Ecto-ATPase mRNA Is Regulated by FSH in Sertoli Cells

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ABSTRACT: A putative messenger RNA (mRNA) sequence, designated C8, that was up-regulated in Sertoli cells prepared from hypophysectomized rats treated with testosterone, was isolated from a Sertoli cell complementary DNA (cDNA) library. The coding region of C8 exhibited 99% identity with rat brain ecto-ATPase and expressed a 60-kilodalton protein following in vitro transcription/translation. Transfection of COS7 cells with C8 cDNA resulted in a marked increase in Ca^{2+}- and Mg^{2+}-dependent ATPase activity in both whole cells and cell homogenates, which is consistent with localization of this enzyme in the plasma membrane. C8 ecto-ATPase steady state mRNA levels were increased within 6 hours and for 3 days by follicle-stimulating hormone (FSH) in Sertoli cells but not in peritubular cells. In contrast, dibutyryl-cyclic adenosine monophosphate (cAMP) increased ecto-ATPase in both Sertoli and peritubular cells. Testosterone had no significant effect under these conditions. These data indicate that ecto-ATPase mRNA is positively regulated by FSH in Sertoli cells and by cAMP in both Sertoli and peritubular cells. This enzyme may play a role in the control of extracellular signaling by ATP, adenosine, or both in the cells of the seminiferous epithelium.

Key words: Testosterone, cDNA, testis, purine.

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The pituitary hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are required for quantitative maintenance of normal spermatogenesis; hypophysectomy results in regression of the seminiferous epithelium (Steinberger, 1971; Sharpe, 1994). The Sertoli cell plays a key role in coordinating a number of aspects of testicular function (Griswold, 1998; Lu et al, 1999). FSH stimulates both Sertoli cell division and differentiated function via specific receptors coupled to the cyclic adenosine monophosphate (cAMP) second-messenger pathway (Sharpe, 1994). The effects of testosterone, produced in response to LH, on spermatogenesis are probably mediated by androgen receptors in Sertoli and peritubular cells (Ku et al, 1994; Sanborn et al, 1994; Sharpe, 1994).

The absence of androgen in vivo elevates Sertoli cell androgen messenger RNA (mRNA) and this is reversed by testosterone treatment (Sanborn et al, 1991). Cytoplasmic androgen receptor concentrations are decreased following hypophysectomy, but increased by testosterone in vivo and in vitro (Sanborn et al, 1984; Verhoeven and Cailleau, 1988). However, in culture, relatively few qualitative effects of testosterone on Sertoli cell proteins or mRNA have been observed (for a review, see Sanborn et al, 1994).

We report here the isolation and characterization of a rat Sertoli cell complementary DNA (cDNA) clone coding for an mRNA that is up-regulated in cells prepared from hypophysectomized rats treated with testosterone. This clone has been identified by sequence and function as rat ecto-ATPase. Ecto-ATPase has been cloned from several species (Chadwick and Frischauf, 1997; Kegel et al, 1997; Kirley, 1997) and has been postulated to participate in cell-cell adhesion and signal transduction (Dubyak and el-Moatassim, 1993; Plesner, 1995; Dombrowski et al, 1998). In this report, we demonstrate that although ecto-ATPase mRNA is increased by testosterone in prostates of castrated rats, it is marginally regulated by testosterone in Sertoli cells in culture. However, it is markedly up-regulated in Sertoli cells by FSH and in Sertoli and peritubular cells by cAMP.

Materials and Methods

Animals and In Vivo Hormone Treatment

For library generation and screening, Sprague-Dawley male rats (Hormone Assay Co, Chicago, Ill) were hypophysectomized at 20 days of age, injected once daily from the day of surgery for 15 days with sesame oil (H) or testosterone propionate (HT; Steraloids, Wilton, NH; 0.5 mg/d in oil, subcutaneously), and

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C
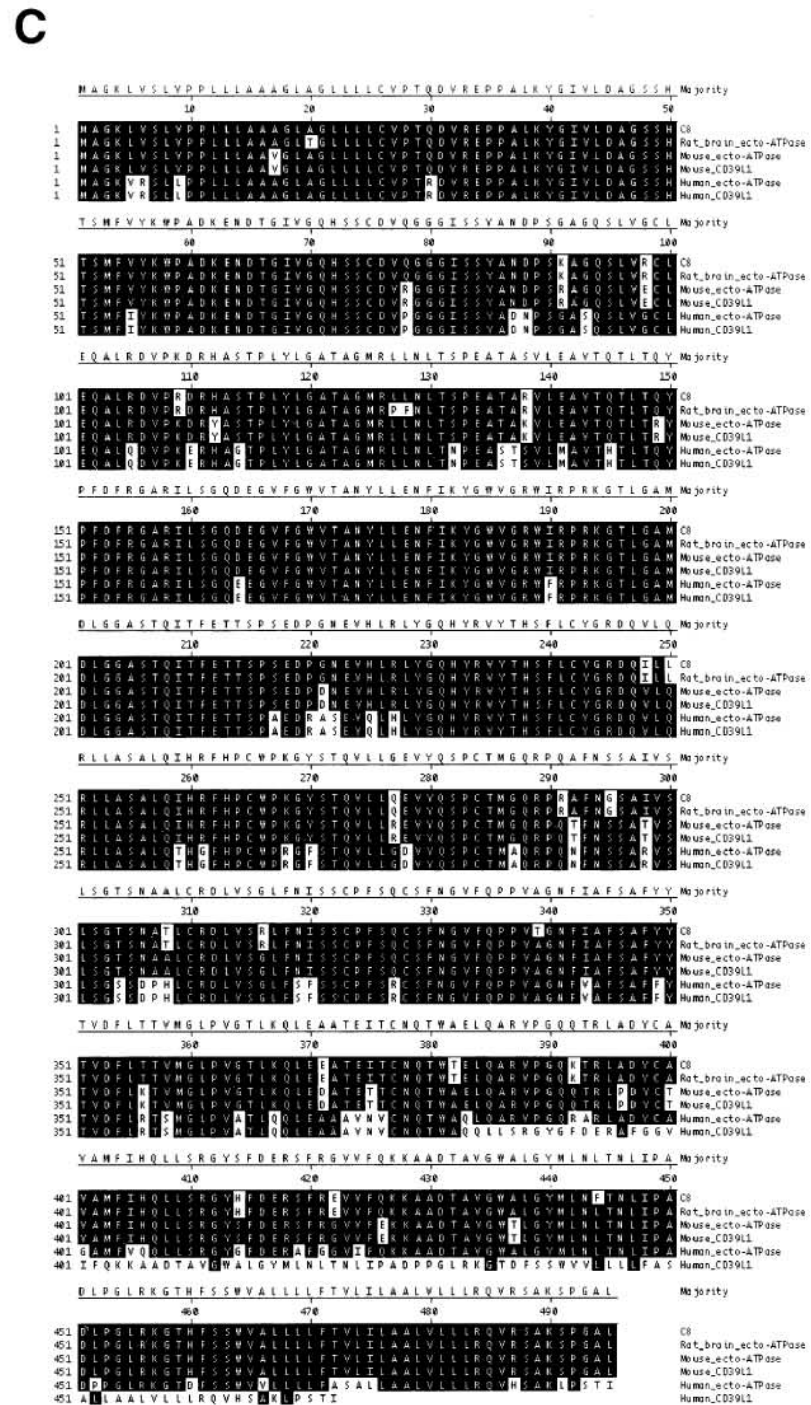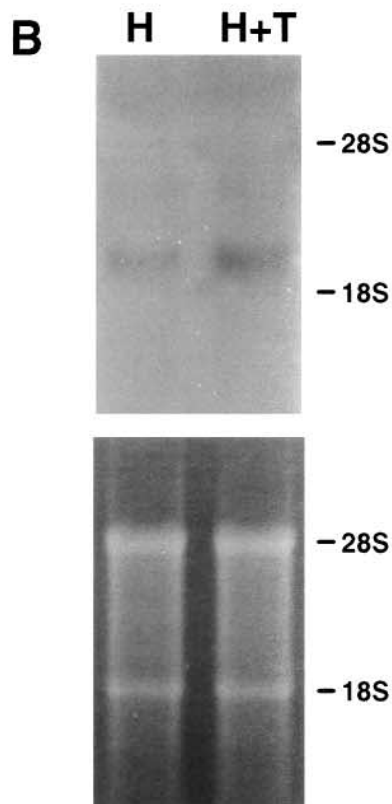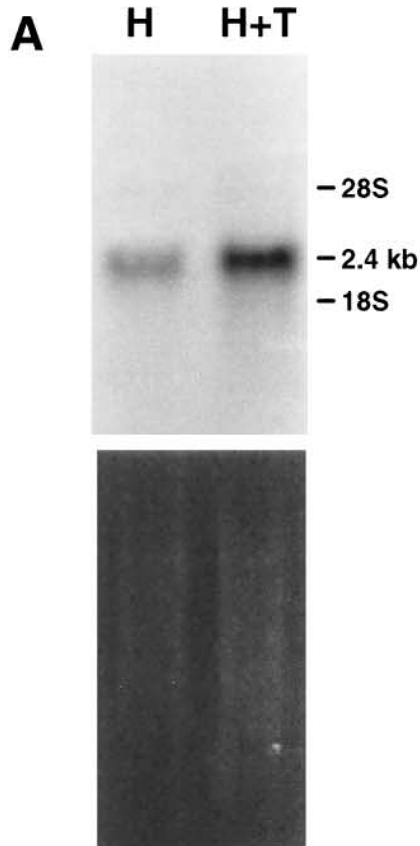
killed at 35 days of age. Sertoli and peritubular cells were isolated from the testes.

