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Androgen Binding Protein is Tissue-specifically Expressed and Biologically Active in Transgenic Mice

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In view of the inconclusive data concerning the role of androgen-binding protein (ABP) in male reproductive physiology, we thought it would be pertinent to make several transgenic mouse lines overexpressing the rat ABP gene to unravel its role in Sertoli cell and epididymal homeostasis. Heterozygote transgenic mouse lines carrying the 5.5 kb ABP rat genomic DNA were produced by pronuclear microinjection. Northern blot analysis showed overexpression of rat ABP (rABP) mRNA in the testis of transgenic mice compared to rat testis control. rABP was appropriately expressed in Sertoli cells as demonstrated by *in situ* hybridization analysis. Sertoli cell number is increased in the seminiferous tubules of mice overexpressing rABP compared to non-transgenic littermates and scattered Sertoli cells present vacuolated-like cytoplasms, PAS and osmium negative. Compared to the wild type, the transgenic mice exhibited reduced fertility and focal damage in seminiferous epithelium characterized by morphological features compatible with programmed cell death.

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INTRODUCTION

Sertoli cells of the mammalian testis produce an androgen-binding protein (ABP) that binds androgens produced by Leydig cells with high affinity. ABP is secreted into the lumen of seminiferous tubules and transported to the epididymis where it is taken up by the epithelial cells [1–3]. Nevertheless, a small fraction of ABP is simultaneously secreted into the blood plasma [4]. *In vivo* and *in vitro* studies have demonstrated that synthesis and secretion is regulated by androgens and FSH [5, 6]. Rat ABP (rABP) was one of the first Sertoli cell proteins identified and it has been widely used to study Sertoli cell function. It has been proved to be a useful marker of testicular physiology and pathology, including the regulation of Sertoli cell by hormones [7–10].

ABP cDNA was isolated from a rat testis cDNA library [11] and the regulation of its mRNA by testosterone (T) and FSH studied in enriched Sertoli cell cultures [11] and in hypophysectomized rats [12].

ABP is closely related with sex-hormone-binding globulin (SHBG), a liver secreted protein and the major carrier of androgens and estrogens of plasma [12]. It was also demonstrated that both proteins result from an alternative mRNA splicing of a single gene [13, 14].

The function of ABP is still unknown, although several theories have been proposed based on its high affinity for T and dihydrotestosterone (DHT). One of them hypothesizes that ABP regulates the bioavailability of androgens in the extracellular space of the testis and epididymis, regulating spermatogenesis and sperm maturation [8, 15]. ABP may protect androgens from metabolism and/or facilitate the uptake of androgens in the male reproductive tract [16]. It has also been suggested that ABP could act as a hormone or growth factor through its plasma membrane receptor [17]. A recently described sequence homology between extracellular matrix proteins, laminin and merosin, and ABP/SHBG suggest that ABP/SHBG could function as a developmental growth factor [18].

Transgenic mice carrying a genomic DNA clone for rat ABP/SHBG [13] were developed by pronuclear microinjection [19]. The 5.5 kb genomic DNA

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fragment contained the entire coding region of testicular ABP and 1.5 kb upstream of the putative transcription start site [13].

In this communication, we use immunological and molecular biology techniques to study the role of the *cis*-acting regions in ABP/SHBG gene expression and characterize the phenotype of the transgenic mice bearing the rABP gene to contribute to the understanding of the role of ABP in testicular homeostasis and male reproductive physiology.

MATERIALS AND METHODS

Identification of transgenic mice

Transgenic mice carrying the 5.5 kb ABP/SHBG rat genomic DNA [19] were produced as described by Gordon *et al.* [20, 21]. In brief, female CD1 mice (Charles Rivers) were induced to superovulate and were mated with male C57BL/6J × DBA/2J F1 mice (Jackson Labs). Fertilized eggs were flushed from the oviducts and the most accessible pronucleus was microinjected with 200–2000 copies of rat ABP/SHBG DNA. The embryos were introduced into the uterus of pseudopregnant mice and the offspring containing rat ABP DNA were identified by Southern blot hybridization of EcoRI-digested DNA [22] or PCR analysis of tail DNA (Amplification with Taq DNA polymerase was for 30 cycles: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. PCR primers represent exons 1 and 7 [19]. The product amplified was visualized in an agarose gel electrophoresis, transferred to a nylon membrane and hybridized with a ABP probe). The rABP cDNA [12] was used as probe for all hybridization reactions. Founders were bred with C57BL/6J × DBA/2J F1 mice and the offspring analyzed for rABP gene. The mice that did not carry the genomic DNA clone for rat ABP/SHBG were used as negative controls in all the experiments.

