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Detection of Spatially Regulated Gene Expression by Hybridization Histochemistry

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Steady state measurements of kidney androgen-regulated protein (KAP) mRNA suggested that KAP gene expression was unusually sensitive to low hormone-receptor levels. Two of the criteria used to reach this conclusion involved relative insensitivity to treatment with a competitive antiandrogen and a partial androgen response of the gene in Tfm/Y androgen receptor (AR) deficient mice. These data may indicate the ability of the KAP gene to respond to an extremely low level of androgen-AR complex or that the effect of androgens is, at least in part, indirect. Hybridization *in situ* revealed that KAP mRNA expression was restricted to proximal tubule epithelial cells in the juxtamedullary region of castrated animals rather than throughout the cortex as in intact males. Examination of sections of kidneys from Tfm/Y mice before and after testosterone (T) treatment revealed that only the juxtamedullary tubules expressed KAP mRNA and that T increased the level of KAP mRNA in these cells. Treatment of Tfm/Y animals with other steroids showed that β-estradiol treatment mimicked the effect of T while dihydrotestosterone (DHT) had no effect. The facts that DHT and T both stimulate cortical expression of KAP mRNA in normal animals but DHT has no effect on the juxtamedullary cells in the Tfm/Y variant may indicate that the action of T is due to an estrogenic metabolite. Castrated, hypophysectomized males exhibited no KAP gene expression, while in the presence of T, expression was observed throughout the cortex as in intact males. These results clearly indicate the involvement of pituitary hormones in KAP gene expression in the juxtamedullary tubules. These studies have shown that the regulation of KAP gene expression in the mouse kidney is much more complex than originally believed. Future studies will further investigate the roles of estrogen and specific pituitary hormones in KAP gene expression.

Key words: Androgen, Gene expression, *in situ* hybridization.

Localization of individual mRNAs by *in situ* hybridization is a valuable approach to a broad range of questions in research areas as diverse as gene expression, chromosome structure and development (1, 13, 14). Hybridization histochemistry is a technique developed to detect specific nucleic acid sequences in individual cells. In principle it is identical to immunohistochemistry but a labeled DNA or RNA hybridization probe is

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used to identify the target molecule rather than an antibody. Only immunological techniques and *in situ* hybridization allow identification of individual cells expressing specific proteins or genes, respectively. Localization of a protein is not definitive in regard to its actual site of synthesis (18). Proteins detected in cells may have arrived there other than from the *de novo* synthesis. To determine without doubt in which cell a gene is being expressed, *in situ* hybridization is the method of choice.

When studying levels of expression for a particular gene under various conditions, *in situ* hybridization presents some advantages over classical Northern blot techniques. Besides the advantage of determining the spatial localization of the mRNA, the *in situ* hybridization method is more sensitive (19). In addition, Northern blot analysis requires laborious extraction of RNA from the tissues which frequently results in loss of sequences by degradation.

Studies on the regulation of the expression of two androgen-regulated genes in mouse kidney, those coding for β-glucuronidase (β-gluc) and kidney androgen-regulated protein (KAP) by Northern blot analysis revealed that they were differentially regulated by testosterone (8, 11). The KAP gene appeared to be more sensitive to induction of testosterone (T) by several criteria. First, the KAP gene was maximally induced at physiological doses of T. Second, it was expressed and induced by T treatment in Tfm/Y animals. Third, the KAP gene was less sensitive to the action of a non-steroidal antiandrogen. The differential regulation exerted by testosterone on the expression of these two genes in mouse kidney could be explained by their different sensitivities to the hormone-receptor complex or alternatively, simply due to different cellular sites of synthesis, possibly in cells exposed to different levels of androgen or other nuclear factors required in the transcriptional process. By applying *in situ* hybridization we have localized the site(s) of synthesis of KAP mRNA and studied the spatial expression and multihormonal regulation of the gene (20, 21).

Materials and Methods

Testosterone, 5α-androstan-17β-OL-3-one (DHT), β-estradiol, and Poly-L-lysine homobromide (> 300.000 Mr) were obtained from Sigma. All other reagents were obtained as previously reported (20, 21).