In experiments examining mRNA from prostate, kidney, and liver, 45-day-old Sprague-Dawley rats (Harlan, Houston Tex) were castrated and, after 2 days, separated into 2 groups. Animals were injected either with sesame oil or testosterone propionate as described above for 3 days prior to being killed. Tissue was collected and RNA was prepared.

Cell Culture

Sertoli and peritubular cells were isolated from rats in the H and HT groups or from 20-day and 28-day-old intact rats by sequential enzymatic digestion as described previously (Caston and Sanborn, 1988). Sertoli cells were cultured in Dulbecco modified Eagle medium/F12 (DMEM/F12; 1:1) plus 15 mM Hepes, 4 mM glutamine, 10 μg/mL insulin, 5 μg/mL transferrin, 100 U/mL penicillin, and 100 μg/mL streptomycin. Sertoli cells from HT rats also received 100 ng/mL testosterone continuously from the time of plating. Tissue culture reagents and enzymes were obtained from Life Technologies (Grand Island, NY). After 2 days, the cells were subjected to hypotonic treatment to remove residual germ cells (Wagle et al, 1986). As a result of this treatment, the purity of the T and HT Sertoli cell cultures were essentially equivalent by examination under the microscope. After 3 days in culture, these cells were used to prepare RNA. When indicated, cells from normal animals were cultured and subjected to hormonal treatment (300 ng/mL ovine FSH [NIH ovine S16], 100 ng/mL testosterone, or 0.4 mM dibutyryl-cAMP [Sigma Chemical Co, St Louis, Mo]) as indicated 24 hours after hypotonic treatment. In all cases, medium was changed 24 hours before collection of cells for RNA preparation.

Peritubular cells isolated by collagenase/hyaluronidase digestion (Caston and Sanborn, 1988) were plated in DMEM/F12 plus 10% fetal bovine serum (FBS); 10 μg/mL insulin; and transferrin, penicillin, and streptomycin. Five hours after plating the medium was changed to remove remaining suspended Sertoli and germ cells, leaving the peritubular cells attached on the bottom of the dishes. Cells were cultured for up to 12 days before use. Medium containing charcoal-stripped serum was substituted 24 hours before the addition of FSH or testosterone in peritubular cells. Medium was changed 24 hours before collection of cells for RNA preparation.

RNA Isolation and Northern Analysis

Total RNA was isolated from both cultured cells and tissue by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). The concentration of RNA in the samples was estimated by absorbance at 260 nm. The poly A$^+$ RNA for cDNA library construction was isolated using a Fast Track RNA isolation kit (Invitrogen, San Diego, Calif).

RNA was subjected to electrophoresis in 1.2% agarose gels containing 0.65 M formaldehyde. To assess equality of loading, the gels were stained with ethidium bromide and photographed prior to transfer to Nytran filters (Schliecher and Schuell, Keene, NH). Probes were labeled by random priming of DNA inserts isolated by Geneclean (Bio101, La Jolla, Calif). The filters were incubated with [^{32}P]-labeled probes in 50% formamide, 2× Denhardt solution, 5× SSPE (Sambrook et al, 1989), 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 1 μg/mL denatured salmon sperm DNA (Sigma) for 24 hours at 42°C. Filters were washed twice in 2× SSPE/0.1% SDS for 10 minutes at room temperature, then in 0.1× SSPE/0.1% SDS for 15 minutes at 50°C, then in 0.1× SSPE/0.1% SDS for 15 minutes at 65°C, and were then subjected to autoradiography at −70°C. Probes were removed following the manufacturer's instructions and blots were reprobed with β-actin or cyclophilin probes for normalization. For the same samples, normalization using these 2 probes produced equivalent results within experimental error. Autoradiographs were scanned on a soft laser scanning densitometer (Zeineh, Fullerton, Calif) and analyzed by a Hewlett Packard–based program (Palo Alto, Calif). Normalized data were analyzed by analysis of variance and Duncan's modified range test when appropriate.

