIH guidelines for the care and use of laboratory animals using protocols approved by the institutional Animal Care and Use Committee.

Northern Blot Analysis

Mature Sprague-Dawley rats were killed and appropriate tissues excised and rapidly frozen in liquid nitrogen. All tissues were stored at −70°C until RNA isolation. Total RNA from tissues was prepared using the TRI REAGENT isolation system (Molecular Research Center, Cincinnati, OH). Northern blot analysis was performed as described previously (Meseguer and Catterall, 1987). Briefly, 10 or 20 μg of total RNA from each tissue was fractionated on 1% denaturing formaldehyde/agarose gels, transferred onto NYTRAN membranes (Schleicher and Schuell, Keene, NH), crosslinked in a Stratagene UV crosslinker oven, and hybridized with a ^{32}P-labeled rat KAP cDNA probe that extends from nucleotide 158 to 529 (Fig. 1). Equal loading of samples was controlled by ethidium bromide staining of the 18 and 28S RNAs. Autoradiograms were quantified by phosphorimaging and levels of KAP mRNAs normalized relative to glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) signals (probe from Clonetech, cat. no. 9805.1).

Data Analysis

The number of animals analyzed for each experimental condition was variable, and except for hypophysectomized animals, consisted of at least three rats. RNA samples from one or more animals were pooled together in a given Northern blot hybridization, and each sample was run on at least two independent blots in the course of the study. For each blot, the ratio of KAP/G3PDH for the intact male kidney (Fig. 5) or the intact female uterus (Fig. 6) was given the value of 1. All other samples were expressed as a ratio of this control, and were consistent with the representative values given in Figs. 5 and 6.

Acknowledgments

The authors wish to thank Jean Schweis and Evan Read for their assistance in preparation of the manuscript. This work was supported by NIH grant number HD-13541.

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