Animals and Hormone Treatment. Mice (strain A/J), testicular feminized (Tfm/Y) variant mice (strain C57BL/6JA$^{W^J}$/A$^{W^J}$-Ta+/+ Tfm/Y) and normal control animals (strain C57BL/6J-A$^{W^J}$) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice (strain BALB/c) control and hypophysectomized animals were obtained from Charles River (Wilmington, MA).

Normal males were castrated under light ether anesthesia and allowed to recover for 8 days before hormone treatment. Hormonal treatment was accomplished by implantation with Silastic rods as previously described (20, 21). All treatments were for 8 days which is sufficient for complete induction of KAP gene expression. Hypophysectomy of BALB/c mice was performed by the Charles River laboratories. Animals were killed by cervical dislocation between 8-10 weeks of age.

Preparation of Tissue Sections and Synthesis of RNA Hybridization Probes. — Kidneys were removed, decapsulated and placed in a cryomold containing O.C.T. compound and frozen in 2-methylbutane previously cooled in liquid nitrogen. Seven-um sections were cut in a cryostat (Bright Instrument Co., Ltd., Hungtinton, UK) at −20 °C and placed on glass slides treated as already described (21).

Glass slides containing kidney sections were kept at −70 °C for no longer than 4 weeks before *in situ* hybridization was performed. Synthesis of RNA hybridization probes has been already described (20).

Formaldehyde Gel Electrophoresis and Northern Blotting. — These procedures were performed as previously reported (20).

In situ *Hybridization.* — Slides kept at −70 °C were allowed to dry at room temperature for 20 minutes and were subsequently fixed in PBS containing 4 % of glutaraldehyde at room temperature for another 20 minutes. After fixation, slides were washed twice in PBS, dehydrated in increasing concentrations of ethanol (30 %, 50 %, 70 % and 100 %) containing 0.3 M ammonium acetate and air dried. Hybridization conditions were reported previously (20).

Autoradiography. — After hybridization, slides were dehydrated, air dried, and exposed to XAR film; exposures were made at −70 °C. After exposure, slides were immersed in Kodak NTB-2 photographic emulsion diluted 1:1 with distilled H_2O at 39-42 °C and air dried at room temperature for 1 hour. Autoradiography was performed at 4 °C in a dry chamber. After appropriate exposure times, the slides were developed in Kodak D-19 developer for 2 minutes and fixed for 5 minutes in Kodak fixative. Sections were dehydrated and examined by light microscopy.

Results

The KAP cDNA insert of plasmid pKAP 17 (16) was isolated and subcloned in the pT7/T3-18 plasmid (BRL, Gaithersburg, MD). The upper part of figure 1 shows the position of the sequences used as hybridization probes. Clones containing both orientations of the cDNA insert were identified so that both sense and antisense RNA strands could be generated using bacteriophage T7 RNA polymerase. Restriction digestion analysis of the resultant clones predicted that the pKAP 3 orientation would generate cRNA while that of pKAP 4 would result in the sense strand to be used as an internal control for *in situ* hybridization experiments.

^{32}P-labeled KAP 3 and KAP 4 RNA probes were hybridized to poly(A) RNA extracted from kidneys of control or T-induced female mice, using Northern blot analysis. Results in figure 1 B confirmed the predicted specificity of both probes. When mouse testis poly (A) RNA was used, neither of the probes resulted in any specific hybridization signal.

The same probes, ^{35}S-labeled, were hybridized to frozen kidney sections from castrated, non-treated control, or T-induced male mice. After hybridization, the slides were exposed without further treatment to x-ray film. Such direct autoradiography constitutes a quick method to assess the background, distribution, and strength of the hybridization signal, prior to application of photographic emulsion and autoradiographic exposures.

