A 5-kilobase pair promoter fragment of the murine epididymal retinoic acid-binding protein gene drives the tissue-specific, cell-specific, and androgen-regulated expression of a foreign gene in the epididymis of transgenic mice

Jean-Jacques Lareyre, Tania Z. Thomas, Wen-Li Zheng, Susan Kasper, David E. Ong, Marie-Claire Orgebin-Crist, Robert J. Matusik

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 12, Issue of March 19, pp. 8282–8290, 1999

Printed in U.S.A.

A 5-Kilobase Pair Promoter Fragment of the Murine Epididymal Retinoic Acid-binding Protein Gene Drives the Tissue-specific, Cell-specific, and Androgen-regulated Expression of a Foreign Gene in the Epididymis of Transgenic Mice*

(Received for publication, November 6, 1998, and in revised form, December 30, 1998)

Jean-Jacques Lareyre‡§, Tania Z. Thomas¶, Wen-Li Zheng‖, Susan Kasper¶‡‡, David E. Ong‖‡‡, Marie-Claire Orgebin-Crist‡**‡‡§§, and Robert J. Matusik¶**‡‡**

*From the Departments of ‡Obstetrics and Gynecology, ¶Urologic Surgery, ‖Biochemistry, and **Cell Biology, and the ‡‡Center for Reproductive Biology Research, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232-2633*

The murine epididymis synthesizes and secretes a retinoic acid-binding protein (mE-RABP) that belongs to the lipocalin superfamily. The gene encoding mE-RABP is specifically expressed in the mouse mid/distal caput epididymidis under androgen control. In transgenic mice, a 5-kilobase pair (kb) promoter fragment, but not a 0.6-kb fragment, of the mE-RABP gene driving the chloramphenicol acetyltransferase (CAT) reporter gene restricted high level of transgene expression to the caput epididymidis. No transgene expression was detected in any other male or female tissues. Immunolocalization of the CAT protein and *in situ* hybridization of the corresponding CAT mRNA indicated that transgene expression occurred in the principal cells of the mid/distal caput epididymidis, thereby mimicking the spatial endogenous mE-RABP gene expression. Transgene and mE-RABP gene expression was detected from 30 days and progressively increased until 60 days of age. Castration, efferent duct ligation, and hormone replacement studies demonstrated that transgene expression was specifically regulated by androgen but not by any other testicular factors. Altogether, our results demonstrate that the 5-kb promoter fragment of the mE-RABP gene contains all of the information required for the hormonal regulation and the spatial and temporal expression of the mE-RABP gene in the epididymis.

During their transit through the epididymis, spermatozoa undergo biochemical and morphological changes to acquire motility and the ability to fertilize an oocyte *in vivo* (1, 2). The maturation process progresses along the epididymal duct and is believed to be dependent on epididymal secretory proteins.

The epididymis displays a highly region-specific pattern of gene expression. However, little is known regarding the molecular mechanisms that are involved in the regulation of tissue- and region-specific gene expression. Gene expression in the epididymis is mainly under androgen control (for review, see Ref. 3). Indeed, androgen withdrawal, either by orchiectomy or by hypophysectomy, prevents the sperm maturation process (4, 5). However, testicular factors (6), estrogen (7), growth factors (8), and retinoic acid (9) have also been demonstrated to be involved in epididymis-specific gene expression. Whether these endocrine and paracrine signal pathways cooperate to restrict gene expression to a narrow segment of the epididymal duct is unclear.

We previously described two proteins (major and minor forms) that are generated by the differential cleavage of a unique precursor, initially named mouse epididymal protein 10 (10). This protein, recently renamed murine epididymal retinoic acid-binding protein (mE-RABP)[1] (11), is specifically synthesized and secreted by the principal cells of the distal caput epididymidis. The mE-RABP protein binds active retinoids (9-*cis*- and all-*trans*-retinoic acid) but not retinol (12) and is the orthologue of two other retinoic acid-binding proteins described in the rat epididymis. These rat proteins were successively named B/C (13), EBP 1 and EBP 2 (14), E-RABP (15), and ESP I (16). Analysis of the amino acid sequence and putative three-dimensional structure show that mE-RABP belongs to the lipocalin superfamily (11).

The mE-RABP protein is encoded by a single-copy gene localized to the [A3-B] region of mouse chromosome 2, a region rich in genes encoding lipocalin and displaying a similar genomic structure to that of the mE-RABP gene (17).

The mE-RABP gene expression is androgen-regulated *in vivo* (11). In addition, transient transfection studies have shown that a functional androgen-specific response region (ARR) is localized within the first 600 bp of the mE-RABP gene promoter (18). The androgen dependence and the strong tissue- and region-specific expression make the mE-RABP gene a good candidate to study the molecular mechanisms that restrict gene expression to a narrow segment of the epididymis under androgen control. In the absence of appropriate epididymal cell lines, *cis*-DNA regulatory elements involved in the tissue-specific and androgen-regulated expression of the mE-RABP gene can only be identified in transgenic mice.

In the present study, we demonstrate that 5 kb of the 5′-flanking region of the mE-RABP gene can drive the specific expression of the chloramphenicol acetyltransferase (CAT) reporter gene to the principal cells of the mid/distal caput epididymidis, thereby mimicking the expression of the endogenous gene. We also demonstrate that the 5-kb DNA fragment

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.This work was supported by National Institutes of Health Grants HD03820, HD05797, HD36900, and HD25206.

§ Present address: Station Commune de Recherches en Ichtyophysiologie Biodiversité et Environnement, Institut National de la Recherche Agronomique, Campus de Beaulieu, 35042 Rennes Cedex, France.

§§ To whom correspondence should be addressed: Center for Reproductive Biology Research, Vanderbilt University, School of Medicine, Medical Center North, Room D2303, Nashville, TN, 37232-2633. Tel.: 615-322-7484; Fax: 615-343-7797; E-mail: m-c.orgebin-crist@mcmail.vanderbilt.edu.

[1] The abbreviations used are: mE-RABP, murine epididymal retinoic acid-binding protein; ARR, androgen-specific response region; bp, base pair(s); kb, kilobase pair(s); CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

contains most, if not all, of the information required for the temporal and hormonal regulation of the mE-RABP gene *in vivo*.

EXPERIMENTAL PROCEDURES

Animals—All experiments were conducted in accordance with the National Institutes of Health Guidelines for Care and Use of Animals in the Laboratory. Castration or efferent duct ligation was performed by the abdominal route under light methoxyflurane (Mallinckrodt, Mundelein, IL) anesthesia. When required, hormone replacement was begun 30 days after castration with daily subcutaneous injections of testosterone propionate (2 μg/g), β-estradiol-3-benzoate (2 μg/g), hydrocortisone 17-butyrate (2 μg/g) dissolved in sesame oil. Treatments were conducted for 9 days, and mice were killed 1 day after the last injection. Organs were excised, immediately frozen in liquid nitrogen, and stored at −80 °C.

Chimeric Constructs—DNA fragments encompassing the mE-RABP gene promoter were generated from the pHindIII genomic clone (17) using appropriate restriction enzymes. DNA fragments were purified on a 1% (w/v) agarose gel and then ligated into the promoterless pBLCAT3 plasmid (19) using standard methods (20) in such a way that the mE-RABP promoter DNA fragment would drive the CAT reporter gene.