cDNA Library Construction and Clone Isolation

Sertoli cell mRNA from rats in the HT group was used to prepare an oligo-dT-primed cDNA library using methods described previously (Ku et al, 1991). Briefly, cDNA was synthesized and ligated to Bst*I* linkers. Constructs of >800 base pairs (bp) were cloned in random orientation at the Bst*I* site in pCDM8 (Invitrogen, San Diego, Calif), creating a library of ~5 × 10^5 colonies. A negative selection strategy devised by Palazzolo et al (1989) was used to select clones more abundantly expressed in HT than in H Sertoli cell mRNA. In brief, this method generates a cDNA probe directly from reference mRNA (H in this case) with which to probe a cDNA library from the treated (ie, HT) source. Colonies or plaques that do not hybridize are candidates for sequences unique to or up-regulated in the treated HT sample, which in the present case, was about 15% of the colonies screened. Library colonies that are detected with the reference H probe represent relatively abundant sequences common to both H and HT samples and are eliminated or "subtracted" from further analysis by picking only negative colonies and scanning these again separately. For the data reported here, only a fraction (600 colonies) of the HT library was screened (Sambrook et al, 1989). The [^{32}P]-labeled complex H cDNA probe was synthesized for differential screening as described by Craig et al (1989) except that the template for probe synthesis was H mRNA and priming was accomplished using random oligodideoxynucleotides (Sambrook et al, 1989). Several exposures to film were

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Figure 1. **(A)** cDNA clone C8 is more abundant in testosterone propionate (HT) than in sesame oil (H) Sertoli cell RNA. Three μg mRNA isolated from HT and H Sertoli cell RNA, prepared as described in "Materials and Methods," was loaded per lane, transferred to Nytran, and probed with random primed ^{32}P-labeled C8 probe. Ethidium bromide staining prior to transfer is shown below. The positions of 28S and 18S ribosomal RNA in total RNA samples run in the same gel are indicated. **(B)** Similar Northern analysis performed using 10 μg of H or HT total RNA that was obtained from different groups of animals than those used to generate the RNA in **(A)**. The ethidium bromide staining pattern prior to transfer is shown below. **(C)** Alignment of protein sequence derived from the C8 clone (BankIt 341069, AF276940) with those reported for rat brain ecto-ATPase, mouse ecto-ATPase, mouse CD39L1, human ecto-ATPase, and CD39L1 clones (accession numbers Y11835, AF042811, NM009849, AF144748, and U91510, respectively).
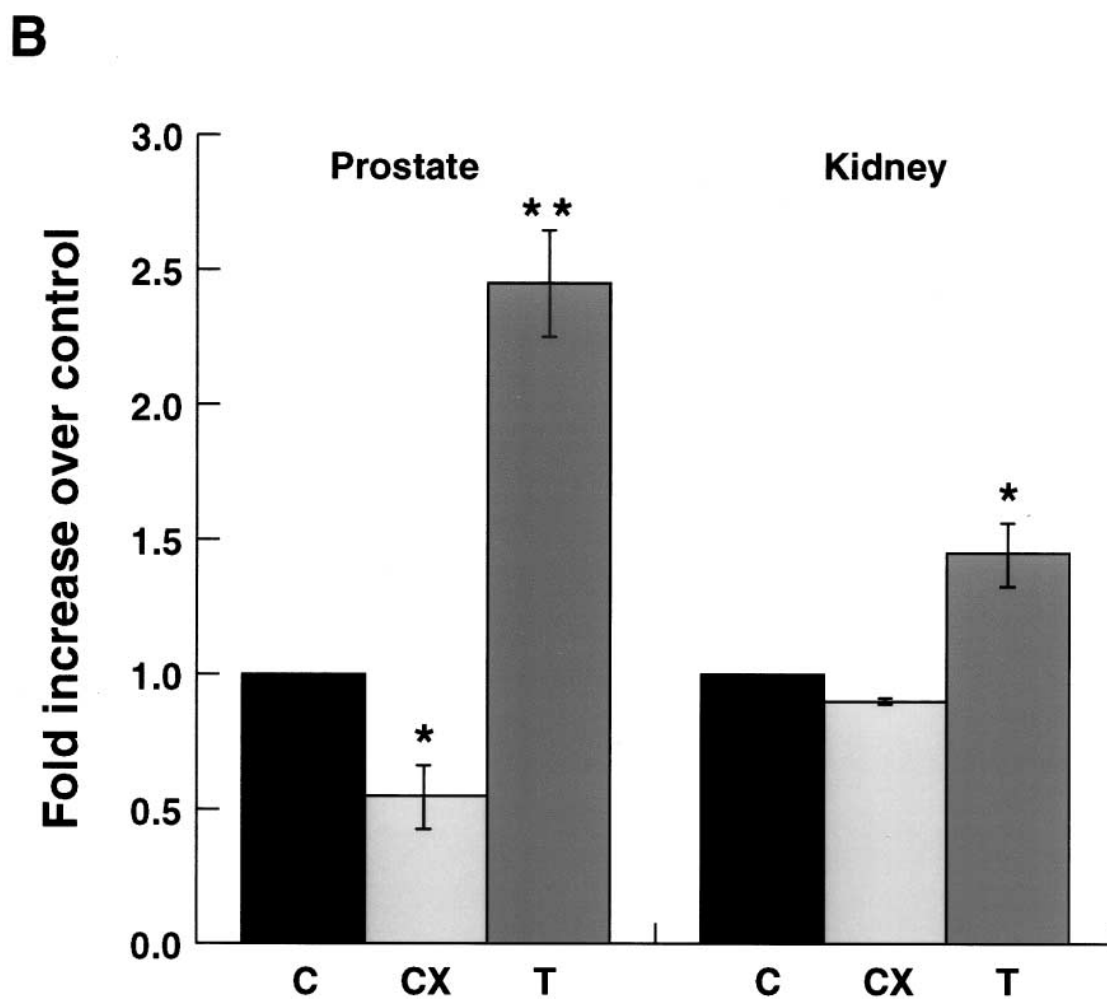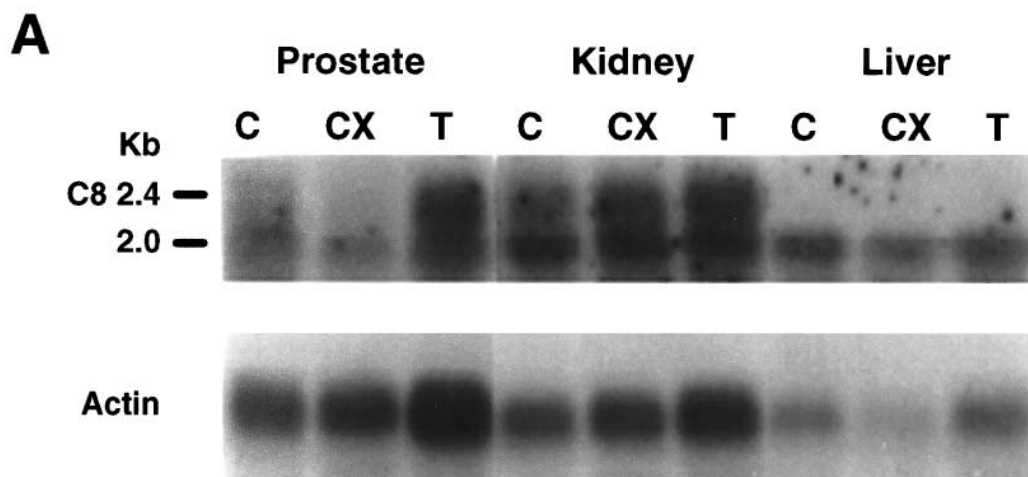
made so that the data could be viewed through the linear range of detection of the film up to the point of signal saturation. Colonies (96 colonies) with little or no signal above background were selected and rescreened as described earlier. A group of these clones (26 ''negative'' clones) containing sequences absent or of low abundance in the H cDNA probe, but still potentially highly expressed in the HT Sertoli cell cDNA library, were selected at random and isolated. These clones were then used to generate individual DNA probes that were used in Northern analyses of mRNA or total RNA isolated from H and HT Sertoli cells. Two different RNA preparations derived from separate groups of animals for both H and HT Sertoli cells were used in the Northern analysis. From these 26 clones, 2 were found to exhibit higher expression in HT than in H. C8 is one of these clones.