Northern blot hybridization

Total RNA from several tissues of adult male ABP transgenic mice was extracted using the Chomczynsky and Sacchi method [23]. 10 μg of RNA were fractionated in a denaturing agarose gel electrophoresis, transferred to a nylon membrane [24] and hybridized with a ^{32}P-labeled rABP cDNA probe [12].

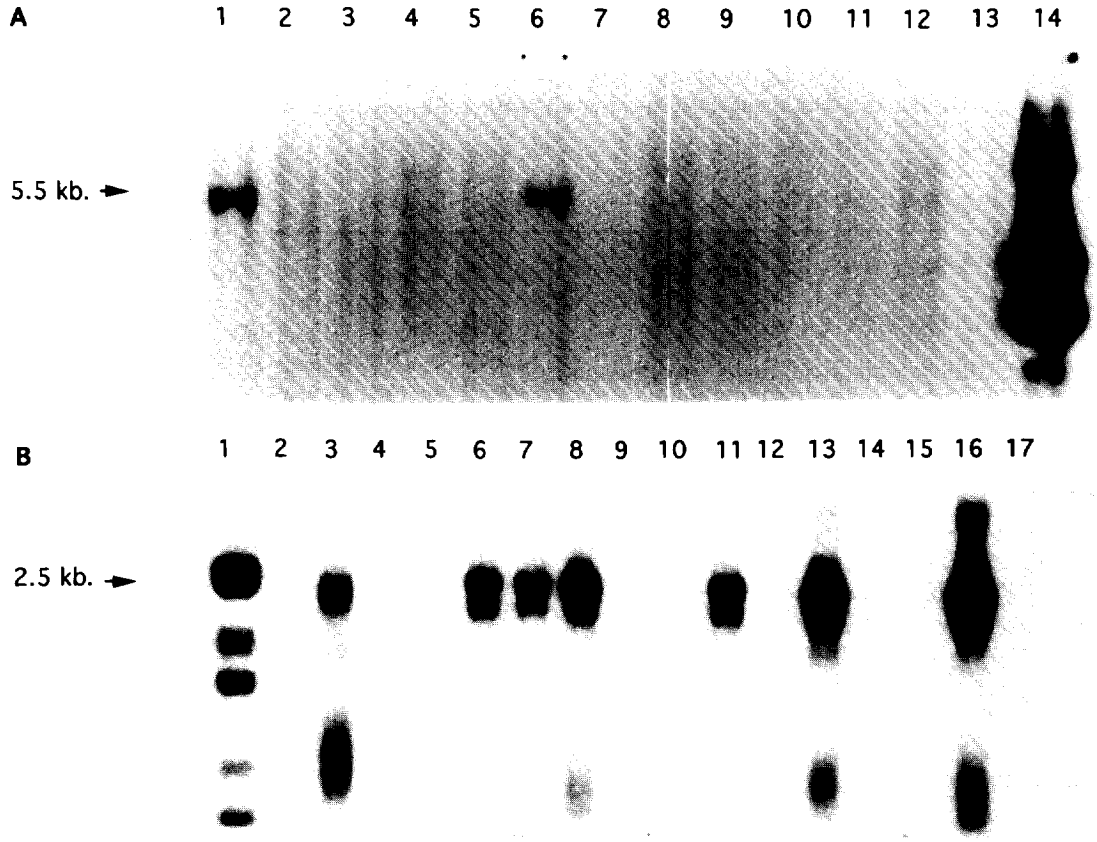

Fig. 1. DNA analysis of ABP transgenic mice. (A) Identification of founders by Southern blot analysis. Spleen DNA (10 μg) from each animal was digested with EcoRI, fractionated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with a ^{32}P-labeled ABP cDNA probe. The bands were visualized by autoradiography. (B) Identification of heterozygote carriers by PCR analysis. Amplification of genomic DNA obtained from the tails of the animals were fractionated by electrophoresis, transferred to a nylon membrane and hybridized with ABP cDNA probe. Lane 1, molecular weight marker DNA (pGEM marker, Promega), lane 2, water as negative control, lane 3, positive control, lanes 6, 7, 8, 11, 13, 16, mice that contained the ABP/SHBG gene, lanes 4, 5, 9, 10, 12, 14, 15, 17, normal littermates.