Figure 2 shows the direct autoradiographic images of kidneys hybridized with antisense and sense RNA probes. Clearly, a different distribution of KAP mRNA appears to correlate with the T levels of the animals. In the presence of a physiological level of T, KAP mRNA was expressed in the renal cortex. Exogenous T further induced this effect by increasing the signal in the same area while castration limited the expression of KAP mRNA in a very specific area of the kidney. The slides were coated with emulsion, and developed after a few days of exposure. Subsequently, they were counterstained using the PAS reaction procedure. Carbohydrates in the luminal brush border of the proximal convoluted tubules react with the Periodic acid and form a Schiff base.

As a consequence of the reaction, a very strong pink color develops and proximal tubules can be distinguished clearly from distal ones.

When analyzed under the light microscope it appeared that epithelial cells of the proximal convoluted tubules were responsible for the expression of KAP mRNA. Furthermore, it was established that the epithelial cells of tubules lying in the outer stripe of the medulla expressed KAP mRNA in the presence of low circulating

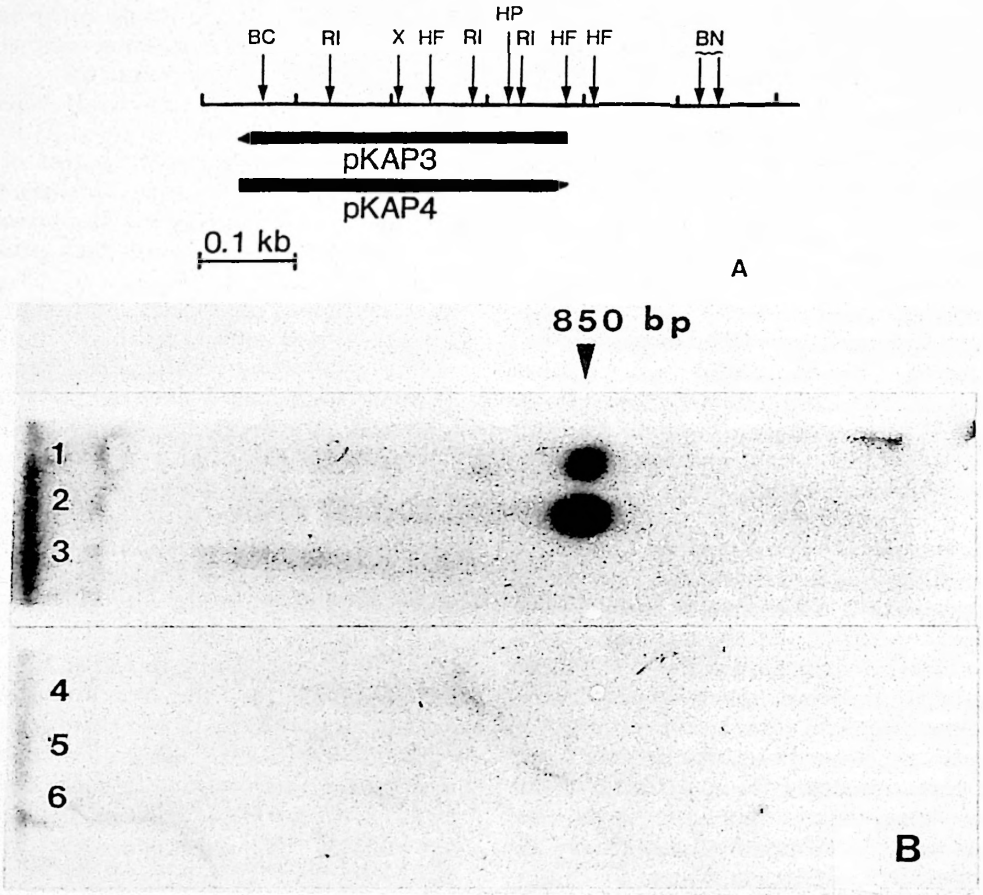


Fig. 1. *KAP cDNA restriction map and RNA probe specificity.*