Construction of C8 Expression Clone, Transfection, and Ecto-APTase Expression

The insert containing the recombinant C8 clone was sequenced on both strands using conventional methods. As a new sequence was derived it was scanned for related sequences in GenBank with FASTA and BLAST programs. The expression clone, C8-pCDM8, under the control of the CMV promoter was constructed by excision of the clone with Xho*I*, religation into the Xho*I* site of C8-pCDM8, and selection of the clone in the proper orientation by restriction digest analysis and confirmation by sequencing.

COS7 cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. At 80% confluency the cells were washed twice with $1\times$ phosphate-buffered saline and removed with 0.05% trypsin/0.53 mM EDTA at 37°C for 10 minutes. Trypsin was inactivated by addition of an equal volume of medium plus serum. The cells were collected by centrifugation at $1000 \times g$ for 7 minutes and washed once with electroporation buffer (10 mM Hepes pH 7.2, 260 mM sucrose, 1 mM $MgCl_2$, and 15 mM $NaPO_4$). 10^6 Cells were mixed with 10 μg of C8-pCDM8 or 6 μg of pCMV-sport-β-gal (Life Technologies, Gaithersburg, Md) plasmid DNA in 200 μL of electroporation buffer. Electroporation was performed with a Bio-Rad Gene Pulser II (BioRad, Richmond, Calif) electroporation system connected in RF Module at 200 V, 2-millisecond burst duration, 100% modulation, 40 KHz, 5 bursts, 1-second burst interval. After electroporation the cells were immediately diluted with warm medium and plated at 10^5 cells per 100-mm dish. The cells were incubated at 37°C, 5% CO_2, and medium was changed 24 hours later. Transfection efficiency was estimated at 75% by β-gal staining (MacGregor et al, 1991). Ecto-ATPase activity was assayed 48 hours after transfection.

Ecto-ATPase Assay

Ecto-ATPase activity was assayed in transiently transfected COS7 cells by measuring the amount of [32P]P$_i$ released from [γ32P]ATP substrate after adsorption of intact nucleotide with activated charcoal (Dombrowski et al, 1993). COS7 cells containing no plasmid or transfected with pCMV-sport-β-gal expression vector were used as negative controls. COS7 cells were harvested by repeated pipetting with Hanks balanced salt solution (HBSS, Life Technologies, Grand Island, NY) containing 10 mM EDTA. For assay of total cell activity, cells were washed 3 times with enzyme reaction buffer minus [32P] ATP prior to assay. For assay of activity in cell lysates cells were washed 3 times with HBSS at 4°C, harvested by scraping in the reaction buffer, and homogenized with a Dounce homogenizer. Cell lysates from 5×10^5 cells were used for each data point and assayed in triplicate. The assay was carried out in 200 μL of reaction buffer (10 mM Hepes pH 7.4, 135 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1% BSA, 0.3 mM ATP, and 0.3 μCi [γ32P]ATP). To assess the Ca^{2+} and Mg^{2+} dependence of the enzyme activity (Lin, 1989), $CaCl_2$ and $MgCl_2$ were replaced with 2 mM EDTA. The reaction was incubated at 37°C and stopped by the addition of 0.5 mL of cold 20% (w/v) activated charcoal in 1.0 M HCl at the time points indicated. After 10 minutes on ice the charcoal pellet with absorbed nucleotide was separated from the released [32P]P$_i$ by centrifugation at $10\,000 \times g$ for 10 minutes. Two hundred microliters of the supernatant containing the released [32P]P$_i$were counted on a Beckman scintillation counter.

Transcription/Translation

C8-pCDM8 DNA (40–100 ng) was used to generate [35S]-Met labeled protein using a transcription/translation kit from Promega (Madison, Wis). An empty vector served as the control. The products of the reactions were subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) analysis in 12% acrylamide gels and subjected to autoradiography to detect [35S]-labeled protein.

Results

Isolation of Hormonally Regulated cDNA Clones from a Sertoli Cell cDNA Library

We had found that alterations in hormone treatment in vivo were reflected in changes in Sertoli cells in culture, at least for short periods (Sanborn et al, 1991). We used a negative selection strategy (Palazzolo et al, 1989) to isolate clones corresponding to mRNAs present at higher

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Figure 2. Comparison of C8 mRNA level in organs of intact control (C), castrate (CX), and castrate rats treated with testosterone (T). **(A)** A representative Northern blot using 15 μg total RNA from each sample. The blot was prepared as described in ''Materials and Methods'' and probed with a mixture of 32P-labeled C8 and 18S rRNA inserts. Following autoradiography, the blot was stripped and reprobed with a 32P-labeled β-actin probe. **(B)** Summary of data from 3 separate experiments, expressed as fold increase over control and normalized to actin. In each experiment, kidney and liver samples and control prostates were taken from 3 animals. In the CX and T groups, prostate samples were 3 pools of 3 organs each. Data were analyzed by ANOVA and Duncan's test. Significant differences versus control are designated by *$P < .05$. **$P < .01$.