Hybridization was performed at 42°C in the presence of 50% formamide and denatured salmon sperm DNA [24]. After the final wash in 0.1 × SSPE at 65°C, specific bands were visualized by autoradiography after 3 days of exposure.

In situ hybridization

In situ hybridization experiments were carried out after the mouse testes were removed and frozen in liquid nitrogen. Afterwards, they were cut in cryostatic sections of 3–5 μm. In order to minimize the non-specific binding, the following prehybridization solution was used (per ml): 200 μg of denatured sonicated salmon sperm DNA, 250 μg yeast RNA, 0.2% SDS, 40 mM EDTA, 40 mM piperazine ethane sulfonic acid (Pipes), 40% deionized formamide, 0.5 M NaCl in Denhart's solution. Prehybridization was carried out at 37°C for 2 h.

The hybridization components were the same as prehybridization mixture adding 10% dextran sulfate, 1 mM dATP, 0.01% NaN_3 and 10 ng per sample of labelled probe. Hybridization was carried out with a ^{35}S-labeled rABP cDNA probe (10 ng per slide SA: 5×10^7 cpm/μg). Slides were embedded into photographic emulsion and exposed for 30 days.

Dihydrotestosterone-binding assay in mouse testis

Binding of [^{3}H]DHT in tissue extracts was determined using the dextran-coated charcoal (DCC) method [25]. In brief, 50 μl of testicular extracts of transgenic mouse versus control mouse were incubated with [^{3}H]DHT (2×10^{-9} M) with and without an excess of DHT (4×10^{-7} M) for 24 h at 4°C. The unbound DHT was removed by adding DCC for 2 or 30 min, and the radioactivity in the supernatant fluid was determined by liquid scintillation spectrometry. Specific binding was calculated as previously described [25].

Microscopy analysis

Light microscopy analysis was performed after testis were fixed in Bouin and embedded in paraffin. Testicular parenchyma was cut in cross sections of 3 μm and counter stained in hematoxylin-eosin solutions.

RESULTS AND DISCUSSION

Since its discovery more than 30 years ago, the Sertoli cell product ABP has been extensively used as a marker of the hormone regulation of the above cell type, as well as of the cell-to-cell interactions in the testis of different species [25–27]. The transport and internalization of ABP in the epididymis has also suggested an important role of this protein in sperm maturation during the transit of the latter through the epididymis [8, 28]. Nevertheless, no definitive data have ever defined the specific role of ABP in male reproductive physiology. In view of this lack of information, we have developed several transgenic mouse lines (ABP 1, 7 and 24) bearing the rat genomic ABP/SHBG [19].

The animals analyzed in this report were obtained by crossbreeding of two heterozygous mice of line 24 [19]. The expected fragments of 5.5 kb corresponding to the integrated DNA in head-to-tail tandem arrays were identified by Southern blot hybridization [Fig. 1(A)]. PCR amplification analysis [Fig. 1(B)] was also performed, as was explained in the Materials and Methods section, using primers corresponding to the exons 1 and 7 resulting a major band of 2.5 kb [Fig. 1(B)].