A) The upper line shows a KAP cDNA restriction enzyme map. The entire KAP cDNA length is shown excluding the poly(A) tail which is responsible for the discrepancy in the length of the cDNA and the mRNA shown below. The heavy bars beneath the cDNA map illustrate the position of transcripts used as hybridization probes for *in situ* hybridization. The arrowhead on each bar indicates the 5'- to 3'-orientation of each transcript and the plasmid template from which each was made is shown. Restriction endonuclease sites included are: BC-BclI, HF-HinfI, HP-HpaII, RI-EcoRI, X-XbaI. b) Northern blot hybridization of kidney and testis poly(A) RNA. Samples (1 μg) of kidney poly(A) RNA from kidneys of untreated female mice (lanes 1 and 4), androgen-treated female mice (lanes 2 and 5), and testicular poly(A) RNA (lanes 3 and 6) were analyzed as described in Materials and Methods. The nitrocellulose bound RNA was hybridized to ^{32}P-labeled pKAP3 transcript (lanes 1-3) or pKAP4 transcript (lanes 4-6). kb, Kilobase; bp, base pair. (From MESEGUER and CATTERALL (20).)

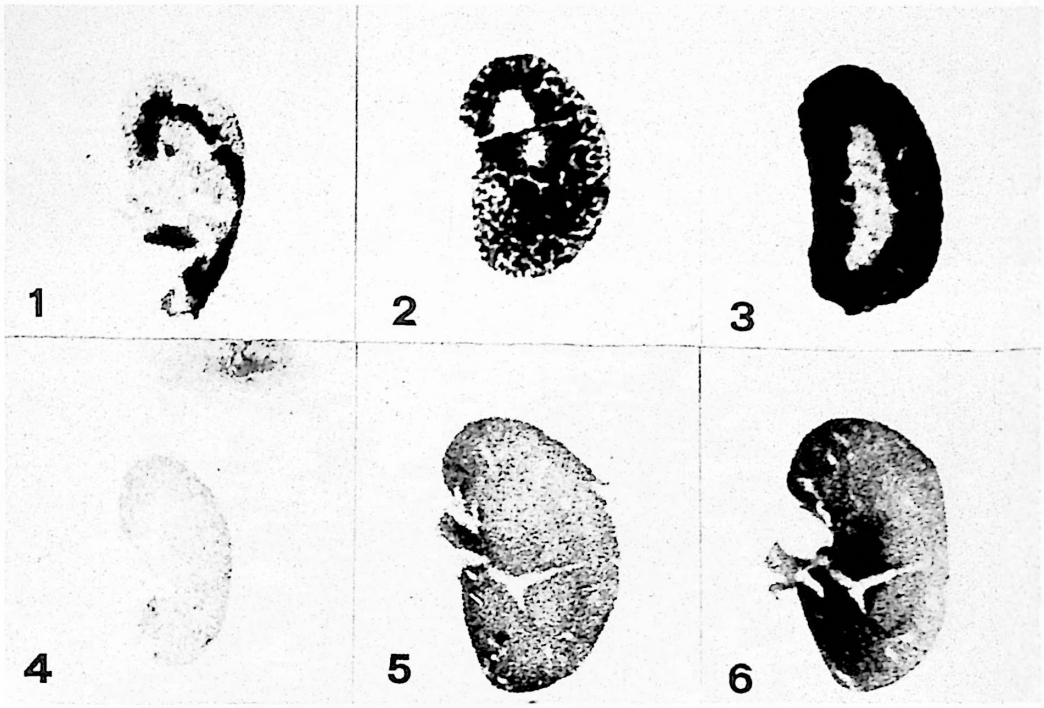


Fig. 2. *Direct autoradiography of kidney sections after* in situ *hybridization.* Kidney sections from castrated male mice (panels 1 and 4), intact males (panels 2 and 5), and testosterone-treated males (panels 3 and 6) were exposed to x-ray film after hybridization with ^{35}S-labeled pKAP3 (panels 1-3) or pKAP4 (panels 4-6) transcripts. (From MESEGUER and CATTERALL (20).)