Transgenic Mice—The m0.6Kb-CAT and m5Kb-CAT DNA fragments, containing 0.6 or 5 kb, respectively, of the mE-RABP gene promoter driving the CAT reporter gene were excised from the pUC18 vector by restriction enzyme digest. DNA fragments were purified on a 0.8% (w/v) agarose gel using the Agar*ACE*® enzyme (Promega, Madison, WI). Transgenic mice (strain B6D2; Harlan Sprague-Dawley) were generated by microinjection of the DNA into the male pronucleus of a fertilized oocyte using standard techniques (21). Transgenic animals were identified by PCR-based screening assay using isolated tail DNA. Approximately 1 cm of the tail was digested overnight at 55 °C in a Proteinase K digestion mix (10 mM Tris-Cl, pH 7.5, 75 mM NaCl, 25 mM EDTA, 1% SDS, 0.5 mg/ml Proteinase K). Then DNA was extracted with 1 volume of phenol/chloroform/isoamyl alcohol (25/24/1) and precipitated at room temperature with 2 volumes of absolute ethanol. Samples were centrifuged at 10,000 × *g* at 4 °C for 15 min, washed with 70% ethanol, centrifuged at 10,000 × *g* at 4 °C for 15 min, and dried for 2 h at room temperature. 500 ng of genomic DNA were mixed with 1× PCR buffer II (Perkin Elmer, Foster City, CA), 2 units of *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$, 1 μM concentration of each primer (primer 1, 5′-TGGATGGATAGATGCATACATGAG-3′; primer 2, 5′-CAACGGTGGTATATCCAGTG-3′; casein forward, GATGTGCTCCAGGCTAAAGTT-3′; and casein reverse, AGAAACGGAATGTTGTGGAGT-3′) and 0.2 mM dNTP. DNA fragments were amplified for 30 cycles (95 °C, 1 min; 50 °C, 45 s; 72 °C, 45 s) and one cycle (95 °C, 1 min; 50 °C, 45 s; 72 °C, 10 min). PCR products were analyzed on a 2% (w/v) agarose gel. To monitor CAT activity, organs were homogenized with 20 strokes with a B pestle in a glass Dounce homogenizer in 200 μl of 0.1 M Tris-HCl, 0.1% Triton X-100, pH 7.8. Insoluble material was removed by centrifugation (14,500 × *g*, 15 min, 4 °C), and CAT assays were performed as described previously (22).

Southern Blot of Genomic DNA—Genomic DNA was extracted from the liver of adult male mice as described previously (23). Aliquots (25 μg) were digested with 40 units of *Hin*dIII restriction enzyme (Promega), electrophoresed on a 0.8% (w/v) agarose gel, and then incubated in 0.25 N HCl for 10 min, in 0.5 N NaOH, 1.5 M NaCl for 30 min and twice in 0.5 M Tris-HCl, pH 7.5, 1 mM EDTA, 1.5 M NaCl for 15 min. DNA fragments were transferred overnight to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) by blotting (24). The membrane was baked 2 h at 80 °C and prehybridized for 3 h at 42 °C in 6× SSC, 1% (w/v) SDS, 100 μg/ml salmon sperm DNA, 50% (v/v) formamide, 5% (v/v) dextran sulfate. The random-primed ^{32}P-radiolabeled probe was synthesized using the Rediprime DNA labeling system (Amersham Pharmacia Biotech) and incubated overnight (10^6 cpm/ml). The filter was washed once in 2× SSC for 15 min; once in 2× SSC, 0.1% (w/v) SDS for 15 min; once in 2× SSC, 0.1% (w/v) SDS for 30 min; once in 0.2× SSC, 0.1% (w/v) SDS for 15 min at 65 °C; and once in 0.1× SSC, 0.1% (w/v) SDS for 15 min at 65 °C before being autoradiographed for 0.5–4 days at −80 °C with Hyperfilm MP film (Amersham Pharmacia Biotech). The relative density of the transgenes and endogenous mE-RABP gene was determined using an imaging densitometer (Bio-Rad model GS-670) and Molecular Analyst software.

Immunohistochemistry—Tissues were fixed in 4% paraformaldehyde, 1× PBS (pH 7.4) overnight at room temperature, dehydrated, and embedded in paraplast. Tissues were sectioned at 7 μm and rehydrated. The slides were washed in H_2O for 5 min, and endogenous peroxidase activity was quenched in the presence of 3% H_2O2 for 30 min. Then the slides were washed with 1× PBS for 5 min and incubated with 1% blocking reagent (Boehringer Manheim) for 30 min. Sections were incubated at 4 °C overnight with polyclonal rabbit IgG anti-CAT (5 Prime → 3 Prime, Inc., Boulder, CO) (1:1000). The slides were subsequently washed in 1× PBS, incubated at room temperature for 2 h with anti-rabbit IgG antibody (DAKO, Carpinteria, CA), washed in 1× PBS for 5 min, and incubated at room temperature with rabbit PAP (DAKO) for 1 h. The slides were washed again in 1× PBS for 5 min and equilibrated in 50 mM Tris-Cl, pH 7.5. Peroxidase activity was revealed using the DAKO liquid DAB substrate kit. The staining was monitored under the microscope and stopped in tap water. Sections were dehydrated and mounted with Permount (Fisher) for photography.

Western Blotting—Tissues were homogenized in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl in the presence of protease inhibitors (1 μg/ml leupeptin, 1 μg/ml chymostatin, 1 μg/ml aprotinin, 2 μg/ml antipain, 10 μg/ml benzamidine). Samples were centrifuged at 40,000 × *g* for 30 min and the supernatants were stored at −80 °C before use. 10 μg of total protein were separated by SDS-polyacrylamide gel electrophoresis (17% polyacrylamide gel) and transferred to a Protran™ nitrocellulose membrane as described previously (25). Nitrocellulose membranes were incubated overnight at 4 °C in PBS, 1% (w/v) bovine serum albumin, washed five times in PBS, 0.1% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20, prior to incubation for 1 h with the immune rabbit IgG anti-mE-RABP (10). The membranes were washed five times in PBS, 0.1% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20; incubated for 1 h at room temperature with biotinylated anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA); washed again; and incubated for 1 h at room temperature with ABC-peroxidase reagent (Vector Laboratories). After washing five times in PBS, 0.1% (v/v) Tween 20 and once in 1× PBS, reactive bands were visualized using a solution containing 0.5 mg/ml diaminobenzidine, 0.02% H_2O_2, 0.04% $NiCl_2$ in 0.05 M Tris-HCl, pH 7.5. The reaction was stopped with H_2O, and the membrane was air-dried. The relative density of the mE-RABP bands was determined using densitometry (Bio-Rad model GS-670) and Molecular Analyst software.

In Situ Hybridization—The epididymis was fixed overnight in freshly prepared 4% (v/v) paraformaldehyde/phosphate-buffered saline (pH 7.4), rinsed with PBS, and then dehydrated through a series of increasing concentrations of ethanol for a period of 3–5 h prior to embedding in paraplast. Tissues were sectioned at 7 μm. The CAT reporter gene was excised from the pBLCAT3 vector (19) and ligated into the pGEM7Zf(−) vector (Promega) to obtain the pGEM-CAT construct. CAT RNA probes were transcribed from pGEM-CAT and labeled with [^{35}S]UTP to a specific activity of $1.2 \cdot 10^9$ cpm/μg using the *in vitro* transcription kit MAXIscript™ (Ambion Inc., Austin, TX). Hybridization was carried out at 55 °C with $2 \cdot 10^4$ cpm/ml riboprobe overnight in 50% (v/v) formamide, 300 mM NaCl, 10 mM Tris (pH 7.4), 10 mM NaH_2PO_4 (pH 6.8), 5 mM EDTA (pH 8), 0.2% (w/v) Ficoll 400, 0.2% (w/v) polyvinyl pyrrolidone, 10% (w/v) dextran sulfate, 200 μg/ml yeast transfer RNA, and 50 μM dithiothreitol (DTT). Excess of riboprobe was removed by washing in 2× SSC, 20 mM β-mercaptoethanol for 15 min at 50 °C, once followed by two washes in 4× SSC, 50% (v/v) formamide, 20 mM β-mercaptoethanol for 30 min each at 55 °C, and two washes in 4× SSC, 20 mM Tris-Cl, pH 7.5, 2 mM EDTA for 10 min each at 37 °C. Single-stranded RNA was digested in 4× SSC, 20 mM Tris-Cl, pH 7.5, 2 mM EDTA, and 20 μg/ml RNase A for 30 min at 37 °C. The reaction was stopped by two washes in 4× SSC, 20 mM Tris-Cl, pH 7.5, 2 mM EDTA for 10 min each at 37 °C and two washes in 4× SSC, 50% (v/v) formamide, 20 mM β-mercaptoethanol for 30 min each at 55 °C. Slides were quickly rinsed twice in H_2O and air-dried. Slides were dipped in NTB-2 Kodak emulsion and exposed for 23 days at 4 °C, developed, fixed, and mounted with Permount (Fisher) for photography.