To assess the expression of the transgene, total RNA was extracted from several tissues from transgenic mice and non-transgenic littermates and analyzed as described in Materials and Methods. These experiments

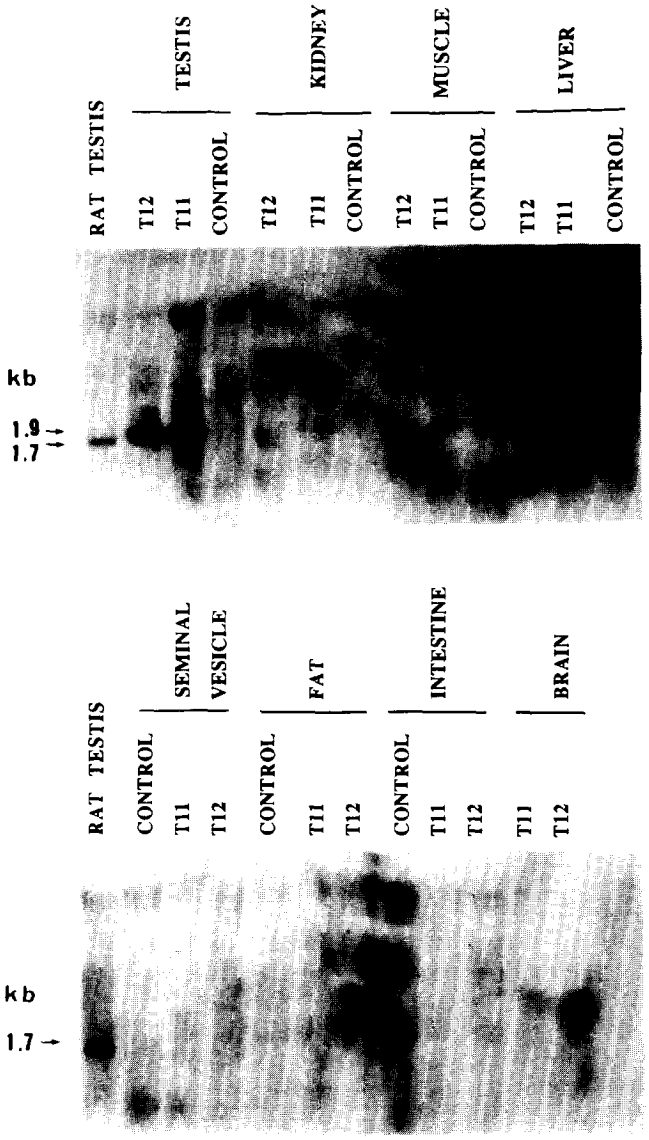

Fig. 2. Tissue distribution of ABP mRNA. Total RNA (10 μg) from several tissues of ABP transgenic mice and their control non-transgenic littermates was analyzed by Northern blot hybridization with ^{32}P-labeled ABP cDNA probe. Autoradiography was exposed for 3 days.