A

MW (Kd)   **C**   **V**   **C8 template**

100 ng   60 ng   40 ng

108 —

80 —

50 —

34 —

27 —

16.9 —

B
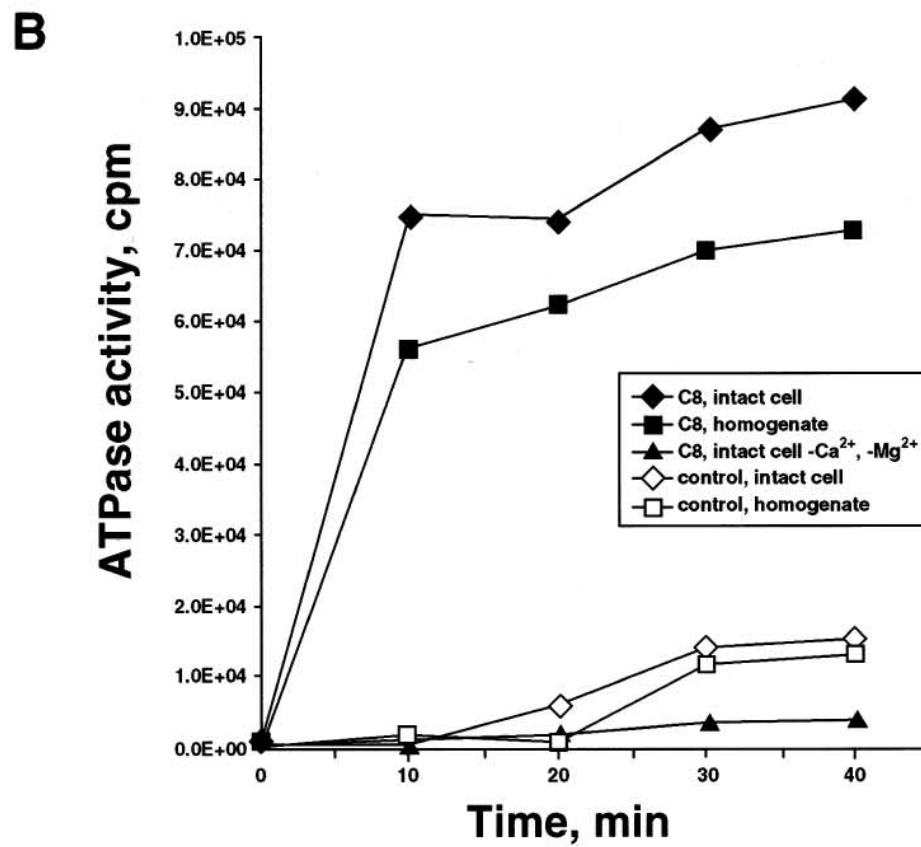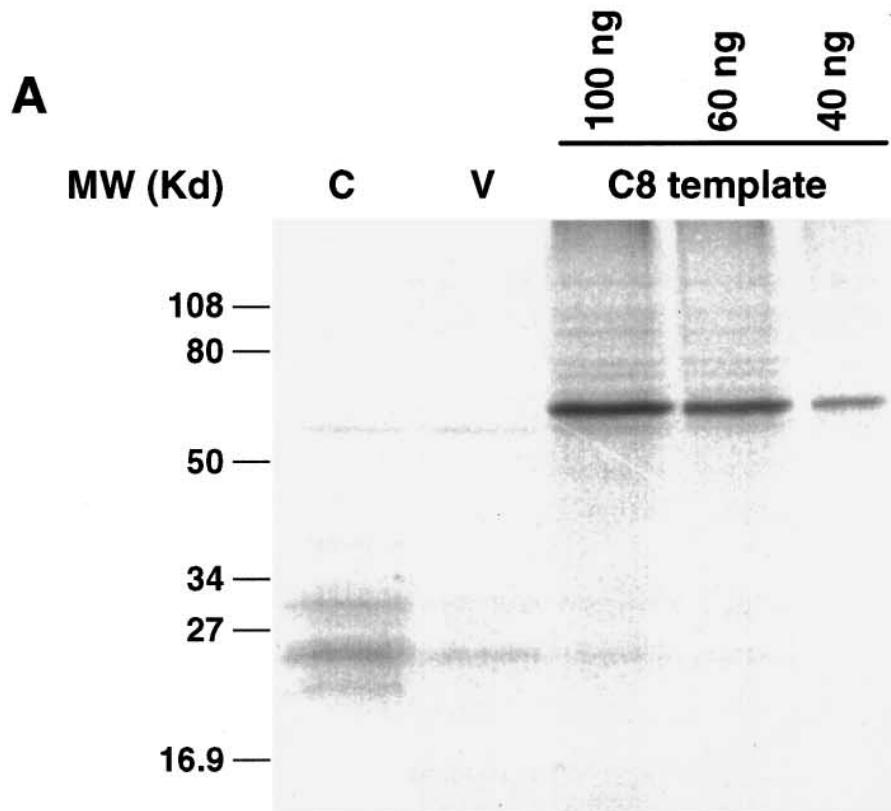
levels in Sertoli cells isolated from hypophysectomized male rats treated with testosterone (HT) relative to cells from untreated hypophysectomized rats (H). The characterization of 1 clone selected for further study on the basis of enhanced expression in HT mRNA and designated C8 is described here. Figure 1A shows the results of 2 Northern analyses using mRNA, or total RNA (Figure 1B); the RNA preparations in each figure were derived from cells isolated from different sets of animals. The mean increase in RNA was 2.2-fold in HT versus H preparations.

To further assess the in vivo regulation of C8 clone by testosterone in androgen-dependent tissues, we performed Northern analysis of C8 mRNA expression in prostate, kidney, and liver from castrates as well as castrates treated with testosterone. In intact animals, C8 was expressed in prostate and kidney as a 2.4-kilobase (kb) mRNA, but not in detectable amounts in a comparable amount of liver RNA under conditions in which 18S ribosomal RNA (rRNA) was clearly detected by a specific probe (Figure 2A). Castration decreased C8 mRNA by 50% in prostate, whereas testosterone administration after castration increased C8 mRNA 2.4-fold over the controls (Figure 2A and B). In contrast, no change in kidney C8 mRNA was observed following castration, although testosterone treatment slightly increased the concentration. Thus there were some tissue differences in the relative expression and regulation of C8 mRNA.

Clone C8 Codes for Ecto-ATPase

Examination of C8 cDNA sequence revealed a putative open reading frame of 1485 bp, which would result in expression of a protein of approximately 54.4 kilodaltons (kd). In vitro transcription/translation produced an [35S]-Met labeled protein of approximately 60 kd from the C8 clone (Figure 3A), which is consistent with this prediction.

The C8 coding region exhibited 99% similarity at both DNA and protein levels (490 of 495 amino acids) with the sequence reported for rat brain ecto-ATPase (Kegel et al, 1997; Figure 1B). In 5 positions (amino acids 20, 127, 128, 339, and 444), the C8 clone differed from the rat brain sequence, but 3 of these (20, 127, and 128) were identical to the mouse and human sequences. Thus, in only 2 positions (amino acids 339 and 444) was the sequence unique to C8. These coding changes in the cDNA sequence were verified by multiple sequencing of both strands in these areas. These changes in the predicted sequence are relatively conservative in the light of the enzyme activity demonstrated below. The nucleotide differences between the brain and Sertoli mRNAs could derive from allelic variation within and between the strains of rat used or errors generated during synthesis of the cDNA. The C8 clone contained only 1 base 5′ to the ATG translational initiation codon and thus may not represent the full-length mRNA sequence. The stop codon at positions 1847–1849 was followed by a 354-bp 3′-UTR, including a 3′ poly(A) tail and a polyadenylation signal (AATAAA) located 14 bp upstream of the poly(A) tail. There was only a 1 base deviation at position 1826 in the 3′-UTR compared with the rat brain ecto-ATPase sequence (Kegel et al, 1997).

The ability of the C8 clone to express ecto-ATPase activity was examined in transiently transfected COS7 cells. As shown in Figure 3B, COS7 cells transfected with C8-pCDM8 showed greatly increased ecto-ATPase activity, relative to cells transfected with pCMV-sport-β-gal, in both intact cell and cell homogenate assays. Enzyme activity was Ca^{2+}- and Mg^{2+}-dependent, consistent with the reported ion dependence for ecto-ATPase (Lin, 1989). Intact cells and cell lysates from the same number of the cells exhibited almost the same amount of activity (Figure 3B). These data are consistent with the expression on the cell surface of ecto-ATPase activity from the C8 clone.