RESULTS

Generation of Transgenic Mice Carrying the CAT Reporter Gene Driven by mE-RABP Gene Promoter DNA Fragments—Two DNA fragments containing either 0.6 or 5 kb of the mE-RABP gene promoter were subcloned from the genomic clone pHindIII (17) and ligated into the promoterless pBLCAT3 vector (19) so that the mE-RABP gene promoter fragments would drive CAT reporter gene expression. The CAT reporter gene was preferred to the *lacZ* reporter gene due to the high endogenous β-galactosidase activity present in the epididymis (26, 27), which cannot be suppressed by the usual modification of

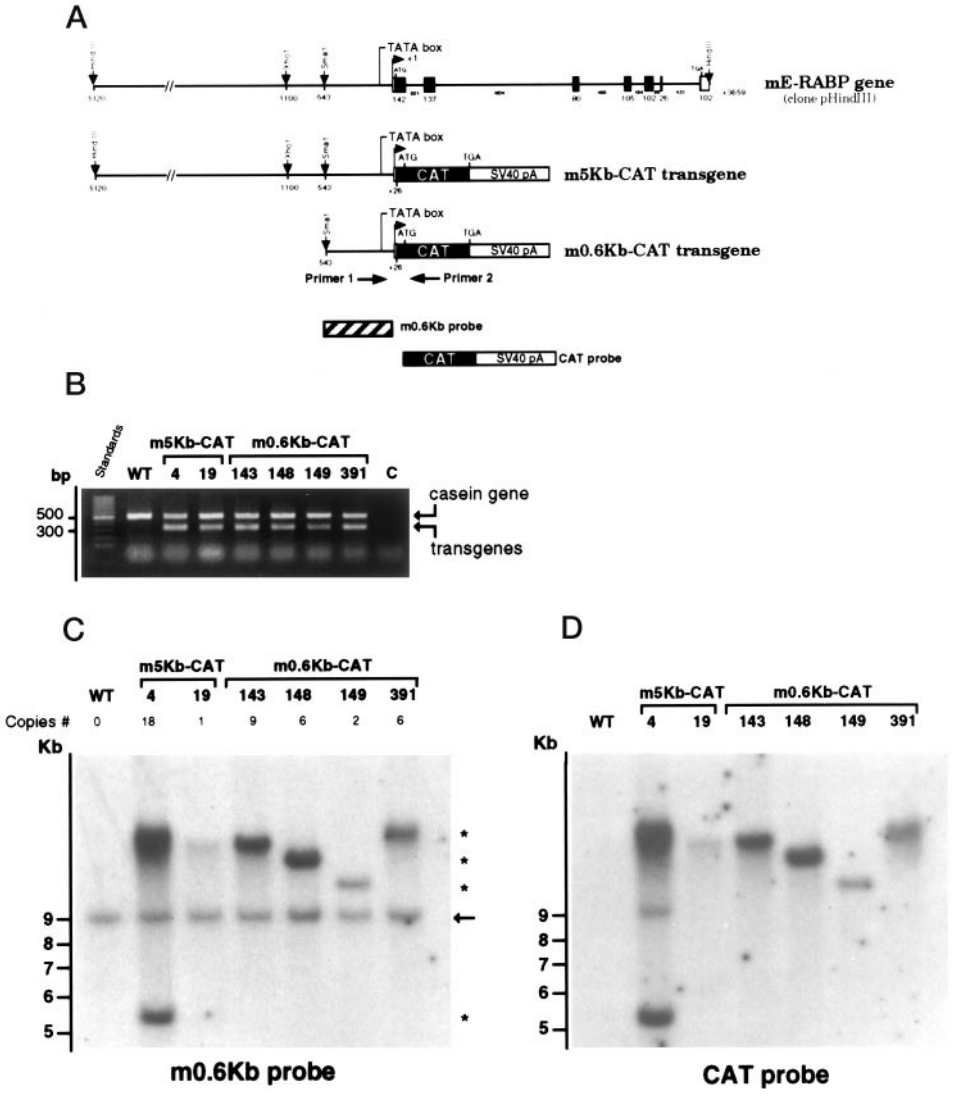


FIG. 1. **Schematic map of the chimeric constructs and characterization of the transgenic mouse lines.** *A*, genomic organization of the mE-RABP gene and schematic maps of the m5Kb-CAT and m0.6Kb-CAT transgenes. Coding and noncoding regions of the mE-RABP gene are represented with *black* and *white boxes*, respectively. The initiation start site is indicated by a *broken arrow*. The size (bp) of the exons and introns is indicated *below*. The nucleotide sequence is numbered according to the major initiation start site of the mE-RABP gene. Positions of the primers used to identify the transgenic mice are represented by *horizontal arrows*. Finally, m0.6Kb and CAT DNA probes used to determine transgene copies number are also illustrated. *B*, characterization of the transgenic mouse lines by PCR of tail DNA. Primers 1 and 2 were used to amplify a 320-bp DNA fragment corresponding to the transgene. The casein forward and casein reverse primers were also used to amplify a DNA fragment (590 bp) of the casein gene as positive control of the PCR. *C*, southern blot analysis of genomic DNA extracted from wild type and transgenic mouse lines. 20 μg of genomic DNA was digested with *Hin*dIII, and resulting DNA fragments were separated on a 0.8% (w/v) agarose gel. DNA fragments were blotted on a nylon membrane and hybridized with the ^{32}P-labeled m0.6Kb probe (*left panel*). Hybridization signals, observed after autoradiography, were quantified using an imaging densitometer (model GS-670, BIORAD). Transgene copy number was determined by calculating the ratio between the intensity of DNA fragments corresponding to transgenes (*asterisks*) and the 9Kb-*Hin*dIII DNA fragment corresponding to the endogenous mE-RABP gene (*arrow*) multiplied by 2. *D*, the identity of DNA bands corresponding to the transgenes was also confirmed using the CAT probe.

the standard protocols of staining for 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (28).

The chimeric genes, named m0.6Kb-CAT and m5Kb-CAT, respectively, were excised from the pUC18 plasmids using the appropriate restriction enzymes (Fig. 1*A*). After purification, the fusion genes were injected into the male pronuclei of B6D2 mouse embryos to generate transgenic mice. Two m5Kb-CAT (one male and one female) and four m0.6Kb-CAT (two males and two females) founder animals were detected by PCR using DNA extracted from the tail and primers 1 and 2 (Fig. 1*B*). All founder animals passed on the transgene to their offspring in a Mendelian fashion. Analysis of the genomic DNA on Southern blot was carried out to confirm random insertion of both transgenes into the mouse genome and to determine the number of transgene copies in each transgenic line (Fig. 1*C*). A probe encompassing the first 600 bp of the mE-RABP gene promoter was used to detect both the endogenous mE-RABP gene as well as the transgenes (Fig. 1*A*). In the nontransgenic control animal (wild type), a 9-kb *Hin*dIII restriction fragment corresponding to the endogenous mE-RABP gene was detected as described previously (17). In addition to the endogenous 9-kb DNA fragment, other DNA fragments of different lengths were detected for each transgenic mouse line, indicating that the transgenes were inserted in a random fashion. Transgene copy number was determined by calculating the ratio between the hybridization signals of the transgenes and the endogenous mE-RABP gene (Fig. 1*C*). The presence of 18 copies and 1 copy of the m5Kb-CAT transgene was detected in mouse lines 4 and 19, respectively. In transgenic mouse lines 143, 148, 149, and 391, 9, 6, 2, and 4 copies of the m0.6Kb-CAT transgene were