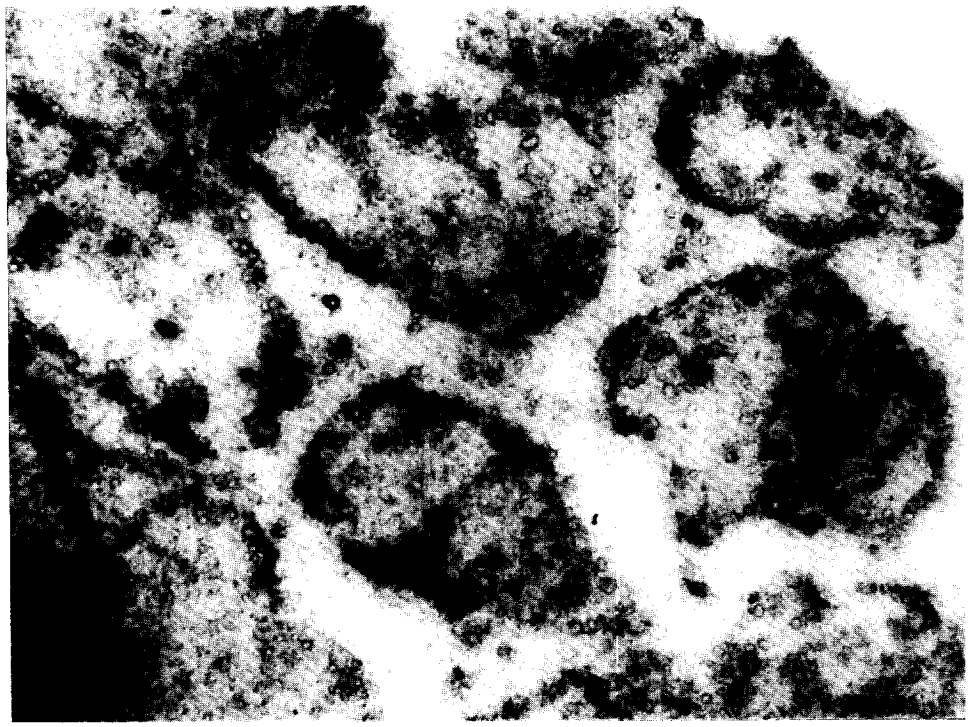

Fig. 3. Detection of rat ABP mRNA on testicular tissue sections of transgenic mice by *in situ* hybridization. Cryostatic sections of mice testis were hybridized with ^{35}S-labeled rABP cDNA probe (10 ng per slide, S.A.: 5×10^7 cpm/μg). Slides were exposed to autoradiography for 30 days. Silver grains are distributed on the edges of the seminiferous tubules where the Sertoli cells are located.

demonstrate a tissue specific expression of rABP in transgenic mice since only the testis contained RNA that hybridized with ABP cDNA (Fig. 2). This ABP mRNA is increased approx. 50-fold when compared to rat testis control (Fig. 2). Nevertheless, rABP mRNA in transgenic mice is processed as a slightly larger band (1.9 kb) when compared to ABP mRNA in control rats (1.7 kb). This may be due to the lack of the full transcription termination sequence in the 5.5 kb DNA which contains 700 bp downstream of the poly(A) addition site [19].

In order to find out which specific cell type was indeed synthesizing rABP in the transgenic testis, we performed *in situ* hybridization analysis using a ^{35}S-labeled rABP cDNA (see Materials and Methods). The data revealed that rABP mRNA sequences were only detected in Sertoli cells of the transgenic mice (Fig. 3). This finding suggests the presence of *cis*-acting promoter/enhancer elements in the genomic DNA injected in the transgenic mice and that these elements direct tissue-specific expression of the gene.

Table 1. Litter size of each ABP transgenic line

Lines	Pups/litter
Control	9
ABP 24	6
ABP 7	3
ABP 1	0

In order to know the biological activity of the overexpressed protein, we analyzed the DHT binding capacity in testicular extracts of transgenic and non-transgenic mice. This experiment revealed a 150-fold increase of DHT binding capacity in transgenic mice compared to controls (800 fmol/mg total protein vs 5 fmol/mg total protein). We conclude from these experiments that the protein is appropriately synthesized and processed since it completely maintains its [^{3}H]DHT binding capacity.

Rat ABP transgenic mice also presented a reduced fertility when compared to wild type C57BL/6J mice (Table 1). ABP 1 transgenic line was interrupted since male founder 1 did not breed successfully. To save this line, sperm from both epididymis of this founder was surgically obtained to perform *in vitro* fertilization which was unsuccessful (data not shown).

Microphotographs of testicular tissue sections of transgenic mice were also prepared and analyzed as described in Materials and Methods. Light microscopy analysis shows important morphological disorders in scattered seminiferous tubules (Fig. 4). These tubules include a general decrease of germ cells in the damaged tubules (right) compared to normal tubules (left). Sertoli cell (arrows) number is increased in the altered tubules when compared to normal (mean of Sertoli cell number per 50 tubule cross sections: control = 6 ± 2.1 vs transgenic = 11 ± 1.8) and are irregularly distributed in the seminiferous tubules. We also noticed the presence of degenerated and modified Sertoli