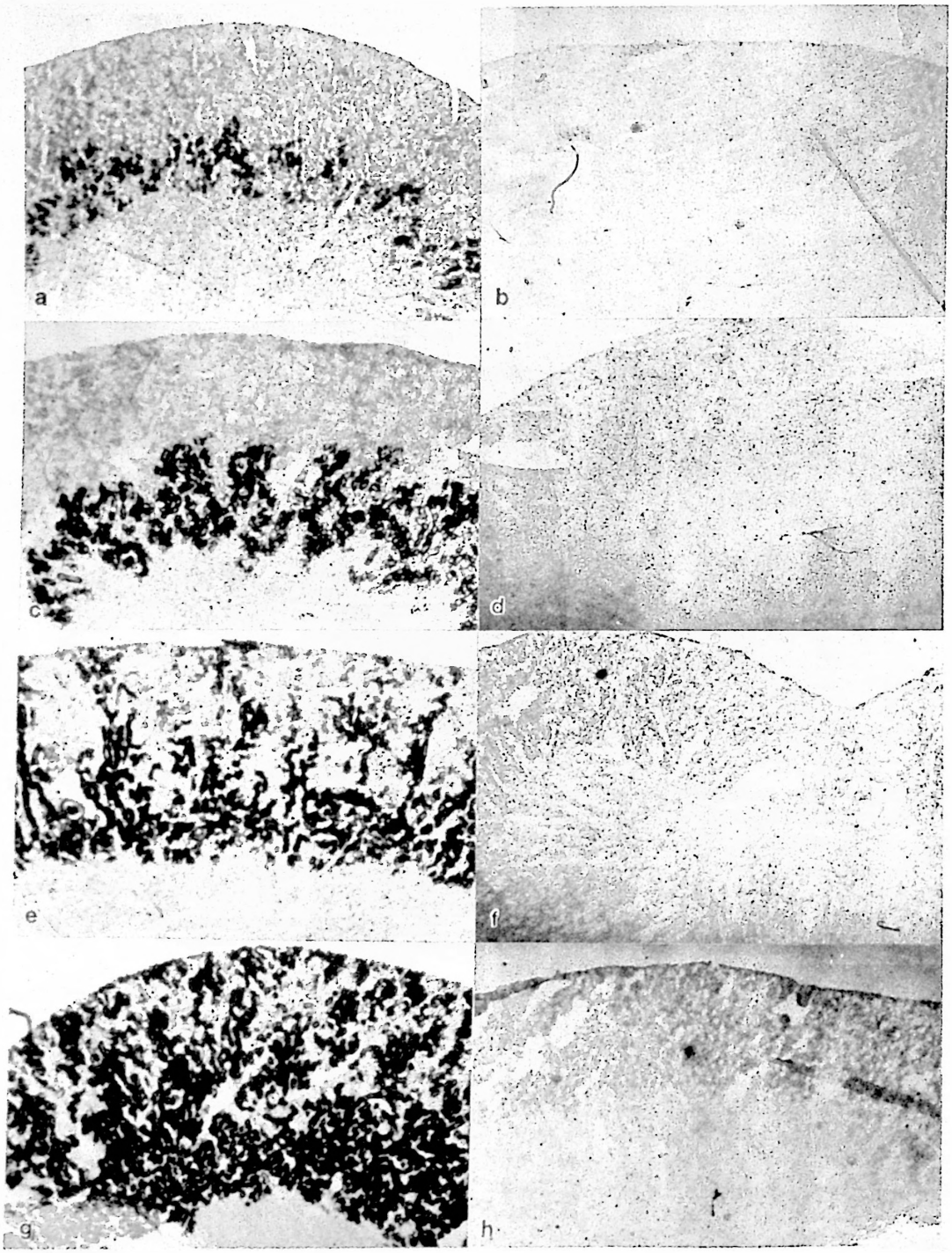
T after castration. These cells, according to the standard nomenclature for structures of the kidney (16) are called S3 cells.