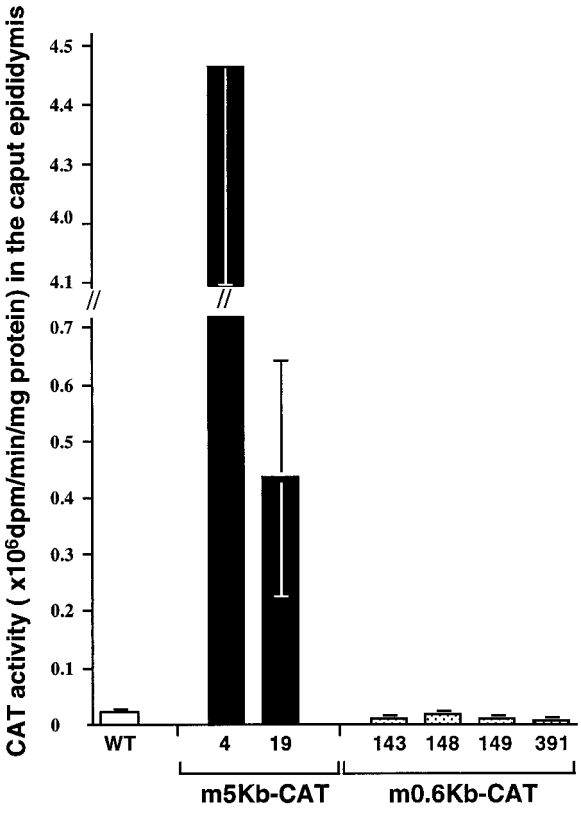


FIG. 2. **m5Kb-CAT and m0.6Kb-CAT transgene expression levels in the caput epididymidis of transgenic mouse lines.** Three adult male mice (60 days old) from each transgenic mouse line and from wild type mice (*WT*) were killed. The caput from each epididymis was dissected and stored individually at −80 °C. CAT activity was determined as described under "Experimental Procedures." To enhance the sensitivity of the CAT assays, different concentrations of total protein were used (line 4, 10 μg; line 19, 100 μg; wild type and lines 143, 148, 149, and 391, 200 μg), but final results are expressed per mg of protein. Values of the CAT activity are presented as the mean ± S.E.

inserted, respectively. The mouse line 4 carrying the m5Kb-CAT transgene exhibited a smaller 5.5-kb band in addition to the full size of the m5Kb-CAT transgene of interest (6.7 kb). The 5.5-kb band probably corresponds to the insertion of a truncated transgene and was not taken into account for determination of copy number. Another probe, specifically designed to detect only the CAT reporter gene (Fig. 1*A*), was also used (Fig. 1*D*). The CAT probe detected the same restriction fragments described above with the exception of the endogenous mE-RABP gene. Only in mouse line 4, a 9-kb restriction fragment was still detectable, suggesting again that a complete or truncated m5Kb-CAT transgene had been inserted at multiple sites.

The 5-kb Promoter Fragment, but Not the 0.6-kb Fragment, of the mE-RABP Gene Targets High Level of Transgene Expression to the Caput Epididymidis—We have previously shown that the mE-RABP gene is specifically expressed in the principal cells of the mid/distal caput epididymidis (11). Therefore, to detect the expression of the m0.6Kb-CAT and m5Kb-CAT transgenes, adult male mice were killed, and the CAT activity was first measured in cell extracts of the caput epididymidis (Fig. 2). When compared with the wild type control animals, no significant CAT activity was detectable in mouse lines 143, 148, 149, and 391 carrying the m0.6Kb-CAT transgene. However, high and moderate levels of CAT activity were found in the caput epididymidis from lines 4 and 19 carrying the m5Kb-CAT transgene. The m5Kb-CAT transgene expression appeared to be correlated with transgene copy number, since line

TABLE I
CAT activity in the epididymis of heterozygous and homozygous m5Kb-CAT transgenic mice

Three adult male mice (60 days old) from B6D2 wild type and m5Kb-CAT transgenic mouse lines were killed. The entire epididymis was dissected and then stored individually at −80 °C. CAT activity was measured as described under "Experimental Procedures." Different amounts of total protein were used to determine the CAT activity in the caput epididymidis as follows: wild type, 200 μg; heterozygous transgenic mice, 10 and 100 μg for line 4 and 19, respectively; homozygous transgenic mice, 5 and 50 μg for line 4 and 19, respectively. Values are presented as the mean ± S.E. Note that CAT activity increases twice on average between heterozygous and homozygous transgenic mice.

	Heterozygous	Homozygous	Increase
	dpm/min/mg protein	*dpm/min/mg protein*	*fold*
Wild type		4446 ± 2560	
Line 4	3772581 ± 151596	6760752 ± 833092	1.8
Line 19	251387 ± 52236	663989 ± 129341	2.6

4 expressed 11-fold greater CAT activity than line 19, and carried the highest copy number (18 *versus* 1, respectively). In addition, homozygous transgenic mice expressed on average a 2-fold higher level of CAT activity than the heterozygous transgenic males (Table I).

The pattern of tissue-specific expression of the m5Kb-CAT and m0.6Kb CAT transgenes was then determined. No CAT activity was detected in the cauda epididymidis and in 12 other tissues, including testis, vas deferens, seminal vesicles, prostate, spleen, kidney, heart, lung, brain, small intestine, liver, and muscle, of adult male mice carrying the m5Kb-CAT transgene (Fig. 3) or the m0.6Kb-CAT transgene (data not shown). In addition, CAT reporter gene expression was undetectable in adult female tissues, including ovary, oviduct, uterus, spleen, kidney, liver, heart, lung, brain, small intestine, and muscle (data not shown). This demonstrates that 5 kb of the 5′-flanking region of the mE-RABP gene is required to target high levels of gene expression to the caput epididymidis.

The m5Kb-CAT Transgene Expression Is Restricted to the Principal Cells of the Mid/Distal Caput Epididymidis—We have previously shown that the mE-RABP gene expression is limited to the caput epididymidis with a highly region- and cell-specific pattern (11). To address the question of whether the m5Kb-CAT transgene contained all of the information required for region- and cell-specific gene expression, *in situ* hybridization of CAT mRNA was carried out (Fig. 4). Serial tissue sections of the epididymis from homozygous m5Kb-CAT transgenic mouse line 4 were incubated either with a CAT antisense or mE-RABP antisense probe. Both CAT (Fig. 4*C*) and mE-RABP mRNA (Fig. 4*E*) were detected only in the principal cells of the mid/distal caput epididymidis. No CAT mRNA was detected in the efferent ducts, the initial segment, the corpus, or cauda epididymidis of m5Kb-CAT transgenic mice. In addition, no hybridization signal was detected in the epididymis of wild type mice using the antisense CAT probe (Fig. 4*A*) or line 4 mice using the sense CAT probe (Fig. 4*B*).

The region- and cell-specific expression of the m5Kb-CAT transgene was further studied by the immunohistochemical localization of the CAT protein (Fig. 5). Sections of the epididymis, from wild type and heterozygous transgenic mouse lines, were incubated in the presence of a rabbit polyclonal antibody directed against CAT (5 Prime → 3 Prime, Inc.). No CAT expression was detected in the efferent ducts and initial segment of the epididymis from heterozygous mouse line 4 (Fig. 5*A*). A faint staining was first detected in the cytoplasm and nucleus of principal cells in segment 2 (Fig. 5*E*) (classification of Abou-Haila and Fain-Maurel (29)). Levels of CAT gene expression progressively increased in segment 3, reaching a maximum in the distal portion of segment 3 and segment 4 (Fig.

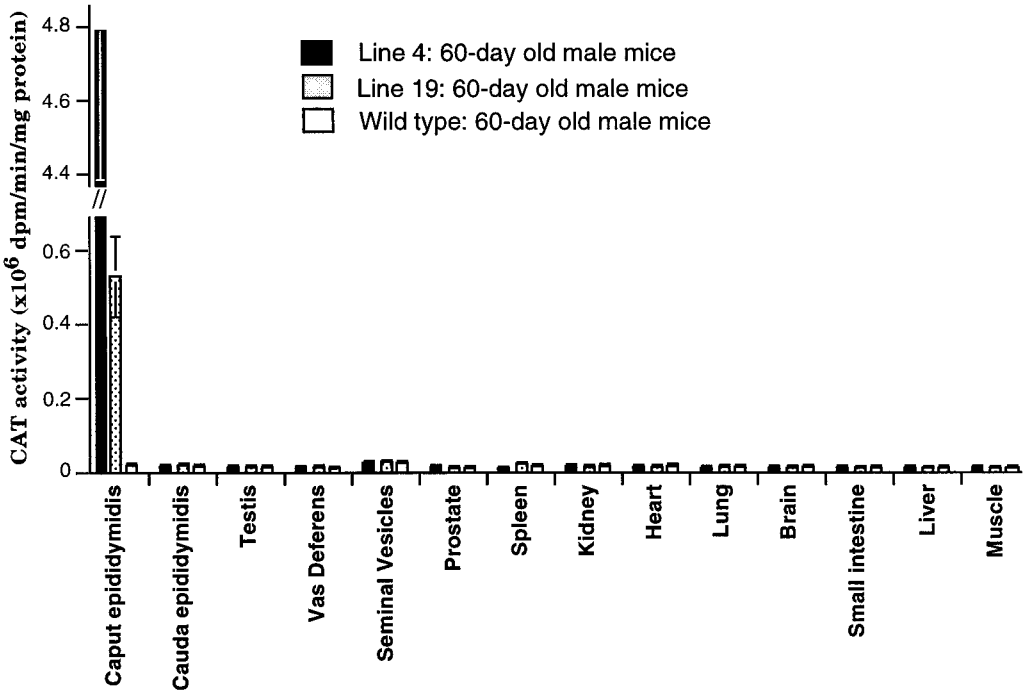


FIG. 3. **Tissue-specific expression of the m5Kb-CAT transgene in the caput epididymidis.** Tissues were dissected from three adult male mice (60 days old) belonging to B6D2 wild type or transgenic mouse lines and then stored individually at −80 °C. CAT activity was measured as described under "Experimental Procedures." 10 and 100 μg of total protein were used to determine the CAT activity in the caput epididymidis of mouse lines 4 and 19, respectively. 200 μg of total protein was used when the CAT activity was assayed in the other tissues. Values are presented as the mean ± S.E. per mg of protein.