In Vitro Regulation of C8 Ecto-ATPase mRNA in Sertoli and Peritubular Cells by FSH

To determine the effects of FSH and testosterone on the steady state level of ecto-ATPase mRNA in Sertoli and peritubular cells in vitro, both cell types isolated from intact rats were exposed to 300 μg/mL ovine FSH or 100 ng/mL testosterone for the times indicated. As shown in Figure 4A, the [32P]-labeled C8 probe detected a major 2.4 kb band in Sertoli cells. Steady state concentrations of 2.4 kb ecto-ATPase mRNA were markedly affected by hormonal treatment in Sertoli cells from 20-day-old rats. FSH stimulation of Sertoli cell ecto-ATPase mRNA was observed at 6 hours, reached a maximum by 18 hours, was still observed at 24 hours, and even persisted at a reduced level after 3 days of FSH treatment (Figure 4A and B). Peritubular cells also expressed a 2.4 kb C8 mRNA, but no significant increase in ecto-ATPase mRNA was observed in peritubular cells over the same period,

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Figure 3. **(A)** In vitro transcription/translation of C8-pCDM8 template. SDS-PAGE electrophoresis pattern of [35S]-Met labeled proteins generated by transcription/translation of C8-pCDM8 as described in "Materials and Methods." Lanes include reactions with no DNA added (C); empty vector (V); and 100, 60, and 40 ng of the C8 template. **(B)** Time course of the Ca^{2+}- and Mg^{2+}-dependent ecto-ATPase activity expressed by the C8 clone transiently transfected into COS7 cells. Cells were transfected with pCMV-sport-β-gal (control) or C8-pCDM8 (10 μg) and assayed as whole cells or homogenates in the absence and presence of Ca^{2+} and Mg^{2+}. Data at each time point represent the mean of duplicates, expressed as enzyme activity over the time-zero background. Similar results were obtained in 3 separate experiments.

A
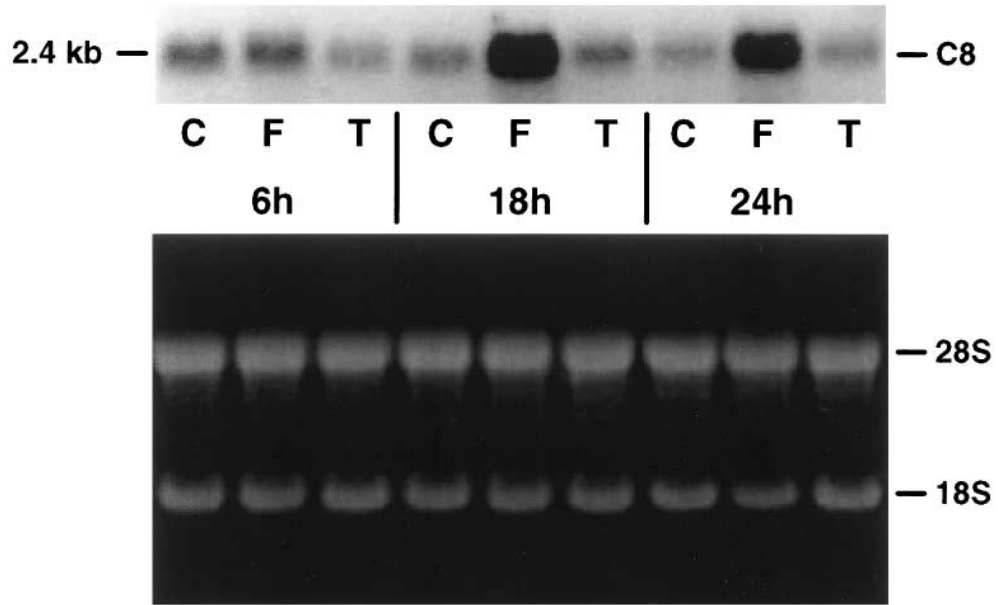
B
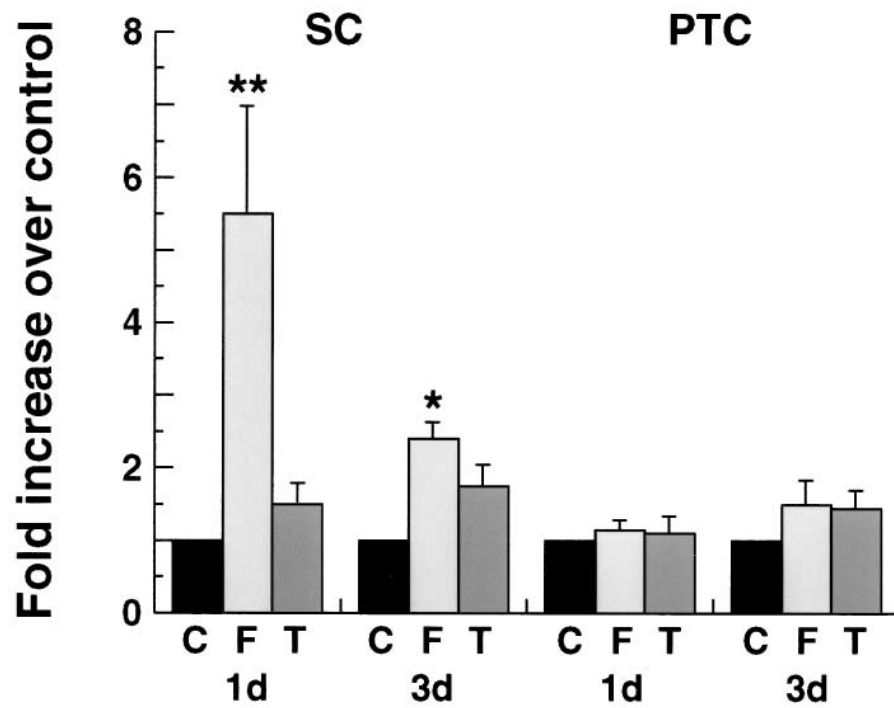
consistent with the absence of FSH receptors in this cell type (Rannikki et al, 1995; Figure 4B). Testosterone had no statistically significant effect on steady state ecto-ATPase mRNA levels in cultured Sertoli cells or peritubular cells (Figure 4B).

In an attempt to increase the magnitude of the androgen response, we also examined the effect of testosterone and FSH on cultured Sertoli and peritubular cells isolated from 28-day-old animals in which the androgen receptor concentration was increased (Buzek et al, 1987). Treatment with FSH or dibutyryl-cAMP for 24 hours resulted in about 4-fold and 11-fold increases, respectively, in ecto-ATPase mRNA in Sertoli cells (Figure 5). Although FSH had no effect on peritubular cell ecto-ATPase mRNA, dibutyryl-cAMP elicited a 5-fold stimulation. Testosterone did not affect ecto-ATPase mRNA in either Sertoli or peritubular cells from 28-day-old animals.