To investigate if the presence of KAP mRNA in S3 cells was due to a higher sensitivity of these cells for the androgen receptor-hormone complexes or was driven by an androgen independent mechanism, KAP gene expression was analyzed in Tfm/Y, androgen receptor-deficient mice. *In situ* hybridization of kidney sections from the Tfm/Y mice showed that S3 cells expressed basal levels of KAP mRNA, which was increased when the animals had been previously exposed to pharmacological doses of T (fig. 3). Figure 3 also shows that the genetically matched castrated control mice, C57BL/6J-A^{WJ}, exhibit increased accumulation of KAP mRNA in proximal tubules of the renal cortex upon T induction.

While androgens are sufficient to fully induce KAP gene expression in cortical tubules of normal castrated males, estrogen (βE_2) affected expression only in the S3 cells (fig. 4).

In Tfm/Y mice the effect of βE_2 on S3 cells was more accentuated than that of testosterone. That the effect of T on S3 cells is mediated by the estrogen receptor after aromatization of T to an estrogenic metabolite is suggested by the lack of any effect of DHT on KAP gene expression in these cells. This non-aromatizable androgen has an identical effect on cortical nephrons as T, but no effect in the S3 cells (fig. 4).

Although estrogen induces KAP gene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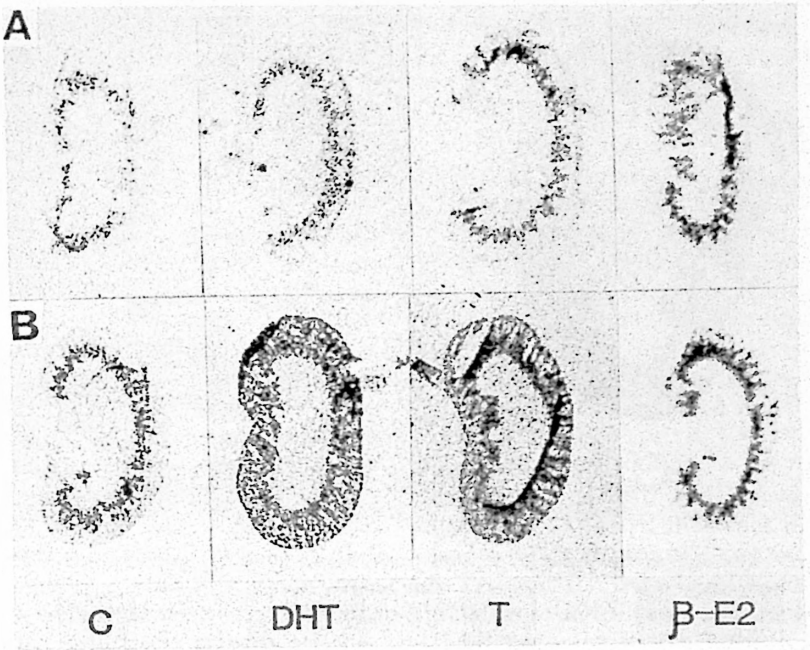

Fig. 4. *Direct autoradiography of kidney sections from Tfm/Y (A) and control (B) castrated male mice (B6/awJ) after* in situ *hybridization.*
Kidney sections from non-treated animals (c) or animals treated with dihydrotestosterone (DHT), testosterone (T), or β-estradiol (β-E_2) were hybridized with ^{35}S-labeled pKAP3 (antisense) probe and exposed to x-ray film.

expression in castrated male mice, ovariectomized females do not exhibit a decline in KAP mRNA in S3 cells and in some cases display a slight increase (unpublished results). This suggested the involvement of other hormones, perhaps of pituitary origin, in the spatial regulation of KAP gene expression.

Fig. 3. *Analysis of KAP gene expression in Tfm/Y Mice by hybridization* in situ.
Kidney sections from untreated (a and b) and testosterone-treated Tfm/Y mice (c and d) and untreated (e and f) and testosterone-treated (g and h) male C57BL/6J-A^{W} control mice were hybridized with ^{35}S-labeled RNA probes. The strand-specific RNA probes contained either the KAP cDNA strand (a, c, e, and g) or mRNA strand (b, d, f, and h) sequences. (From MESEGUER *et al.* (21).)