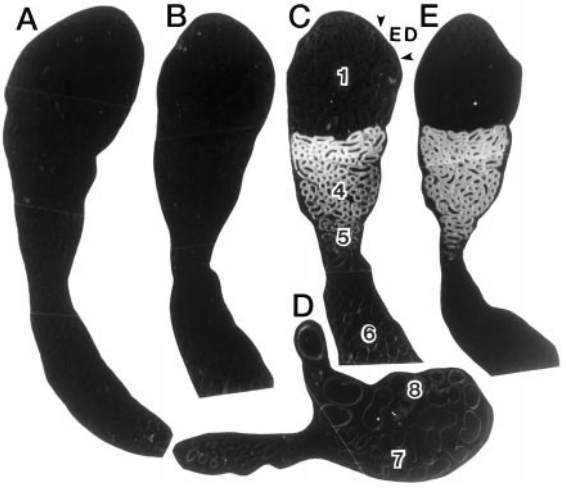


FIG. 4. ***In situ*** **hybridization of the m5Kb-CAT transgene mRNA.** The regions of the epididymis are classified according to Abou-Haila and Fain-Maurel (29). *A*, epididymis from wild type mouse incubated with CAT antisense probe. No CAT mRNA was detected. *B*, epididymis from homozygous mouse line 4 incubated with CAT sense probe. No CAT mRNA was detected. *C* and *D*, epididymis from homozygous mouse line 4 incubated with CAT antisense probe. *ED*, efferent ducts; *1*, initial segment; *4* and *5*, distal caput; *6*, corpus; *7*, proximal cauda; *8*, distal cauda. Note that the plan of section is such that segments 2 and 3 are not visible. High levels of CAT mRNA are detected in the distal caput epididymidis. *E*, epididymis from homozygous mouse line 4 incubated with mE-RABP antisense probe. Note the following: 1) the plan of the section is identical to that treated with the CAT antisense probe, and 2) the localization of the m5Kb-CAT transgene mRNA is identical to that of the endogenous mE-RABP mRNA.

5*F*). Finally, expression decreased in segment 5 and ended with a checkerboard pattern, *i.e.* some principal cells expressed CAT and others did not, at the boundary between the distal caput and the proximal corpus (Fig. 5*G*). No staining was observed in the apical cells, the clear cells, the basal cells of the epithelium, the myoid cells surrounding the tubule, or cells of the connective tissue between the tubules (Fig. 6). No transgene expression was seen in the corpus, the cauda or the vas deferens (data not shown). Thus, the pattern of transgene expression was identical to that described previously for the endogenous mE-RABP gene (11).

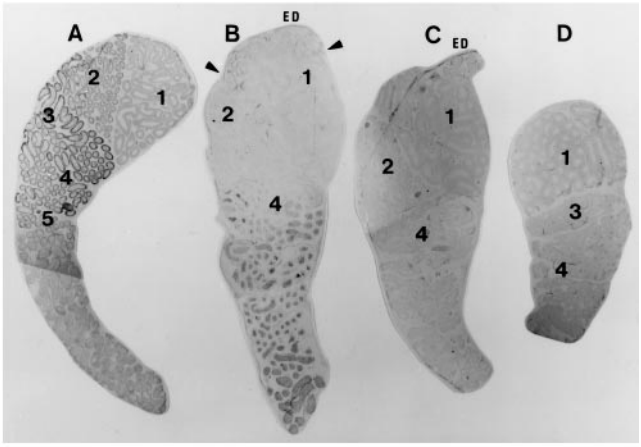
In agreement with our previous results, no CAT staining was detected in the caput epididymidis of wild type mice and transgenic mice carrying the m0.6Kb-CAT transgene (Fig. 5, *C* and *D*). Corpus and cauda epididymidis of wild type or heterozygous mouse line 4 were also negative (not shown).

Altogether, these results demonstrate that the 5-kb promoter fragment contains all of the *cis* DNA regulatory elements required for tissue-, region-, and cell-specific mE-RABP gene expression.

Temporal Expression of the Endogenous mE-RABP Gene and m5Kb-CAT Transgene Is Identical during Postnatal Development—The m5Kb-CAT transgene expression was further studied by monitoring CAT activity in epididymal cell extracts during development (Fig. 7). No CAT activity was found in the epididymis of heterozygous mice from 1 to 20 days of age. The CAT activity was first detected at 30 days and continued to increase until 50 days of age. Finally, a 3-fold increase of CAT activity occurred between day 50 and 60. This temporal expression pattern of the m5Kb-CAT transgene was identical to that of the endogenous mE-RABP gene as demonstrated by Western blot analyses (Fig. 7).

Our results demonstrate that the endogenous mE-RABP gene expression is developmentally regulated and that the 5-kb DNA promoter fragment contains all of the information required for correct temporal expression of the mE-RABP gene.

The m5Kb-CAT Transgene Expression Is Specifically Regulated by Androgens—The appearance of the m5Kb-CAT transgene expression at puberty suggested that androgens might be involved in its regulation. To confirm this hypothesis, adult male mice carrying the m5Kb-CAT transgene were castrated



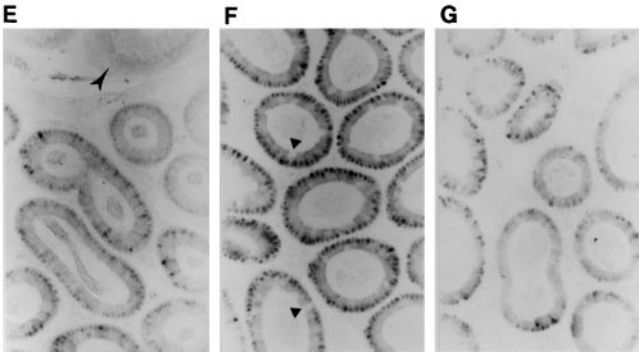


FIG. 5. **Immunolocalization of the CAT and mE-RABP proteins in the m5Kb-CAT transgenic mice.** *A*, the CAT protein is expressed in both the cytoplasm and nuclei of principal cells from segments 2 to 5 of the epididymis in heterozygous m5Kb-CAT transgenic mice (line 4). *E*, note that the principal cells of the initial segment are negative (*large arrow*) and that the intensity of staining increases more distally with some cells displaying a more intense staining (magnification × 125). *F*, principal cells of region 4 are uniformly stained but clear cells (*small arrows*) are negative (magnification × 125). *G*, principal cells in region 5 display a checkerboard pattern with some cells stained and others not stained (magnification × 125). *B*, the mE-RABP protein is secreted and is visible in the lumen of the duct from region 4 to more distal regions. *C* and *D*, immunolocalization of the CAT protein in the epididymis of wild type (*C*) and heterozygous m0.6Kb-CAT transgenic (line 149) mice (*D*). No CAT protein was detected.

for 5, 10, 20, and 30 days (Fig. 8*A*). The m5Kb-CAT transgene expression dramatically decreased during the first 10 days following castration (47% and 5.8% of the intact heterozygous mice, 5 and 10 days after castration, respectively) and then decreased progressively to reach background level 30 days after castration (5.7 and 3.4% of the intact heterozygous mice, 20 and 30 days after castration). Western blot analyses showed that endogenous mE-RABP protein expression appeared to decrease more rapidly after castration than the CAT activity, since the mE-RABP protein was no longer detectable 10 days after castration as reported previously (11).