Discussion

Using a selective cloning approach, we identified an mRNA the steady state level of which appeared to be regulated in vivo in Sertoli cells by androgens. The overall similarity of the nucleotide sequence of clone C8 to rat ecto-ATPase was 99% (Kegel et al, 1997). C8 encoded a protein of the appropriate size capable of hydrolyzing ATP in the presence of Ca^{2+} or Mg^{2+} when transfected into COS7 cells. Both intact cells and cell homogenates from the same number of cells showed relatively similar levels of enzyme activity, consistent with the reported membrane localization for this enzyme (Lin, 1989; Plesner, 1995).

The C8 cDNA clone was selected for study on the basis of enhanced expression in mRNA isolated from Sertoli cells from hypophysectomized rats treated with testosterone relative to cells isolated from untreated hypophysectomized rats. Northern analysis of mRNA from tissues from intact and castrated rats treated with testosterone showed that androgen absence decreased and testosterone treatment up-regulated C8 ecto-ATPase expression in prostate, and testosterone had a smaller positive effect in kidney. However, testosterone treatment in primary cultured Sertoli or peritubular cells did not alter the steady state level of ecto-ATPase mRNA. Although androgen-dependent regulation of reporter constructs are seen in Sertoli and peritubular cells isolated from animals of similar ages as those used in these studies (Ku et al, 1994), the effect of testosterone may be blunted in the culture system. Consistent with this possibility, Sutton et al (1998) have reported androgen regulation of the *pem* homeobox gene in mouse Sertoli cells in vivo but were not able to demonstrate an effect in vitro. Alternatively, Sertoli cell ecto-ATPase may be regulated in vivo indirectly by testosterone as result of the regulation by testosterone of paracrine factors such as pModS in peritubular cells (Skinner, 1991). However, no effect was elicited by testosterone in peritubular cells either, although dibutyryl-cAMP increased C8 mRNA. Although a positive effect of testosterone was observed in the prostate in vivo, it is possible that the effect of testosterone was indirect, reflecting a positive influence of testosterone on the number of cells of a given type that express ecto-ATPase, or was the result of a paracrine effect mediated through another cell type.

In contrast to the lack of a significant effect of testosterone in vitro, FSH markedly increased C8 ecto-ATPase mRNA in cultured Sertoli cells. FSH achieves many effects through a cAMP signaling pathway (Griswold, 1998). Consistent with such a mechanism, dibutyryl cAMP also increased ecto-ATPase mRNA expression in Sertoli cells. The data suggest that the effect of FSH is mediated by cAMP and presumably involves activation of CREB or other transcription factors by protein kinase A, similar to a number of other FSH-regulated genes (Suire et al, 1995; Walker et al, 1995). In peritubular cells where there are no FSH receptors, FSH had no effect but dibutyryl cAMP increased ecto-ATPase mRNA, which again, is consistent with a cAMP-mediated pathway. The putative signal regulating cAMP and ecto-ATPase in peritubular cells is not known.

Ecto-ATPases are transmembrane glycoproteins that hydrolyze extracellular nucleoside trisphosphates. Apyrase enzyme activity is dependent on divalent cations, such as Ca^{2+} or Mg^{2+} (Lin, 1989). Epidermal growth factor or cAMP-elevating agents, such as cholera toxin, increase ecto-ATPase expression in the hepatoma Li-7A cell line (Knowles et al, 1985; Knowles, 1995). Ecto-ATPases have been postulated to function in a variety of physiological events such as cellular adhesion, termination of

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Figure 4. Effects of treatment of cultured Sertoli and peritubular cells with 300 ng/mL FSH or 100 ng/mL testosterone on the concentration of ecto-ATPase mRNA. **(A)** Northern analysis in a representative experiment showing time-dependent effects of stimulation of C8 ecto-ATPase with medium (C), FSH (F), or testosterone (T) in cultured 20-day-old Sertoli cells (SC). Fifteen micrograms of total RNA from each sample was subjected to 1.2% agarose gel electrophoresis. The blots were probed with random primed ^{32}P-labeled C8 insert. Ethidium bromide staining shows the approximately equal loading of RNA samples in the agarose gel. **(B)** Effects of FSH (F) and testosterone (T) treatment for 1–3 days on C8 ecto-ATPase mRNA concentrations in Sertoli (SC) and peritubular (PTC) cells. Data are expressed as fold increase over unstimulated control (C) and represent the mean ± SEM of data using RNA from 3–6 different experiments. Data were analyzed by ANOVA and Duncan's test. Significant differences from control at each time interval are indicated by *$P <$ .05. **$P <$ .01.
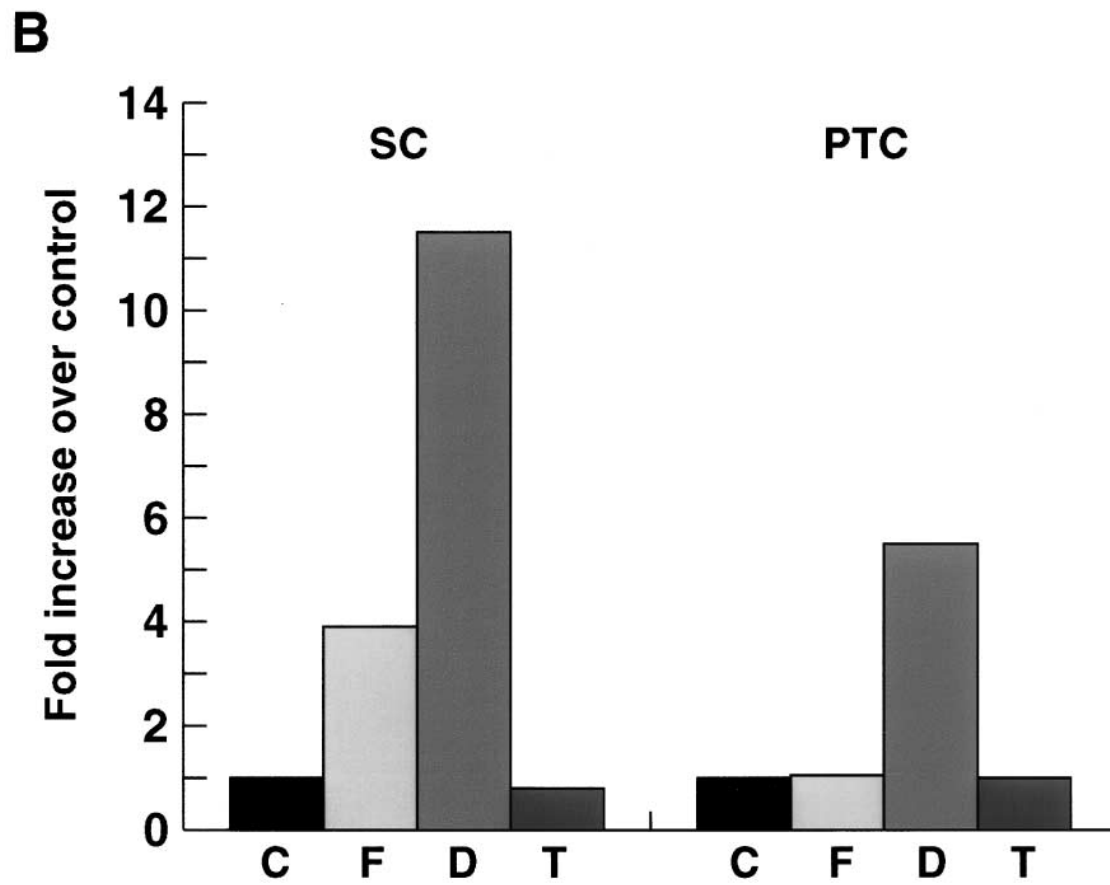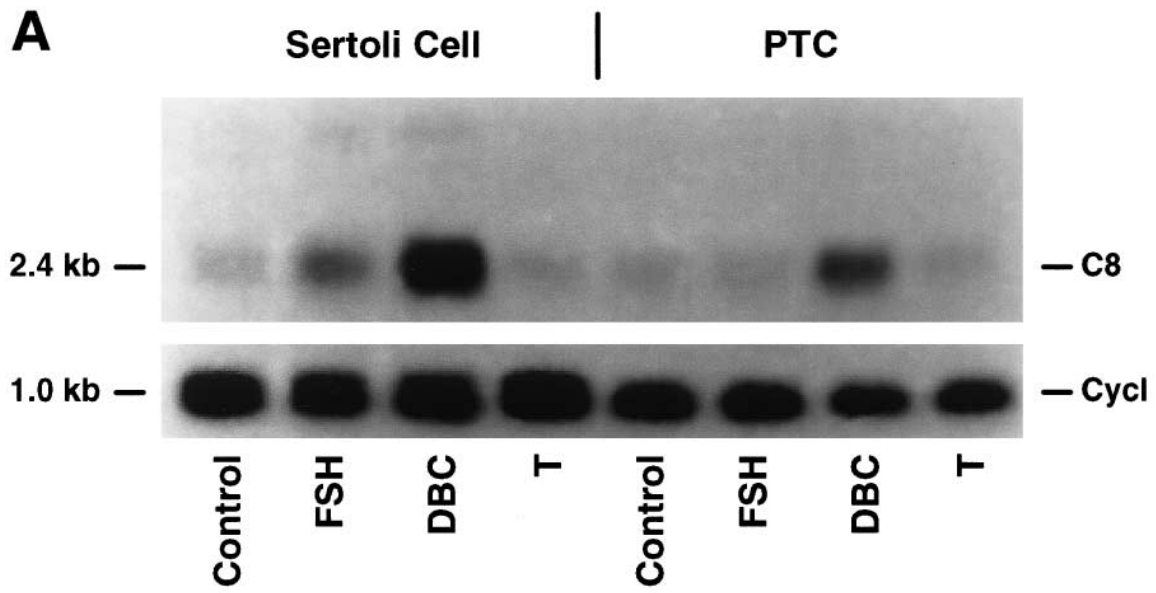
purinergic signaling, vesicular transport, and purine recycling (Kittel and Bacsy 1994; Plesner, 1995). Some cell adhesion molecules possess ecto-ATPase activity (Aurivillius et al, 1990; Dzhandzhugazyan and Bock, 1993). Ecto-ATPase activity is widely distributed through nerve fibers and is regulated during demyelination and remyelination (Felts and Smith, 1996). In the immune system, CD39, a clone with ecto-apyrase activity (Wang and Guidotti, 1996), is implicated in homotypic adhesion of activated B lymphocytes (Kansas et al, 1991; Duensing et al, 1994). Inhibition of ecto-ATPase adversely affects the actions of a number of cells of the immune system (Dombrowski et al, 1993; 1998). Ecto-ATPase is also found in a variety of muscles and may play an important role in the modulation of nucleotide signaling via P_2 purinergic receptors (Westfall et al, 1997; Zinchuk et al, 1999).