The effect of hypophysectomy on the expression of KAP gene in S3 cells, is presented in figure 5. In the absence of pituitary hormones, KAP gene expression is totally turned off. The low levels of T due to the absence of LH in the hypophysectomized animals may account for the lack of expression of KAP mRNA in the cortical tubules. The lack of a presently unknown pituitary hormone, or a product controlled by it, is apparently responsible for the disappearance of the signal in S3 cells.

Our ongoing experiments are currently focused on the identification of the putative pituitary agent involved on the regulation of the KAP gene in S3 cells and on the correlation of the various regulatory phenomena with the structure and DNA sequence of the KAP gene.

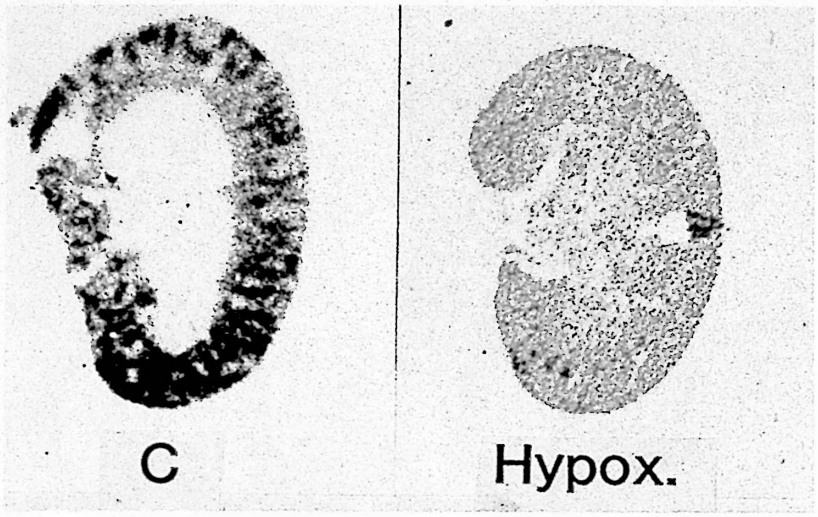

Fig. 5. *Direct autoradiography of kidney sections from control (c) or hypophysectomized (hypox) BALB/c male mice.*
Kidney sections were hybridized with ^{35}S-labeled pKAP3 probe and exposed to x-ray film.

Discussion

Steroid hormone induction of specific eucaryotic genes occurs at the level of gene transcription (4, 5, 27). Hormone receptor complexes act as trans-acting regulatory factors which bind to cis-acting regulatory sequences called steroid response elements (SRE) situated in or near transcription units of steroid-hormone regulated genes (7, 12, 15, 17, 22, 23).

Mouse kidney is profoundly affected by androgenic hormones. Induction of several kidney gene products can be measured following treatment with androgens (2, 3, 6, 25). Comparison of the regulation of the expression of two of these genes, those for β-glucuronidase (β-gluc) (9) and KAP (26), indicated that they are differentially regulated by androgens and that the KAP gene was more sensitive than the β-gluc gene to androgen stimulation (10).

The kidney is a compartmentalized and very complex organ. The possibility that the β-gluc and KAP genes would be expressed in different cell types and consequently be exposed to a different microenvironment was considered. *In situ* hybridization techniques were applied to localize the site of synthesis of KAP and β-gluc mRNAs. Our results demonstrated that both genes were expressed in the same cell type, the epithelial cells of the proximal convoluted tubules (20). Therefore, a gene-specific mechanism may account for the differential expression of these genes in the mouse kidney.

In situ hybridization studies have revealed a level of complexity in the regulation of KAP gene expression that was not detected by analysis of extracted RNA. The unusual pattern of induction is characterized by the expression of the KAP gene in two different areas of the kidney that respond to different stimuli (21). Proximal tubules in the juxtamedullary area (S3 cells) express KAP mRNA in the absence of androgens while in the same cells of cortical nephrons KAP gene expression is clearly androgen dependent.