To study whether transgene expression was regulated by testicular factors present in the luminal fluid, efferent duct ligation was carried out (Fig. 8*B*). No difference of the m5Kb-CAT transgene (90% of the heterozygous intact mice) and endogenous mE-RABP protein expression (98.7% of the intact heterozygous mice) was observed 30 days after efferent duct ligation.

Interestingly, testosterone replacement for 10 days to heterozygous transgenic mice that had been castrated for 30 days restored the expression of the m5Kb-CAT transgene (85% of the intact heterozygous mice) and that of the endogenous mE-

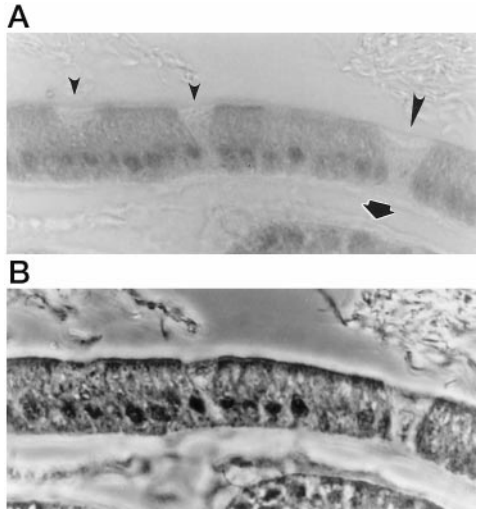


FIG. 6. **Immunolocalization of the CAT protein in the distal caput epididymidis of the m5Kb-CAT transgenic mice.** *A*, principal cells display uniform levels of CAT expression. However, apical cells (*small arrows*), clear cell (*long arrow*), and peritubular cells (basal cells and myoid cells) (*short arrow*) are not stained (magnification × 600). *B*, matched phase contrast photograph (magnification × 600).

RABP protein but to a lesser extent (30% of the intact heterozygous mice) (Fig. 8*B*). Estrogen and glucocorticoid replacement failed to restore m5Kb-CAT transgene and mE-RABP gene expression in heterozygous transgenic mice 30 days postcastration (not shown).

Altogether, our results demonstrate that the expression of the m5Kb-CAT transgene is not dependent on testicular factors present in luminal fluid but is specifically dependent on androgens present in the circulation as described previously for the mE-RABP gene (11).

DISCUSSION

Tissue-, Region-, and Cell-specific Expression of the m5Kb-CAT Transgene—In the present study, we have shown that the 5-kb, but not the 0.6-kb, promoter fragment of the mE-RABP gene was able to direct high levels of CAT reporter gene expression to the epididymis. The m5Kb-CAT transgene expression was not detected in any other tissues examined from male or female transgenic mice, indicating a degree of tissue specificity identical to the native gene. This occurred in the two independent transgenic mouse lines that were established. In contrast, the m0.6Kb-CAT transgene was not expressed in any of the four founder transgenic mouse lines. This strongly suggests that the 5-kb mE-RABP promoter, but not the 0.6-kb fragment, contains all of the information needed for the epididymis-specific expression of the mE-RABP gene.

Numerous studies have reported that promoter fragments are able to direct tissue-specific expression of a reporter gene. For instance, the 0.4-kb promoter fragment of the probasin gene (30, 31) and the 6-kb promoter region of the prostate-specific antigen gene (28) are able to target transgene expression to the mouse prostate. However, gene expression was not correlated with transgene copy number, suggesting that, although these promoter fragments contain tissue-specific *cis*-DNA regulatory elements, they may lack important elements such as matrix attachment regions or locus control regions. Matrix attachment regions are believed to facilitate gene expression and may serve as specific sequence landmarks as they anchor DNA to the nuclear scaffold (32–34). On the other hand, locus control regions are strong enhancers that bind multiple transcription factors that function in a cooperative manner (for review, see Ref. 34). Both of these DNA elements are believed

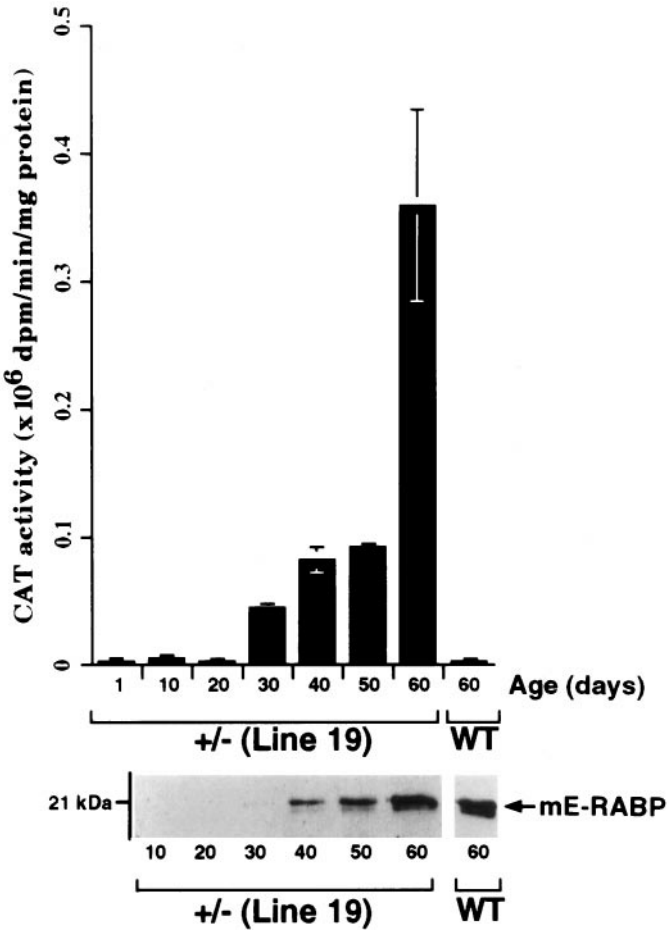


FIG. 7. **Developmental expression of the m5Kb-CAT transgene in the epididymis of mouse line 19.** The CAT activity was determined, as described under "Experimental Procedures," at day 1 from a pool containing 40 epididymides, at day 10 from two pools containing 20 epididymides, and at days 20, 30, 40, 50, and 60 from three animals. 200 μg of total protein was used in CAT assays at days 1, 10, and 20, whereas 100 μg of total protein was used at days 30, 40, 50, and 60. Values are presented as the mean ± S.E. *Lower panel*, Western blot analysis of mE-RABP during postnatal development. Since mE-RABP is a major epididymal secretory protein, only 20 μg of total epididymal proteins was separated on a SDS-polyacrylamide gel electrophoresis (15% polyacrylamide gel) and transferred to a nitrocellulose membrane. The mE-RABP expression was detected using a purified IgG raised against mE-RABP (10).

to ensure a position-independent and copy number-dependent expression of a gene present in their vicinity. Although we generated only two different m5Kb-CAT transgenic mouse lines, transgene expression was independent of site of integration as shown by Southern blot analysis of the genomic DNA (Fig. 1*C*) but was well correlated with transgene copy number. This suggests that matrix attachment regions and/or locus control region-like elements required for the epididymis-specific expression of the mE-RABP gene are contained within 5 kb of the 5′-flanking region.

Within the epididymis, the 5-kb mE-RABP promoter directed CAT gene expression to a narrow region of the epididymis, the mid/distal caput. This mimicked the localization of the expression of the endogenous mE-RABP gene. Region specificity is a prominent feature for gene expression in the epididymis. Although the epididymis is a long convoluted duct lined primarily by the same cell type, a polarized columnar principal cell, genes encoding epididymal proteins display a highly region-specific pattern of expression (for review, see Ref. 3). As examples, the *gpx5* gene is expressed only in the caput epididymidis (35), while expression of the superoxide dismutase gene is restricted to the cauda epididymidis (36). Our results show that all of the *cis*-DNA regulatory elements necessary to confer mid/distal caput-specific expression reside within the 5 kb of the 5′-flanking region of the mE-RABP gene. Therefore, we feel that this 5-kb DNA fragment provides a unique tool to identify region-specific transcription factors that may regulate epididymal genes.