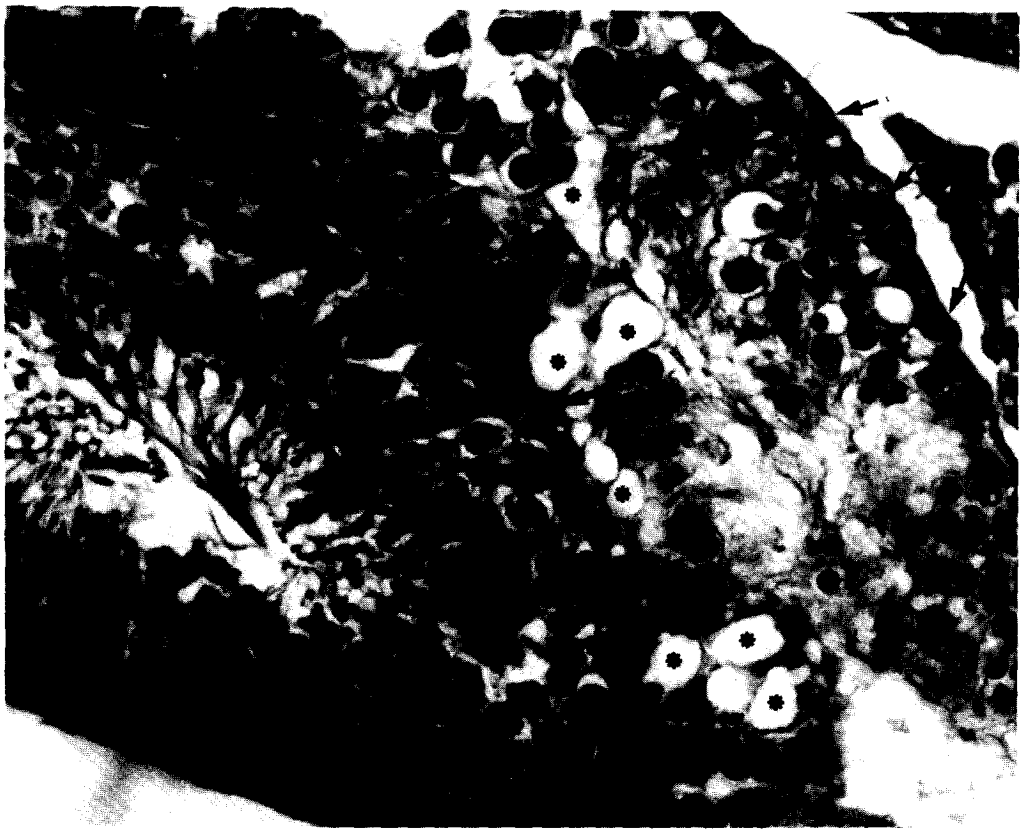

Fig. 4. Light microscopy analysis of a testicular section of an ABP transgenic mouse. Normal (bottom left) and altered (top right) seminiferous tubules of transgenic mice. Abnormal tubules present modified Sertoli cells (arrows) with cytoplasm-associated vacuoles (asterisks).

cells. These modifications are associated with the presence of vacuoles (asterisks) which do not contain carbohydrates or lipids (Pas and Osmium staining negative).

Our results clearly establish the tissue and cell specific expression of the rat ABP gene in transgenic mice. We confirm that a Sertoli cell specific enhancer/promoter is located in the 1.5 kb flanking sequence included in the microinjected rABP genomic clone. Nevertheless, the precise sequence of this enhancer has not been determined yet. We have not been able to identify any consensus homology between any regulatory region of Sertoli cell specific genes and the rABP flanking DNA (data not shown). Furthermore, the microinjected rABP is indeed overexpressed and the protein is biologically active since it maintains its binding capacity.

Testosterone is the principal hormone necessary for insuring normal spermatogenesis, especially the process of testicular spermatid maturation. It has recently been described that the step in spermatogenesis most sensitive to decreased testosterone was the binding of round spermatids to Sertoli cells during mid-spermiogenesis [29]. In hypophysectomized rats, the loss of germ cells is related to a deficient communication between Sertoli cells and the surrounding cells [30].

In our experimental model, there is an incipient degeneration of Sertoli cells with the presence of vacuoles, cytoplasm and organelle disorders. Preliminary results from our laboratory using the terminal-transferase *in situ* labelling assay suggest that Sertoli and germ cells might be undergoing a process of programmed cell death (data not shown). We suggest that the fertility disorders present in the ABP transgenic mice may be due to a permanent excess of rABP in the testis which in turn could reduce the levels of free biologically active androgens. This relative decrease of free androgens would lead to programmed cell death as has been shown by other authors [31].

We conclude that the above modifications in the different transgenic lines bearing the rABP gene indicate that this protein is indeed playing an important role in testicular and epididymal physiology.

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