This constitutive level of expression of KAP mRNA in S3 cells may be responsible for the high basal levels observed in the absence of T. Another feature that distinguishes the KAP gene from other androgen-regulated genes is its expression in Tfm/Y mice.

S3 cells do not respond to androgens directly since DHT does not increase the levels detected in non-treated Tfm/Y animals. Induction of female or castrated, genetically matched normal male mice with androgen resulted in the induction of KAP gene expression in the renal tubules of the cortex. This differential effect of DHT on normal and Tfm/Y mice suggested that estrogens rather than androgens were involved in KAP mRNA expression in these cells. Conversion of T into an estrogenic metabolite by aromatization may be the reason for its action on Tfm/Y mice and in S3 cells in general.

While estrogen treatment of castrated male mice causes an increase in KAP gene expression in S3 cells, ovariectomy does not eliminate this expression in females. In fact, in some strains ovariectomy is associated with a slight increase in KAP mRNA in this region of the kidney. This suggested that estrogen alone could not account for KAP gene regulation in S3 cells. Since glycoprotein hormones of the pituitary have profound effects on gonadal steroid production, we investigated the effect of hypophysectomy on KAP gene expression. Expression is completely abolished in both males and females in the absence of pituitary hormones. Therefore, expression of the KAP gene is not only spatially regulated but also subject to multihormonal control. The particular pituitary hormone responsible for the permissive effect on KAP gene expression presently is not known. Experiments designed to determine its identity employing mice genetically deficient in specific hormones or groups of hormones and reconstitution studies are in progress.

The mechanism of the spatial regulation of the KAP gene is unknown. However, it is possible that there are two KAP genes that are subject to different regulation. There is precedent for gene duplication resulting in differential regulation in the work of STAVENHAGEN and ROBINS (24). These authors have described the androgen regulation of the mouse sex-limited protein (Slp) by the insertion of an upstream element that was identified as the 5' LTR of an ancient provirus. The Slp gene shows 95 % homology in its coding and flanking region with the gene from which it had originated, the fourth component of complement, C4 (24). A similar mechanism could account for the different expression of two KAP genes, one expressed in S3 cells, the other in the cortex under strict androgenic control.

Preliminary data from Southern mapping and DNA sequence analysis of the KAP gene suggest that the KAP gene is unique. However, this has not been rigoroulsy proven. Further structural studies of the KAP gene will allow the correlation of particular sequences with the various regulatory functions associated with this gene.

Resumen

El gen del KAP (kidney androgen-regulated protein) está expresado y regulado por andrógenos en el riñón de ratón. Este gen presenta una gran sensibilidad a niveles bajos de complejo hormona-receptor; no responde a la acción de los antiandrógenos y está expresado en animales Tfm/Y, genéticamente deficientes en el receptor de los andrógenos. La técnica de la hibridización *in situ*, utilizando cortes de riñón y sondas de RNA sintetizado *in vitro*, revela que en ratones castrados, el mRNA del KAP está localizado en los túbulos proximales de la zona yuxtamedular del riñón, mientras que a dosis fisiológicas o farmacológicas de testosterona tal expresión se observaba en todos los túbulos proximales del cortex renal. La hipótesis de que los túbulos de la zona yuxtamedular fueran los responsables de la expresión del gen del KAP en ratones Tfm/Y fue confirmada usando hibridización *in situ*. Se concluye que los túbulos de la zona yuxtamedular son independientes de la ac-

ción de los andrógenos, contrariamente al cortex renal que es andrógeno-dependiente, y que la respuesta del gen del KAP en esa región del riñón depende parcialmente de estrógenos. Asimismo, se observa que en animales hipofisectomizados la expresión del gen del KAP desaparece por completo, indicando que alguna hormona pituitaria debe de estar involucrada en la expresión de la zona yuxtamedular. Actualmente, utilizando ratones hipofisectomizados tratados con distintas hormonas pituitarias y en ratones genéticamente deficientes en esas hormonas se está investigando qué hormonas pueden ser responsables de tal expresión.

Palabras clave: Andrógenos, Expresión génica, Hibridización *in situ*.

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