Within the caput epididymidis, the m5Kb-CAT transgene was expressed specifically in the cytoplasm and nuclei of the principal cells as shown by *in situ* hybridization and by immunohistochemistry. No staining was observed in other cell types either in the epithelium or in the connective tissue between the tubules. The nuclear immunolocalization of the CAT protein may be due to a cryptic nuclear localization signal. Indeed, the CAT protein contains the motif KKNK in its primary amino acid sequence, which is homologous to the nuclear localization signal (KKRK) of the DNA helicase Q1 (37). This cell-specific expression of the m5Kb-CAT transgene mimicked that previously reported for the endogenous mE-RABP gene (11). In addition, as with mE-RABP, there was a checkerboard pattern of expression at the proximal and distal borders of the distal caput epididymidis such that some principal cells exhibited strong expression, whereas adjacent principal cells displayed no or very low levels of expression. A similar pattern has also been observed for other epididymal genes (for review, see Ref. 3). These observations imply that the 5-kb mE-RABP promoter contains all of the information necessary for this highly cell-specific gene expression. A single transcription factor can function as a trigger to determine tissue-specific gene expression. For instance, the critical role of the Pit-1 transcription factor in cell differentiation of the pituitary somatotropes, lactotropes, and thyrotropes is well documented (for review, see Ref. 38). However, to achieve cell-specific gene expression, the tissue-specific transcription factor may combine with other regulatory proteins to control gene expression (39, 40). On the basis of a such model, it is reasonable to anticipate that the m5Kb-CAT transgene contains several *cis*-DNA elements that bind ubiquitous, tissue-, and/or cell-specific transcription factors.

The m5Kb-CAT Transgene Expression Is Specifically Regulated by Androgens—Our study clearly demonstrated that the m5Kb-CAT transgene expression was developmentally regulated and mimicked that of the endogenous mE-RABP gene. The m5Kb-CAT gene expression was well correlated with the increase of DHT and androgen receptor content that occurs in the mouse epididymis during development (41, 42). Castration, efferent duct ligation, and hormonal replacement studies confirmed that androgens, but not other testicular factors, were required to maintain m5Kb-CAT transgene expression in adult animals. Moreover, *in vitro* transient transfection assays had shown that the 5-kb promoter fragment of the mE-RABP gene directing the CAT reporter gene was highly androgen-responsive, indicating that androgens may control mE-RABP gene expression at the transcriptional level (18). In addition, only androgens, but not glucocorticoids or estradiol, were able to increase reporter gene expression *in vitro*. Therefore, the m5Kb-CAT transgene expression during development and its androgen-specific regulation are consistent with our previous *in vitro* studies, demonstrating that the 5-kb promoter fragment of the mE-RABP gene confers androgen-specific responsiveness and that androgens may act directly at the transcriptional level to modulate mE-RABP gene expression *in vivo*.

We have demonstrated in transient transfection studies using HeLa cells that a functional ARR was localized within the first 600 bp of the mE-RABP gene promoter (18). A similar androgen response region was also identified within the 400-bp

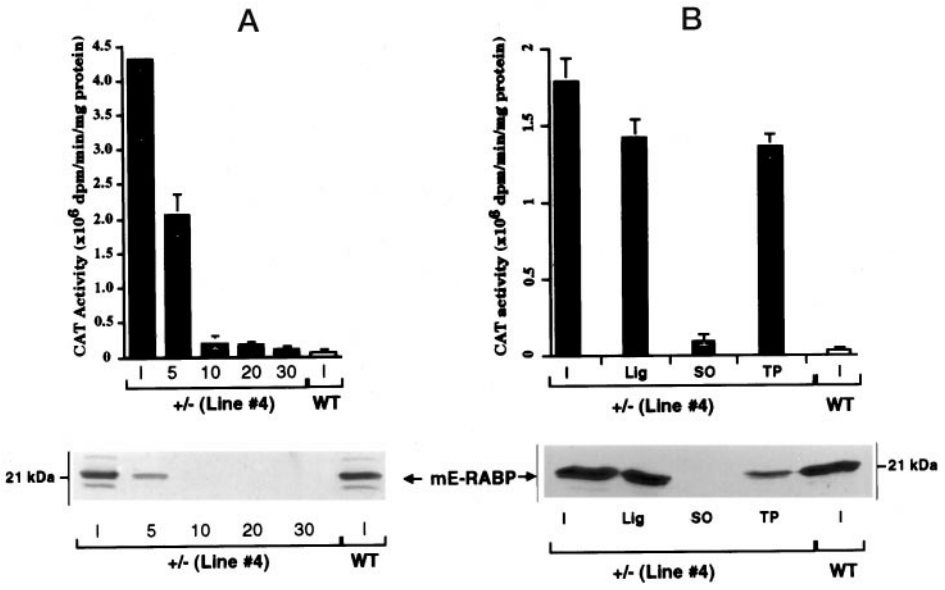


FIG. 8. **Hormonal regulation of the m5Kb-CAT transgene in the epididymis of mouse line 4.** *A*, m5Kb-CAT transgene expression after castration. CAT activity was determined in the epididymis of intact (*I*) and 5-, 10-, 20-, and 30 day-castrated heterozygous (+/−) adult male mice (*Line 4*) and compared with that of intact wild type adult male mice (*WT*). Values are presented as the mean ± S.E. of three individual animals. *B*, m5Kb-CAT expression after efferent duct ligation or androgen replacement. Three 30-day castrated adult heterozygous male mice were injected with heptylate testosterone (testosterone propionate (*TP*); 200 μg/day) or sesame oil (*SO*; 100 μl/day) for 10 days. Three mice had their efferent ducts ligated for 30 days (*Lig*). *Lower panel*, Western blot analysis of mE-RABP expression in intact (*I*), castrated (5, 10, 20, and 30 days postcastration), and 30-day castrated male mice supplemented with testosterone heptylate (testosterone propionate; 200 μg/day for 10 days) or sesame oil (100 μl/day for 10 days). Total epididymal proteins (10 μg/lane) were separated on a SDS-polyacrylamide gel electrophoresis (15% polyacrylamide gel) and transferred to a nitrocellulose membrane. The mE-RABP expression was detected using a purified IgG raised against mE-RABP (10).

promoter region of the probasin gene, which encodes another androgen-regulated lipocalin in the rat prostate (43). In transgenic mice, this short 400-bp promoter fragment was sufficient 1) to restrict gene expression to the prostate and 2) to confer androgen control (30, 31). In the present study, the m0.6Kb-CAT transgene was not sufficient to direct detectable levels of the CAT reporter gene expression in four independent transgenic mouse lines. A similar result has been reported for the human prostate-specific antigen gene encoding an androgen-regulated prostatic secretory protein and belonging to the kallikrein gene family. The first 600 bp of the prostate-specific antigen gene promoter did not target gene expression to the prostate, although two distinct functional androgen receptor binding sites that cooperate were localized within the first 600 bp of the prostate-specific antigen gene promoter (44). However, a 6-kb promoter fragment containing an upstream enhancer was able to place reporter gene expression under androgen control in the prostate of transgenic mice (28). Therefore, it is probable that the ARR localized within the first 600 bp of the mE-RABP gene promoter requires an upstream enhancer present in the m5Kb-CAT transgene to drive gene expression *in vivo*. Further deletions between 0.6 and 5 kb upstream from the transcription initiation site of the mE-RABP gene will be required to identify important cis-DNA elements required for promoter activity *in vivo*. This enhancer may bind to tissue-specific transcription factors, but our results do not exclude the possibility that the 600-bp promoter fragment of the mE-RABP gene may also be required for androgen-regulated and tissue-specific gene expression.

Concluding Remarks—Despite the fact that the epididymis is the site of an important physiological event, the maturation of the male gamete, little is known about the molecular mechanisms regulating its function. The highly region-specific pattern of gene expression observed in the epididymis may be required to coordinate the functions of the different epididymal regions to ensure the efficient post-testicular maturation of spermatozoa. In the present study, we demonstrate that the 5-kb promoter fragment of the mE-RABP gene restricts high levels of gene expression to the principal cells of the mid/distal caput epididymidis in transgenic mice. To our knowledge, this is the first demonstration of targeted expression by an epididymal gene promoter. It is now possible to further dissect and identify the cis-DNA regulatory elements and their associated proteins (steroid receptor coactivators, tissue- and/or cell-specific transcription factors) that are involved in the regulation of the tissue region- and cell-specific expression of epididymal genes.