Sertoli cells have previously been reported to express ecto-ATPase activity on their surface (Barbacci et al, 1996), but its function is not clear. It could play a role in cell-cell communication or in signaling by regulating extracellular contents of ATP or its metabolic products in testis. Extracellular ATP activates cellular responses through P_2 purinergic receptors (Paton and Taerum, 1990; Dubyak and El-Moatassim, 1993), while P_1 purinergic receptors are preferentially activated by adenosine/AMP (Paton and Taerum, 1990). Sertoli cells have P_2-purinergic (P2U) receptors; ATP stimulates phosphatidylinositide turnover and inhibits FSH-dependent cAMP accumulation, shape changes, and a number of other functions (Filippini et al, 1994; Rudge et al, 1995; Meroni et al, 1998). Germ cells also possess P_2 receptors implicated in modulating the activity of the Ca^{2+}-activated K^+ channel (Wu et al, 1998). ATP can also potentially modulate the contractile activity of the peritubular myoid cells, although receptors have not been demonstrated to date in this cell type. Finally, Leydig cells also possess P_2-purinergic receptors and extracellular ATP stimulates testosterone secretion as a result of increasing intracellular Ca^{2+} (Foresta et al, 1996). Because extracellular ATP has a number of potential targets in the testis, control by ecto-ATPase may be critically important in regulating the concentration of this signaling molecule.

On the other hand, Sertoli cell ecto-ATPase may, along with ecto-apyrase and ecto-adenosine diphosphatase and 5′-nucleotidase, also contribute to generation of extracellular adenosine, which could act on adenosine receptors on Sertoli (Monaco et al, 1984) or germ cells (Rivkees,

1994). Adenosine and its analogues inhibit the FSH-dependent cAMP response and associated actions in Sertoli cells (Davenport and Heindel, 1987; Conti et al, 1988). A1 and A3 adenosine receptors that inhibit adenylyl cyclase have been localized to Sertoli cells and to germ cells, respectively (Rivkees, 1994). A2a and A2b adenosine receptors that stimulate adenylyl cyclase are expressed on sperm and influence sperm motility and cAMP levels (Fraser and Duncan, 1993). Hence, contributions of Sertoli cell ecto-ATPase to the generation of adenosine may contribute further to signaling, as well as production of an important metabolic precursor that readily enters cells.

In summary, the data presented here indicate that ecto-ATPase mRNA is positively regulated by FSH in Sertoli cells and by cAMP in both Sertoli and peritubular cells. The regulation of ecto-ATPase mRNA by FSH suggests that acquisition of this enzyme may be an important part of Sertoli cell maturation. Via control of extracellular ATP or adenosine, this enzyme may also influence the sensitivity of the Sertoli cell to FSH.

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Figure 5. **(A)** Northern analysis of the hormonal regulation of ecto-ATPase mRNA in Sertoli and peritubular cells from 28-day-old rats. Total RNA was isolated 24 hours after addition of no hormone (control), 300 ng/mL ovine FSH (FSH), 0.4 mM dibutyryl-cAMP (DBC), or 100 ng/mL testosterone (T). Fifteen micrograms of total RNA from each sample was subjected to 1.2% agarose gel electrophoresis. **(B)** Summary of normalized data derived from (A) and analyzed as described in *Materials and Methods.* Experimental data were normalized to cyclophilin control and the fold increases were calculated by comparison to experimental control.

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