The identification of an androgen-regulated and epididymis-specific gene promoter is not only relevant to identifying individual regions of the gene involved in cell specificity and hormonal regulation but also enables one to study the function of epididymal secretory proteins that are involved in the sperm maturation process. It is possible to disrupt gene function by targeted mutation ("knockout"), but it is also possible to use the antisense RNA or protein engineering technologies as alternative strategies to inhibit gene function. Due to the high level of gene expression achieved with the m5Kb-CAT transgene, the mE-RABP gene promoter will be an appropriate tool to overexpress an antisense-ribozyme RNA or a dominant negative protein to inhibit the expression and/or the function of target proteins *in vivo*.

Acknowledgments—We gratefully acknowledge the Vanderbilt Transgenic/ES cell Shared Resource for generating the transgenic mouse lines. We thank Drs. C. Pettepher and J. Wright for helpful advice throughout the course of these studies. We thank Dr. B. J. Danzo for critical comments on the manuscript. The DNA sequencing was performed by the Cancer Center DNA Sequencing Core, directed by Dr. K. Bhat.

REFERENCES

1. Bedford, J. M. (1967) *J. Exp. Zool.* **166,** 271–281
2. Orgebin-Crist, M. C. (1967) *Nature* **216,** 816–818
3. Orgebin-Crist, M. C. (1996) in *Pharmacology, Biology, and Clinical Applications of Androgens* (Bhasin, D., Gabelnick, H. L., Spieler, J. M., Swerdloff,

R. S., and Wang, C., eds) pp. 27–38, Wiley-Liss Inc., New York
4. Orgebin-Crist, M. C., and Tichenor, P. L. (1973) *Nature* **245,** 328–329
5. Dyson, A. L., and Orgebin-Crist, M. C. (1973) *Endocrinology* **93,** 391–402
6. Douglass, J., Garrett, S. H., and Garrett, J. E. (1991) *Ann. N. Y. Acad. Sci.* **637,** 384–398
7. Toney, T. W., and Danzo, B. J. (1989) *Endocrinology* **125,** 243–249
8. Lan, Z. J., Labus, J. C., and Hinton, B. T. (1998) *Biol. Reprod.* **58,** 197–206
9. Astraudo, C., Lefevre, A., Boue, F., Durr, F., and Finaz, C. (1995) *Arch. Androl.* **35,** 247–259
10. Rankin, T. L., Tsuruta, K. J., Holland, M. K., Griswold, M. D., and Orgebin-Crist, M. C. (1992) *Biol. Reprod.* **46,** 747–766
11. Lareyre, J. J., Zheng, W. L., Zhao, G. Q., Kasper, S., Newcomer, M. E., Matusik, R. J., Ong, D. E., and Orgebin-Crist, M. C. (1998) *Endocrinology* **139,** 2971–2981
12. Rankin, T. L., Ong, D. E., and Orgebin-Crist, M. C. (1992) *Biol. Reprod.* **46,** 767–771
13. Brooks, D. E., Means, A. R., Wright, E. J., Singh, S. P., and Tiver, K. K. (1986) *J. Biol. Chem.* **261,** 4956–4961
14. Ong, D. E., and Chytil, F. (1988) *Arch. Biochem. Biophys.* **267,** 474–478
15. Newcomer, M. E., and Ong, D. E. (1990) *J. Biol. Chem.* **265,** 12876–12879
16. Girotti, M., Jones, R., Emery, D. C., Chia, W., and Hall, L. (1992) *Biochem. J.* **281,** 203–210
17. Lareyre, J. J., Mattei, M.-G., Kasper, S., Ong, D. E., Matusik, R. J., and Orgebin-Crist, M.-C. (1998) *Mol. Reprod. Dev.* **50,** 387–395
18. Lareyre, J. J., Kasper, S., Ong, D. E., Matusik, R. J., and Orgebin-Crist, M. C. (1998) *Xth European Workshop on Molecular and Cellular Endocrinology of the Testis, Capri, Italy, March 28 to April 1, 1998* (Abstr. E14)
19. Luckow, B., and Schutz, G. (1987) *Nucleic Acids Res.* **15,** 5490
20. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) *Molecular cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Palmiter, R. D., and Brinster, R. L. (1985) *Cell* **41,** 343–345
22. Nachtigal, M. W., Nickel, B. E., Klassen, M. E., Zhang, W. G., Eberhardt, N. L., and Cattini, P. A. (1989) *Nucleic Acids Res.* **17,** 4327–4337
23. Blin, N., and Stafford, D. W. (1976) *Nucleic Acids Res.* **3,** 2303–2308
24. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5201–5204
25. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
26. Skudlarek, M. D., and Orgebin-Crist, M. C. (1986) *Biol. Reprod.* **35,** 167–178
27. Skudlarek, M. D., Tulsiani, D. R., and Orgebin-Crist, M. C. (1992) *Biochem. J.* **286,** 907–914
28. Cleutjens, K. B., van der Korput, H. A., Ehren-van Eekelen, C. C., Sikes, R. A., Fasciana, C., Chung, L. W., and Trapman, J. (1997) *Mol. Endocrinol.* **11,** 1256–1265
29. Abou-Haila, A., and Fain-Maurel, M. A. (1984) *Anat. Rec.* **209,** 197–208
30. Greenberg, N. M., DeMayo, F. J., Sheppard, P. C., Barrios, R., Lebovitz, R., Finegold, M., Angelopoulou, R., Dodd, J. G., Duckworth, M. L., Rosen, J. M., and Matusik, R. J. (1994) *Mol. Endocrinol.* **8,** 230–239
31. Greenberg, N. M., DeMayo, F., Finegold, M. J., Medina, D., Tilley, W. D., Aspinall, J. O., Cunha, G. R., Donjacour, A. A., Matusik, R. J., and Rosen, J. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3439–3443
32. Renz, A., and Fackelmayer, F. O. (1996) *Nucleic Acids Res.* **24,** 843–849
33. Gohring, F., and Fackelmayer, F. O. (1997) *Biochemistry* **36,** 8276–8283
34. Oesterreich, S., Lee, A. V., Sullivan, T. M., Samuel, S. K., Davie, J. R., and Fuqua, S. A. (1997) *J. Cell. Biochem.* **67,** 275–286
35. Faure, J., Ghyselinck, N. B., Jimenez, C., and Dufaure, J. P. (1991) *Biol. Reprod.* **44,** 13–22
36. Perry, A. C., Jones, R., and Hall, L. (1993) *Biochem. J.* **293,** 21–25
37. Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., Enomoto, T., and Yoneda, Y. (1997) *J. Biol. Chem.* **272,** 26375–26381
38. Rhodes, S. J., DiMattia, G. E., and Rosenfeld, M. G. (1994) *Curr. Opin. Genet. Dev.* **4,** 709–717
39. Gordon, D. F., Lewis, S. R., Haugen, B. R., James, R. A., McDermott, M. T., Wood, W. M., and Ridgway, E. C. (1997) *J. Biol. Chem.* **272,** 24339–24347
40. Tremblay, J. J., Lanctot, C., and Drouin, J. (1998) *Mol. Endocrinol.* **12,** 428–441
41. Gallon, C., Veyssiere, G., Berger, M., Jean-Faucher, C., De Turckheim, M., and Jean, C. (1989) *J. Androl.* **10,** 188–194
42. Jean-Faucher, C., Berger, M., de Turckheim, M., Veyssiere, G., and Jean, C. (1985) *Int. J. Androl.* **8,** 44–57
43. Rennie, P. S., Bruchovsky, N., Leco, K. J., Sheppard, P. C., McQueen, S. A., Cheng, H., Snoek, R., Hamel, A., Bock, M. E., MacDonald, B. S., Nickel, B. E., Chang, C., Liao, S., Cattini, P. A., and Matusik, R. J. (1993) *Mol. Endocrinol.* **7,** 23–36
44. Cleutjens, K. B., van Eekelen, C. C., van der Korput, H. A., Brinkman, A. O., and Trapman, J. (1996) *J. Biol. Chem.* **271,** 6379–6388