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Regulation of Sertoli Cell Differentiated Function: Testicular Transferrin and Androgen-Binding Protein Expression*

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ABSTRACT. The regulation of Sertoli cell function was investigated through an examination of the effects of various hormones, regulatory agents, and culture conditions on testicular transferrin and androgen-binding protein (ABP) synthesis and steady state levels of mRNA. FSH stimulated both transferrin and ABP production 2-fold above control levels. Interestingly, FSH had a differential effect on transferrin and ABP mRNA levels, with 1.25- and 2.0-fold respective increases in steady state levels of mRNA. Insulin and retinol stimulated both transferrin and ABP synthesis in a similar manner. Testosterone had no significant effect on either transferrin or ABP mRNA levels or synthesis. Maximum stimulation of both transferrin and ABP production occurred when Sertoli cell cultures were treated with a combination of FSH, insulin, and retinol, which resulted in a greater than 4-fold stimulation of synthesis and 2-fold stimulation of gene expression. Optimal transferrin and ABP secretion occurred between days 4–6 of Sertoli cell culture and subsequently declined. Sertoli cell number decreased with time in culture, such that approximately a 50% loss of cells was observed after 10 days of culture. The responsiveness of Sertoli cells to regulatory agents was altered by cell density, with a maximum responsiveness achieved at a density of 12 μg DNA/2 cm^2 for both transferrin and ABP. As the cell density deviated from this level the responsiveness of cells to regulatory agents decreased and approached control values. These observations indicate that the culture conditions and the method of data normalization are important parameters in an analysis of the hormonal regulation of Sertoli cell function. FSH actions on Sertoli cells increased both cellular and excreted cAMP levels but had no effect on cGMP levels. $(Bu)_2$ cAMP affected transferrin and ABP mRNA levels and synthesis in a similar manner, with approximately a 3-fold increase in synthesis and a 1.5-fold increase in steady state levels of mRNA. The minimum and maximum effective concentrations of $(Bu)_2$AMP for both proteins were 1 and 10 μM, respectively. Observations imply that regulatory agents that act via a cAMP-mediated signal transduction mechanism, such as FSH, will probably have similar actions on transferrin and ABP production. In addition, data obtained with insulin and retinol indicate that transferrin and ABP production can be similarly regulated with cAMP-independent signal transduction mechanisms. Results indicate that transferrin and ABP mRNA levels and synthesis are regulated in a coordinate manner with the regulatory agents and culture conditions evaluated. Observations are discussed in regard to the physiological significance of the absence or presence of a differential regulation of Sertoli cell function. (*Endocrinology* **124:** 3015–3024, 1989)

SPERMATOGENESIS requires the integrated activities of several different cell types within the testis. A variety of testicular cell types have been studied *in vitro* to delineate the mechanisms involved in the maintenance and control of testicular function. One of these, the Sertoli cell, forms the seminiferous tubules and creates the blood-testis barrier. These cells provide the proper microenvironment and cytoarchitectural support for the developing germinal cells. Elucidation of Sertoli cell function and the factors that regulate Sertoli cell function is required to understand the mechanisms involved in the maintenance and control of spermatogenesis.

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A number of Sertoli cell secretory products have been identified and used to delineate a variety of cellular functions. Sertoli cells produce energy metabolites that can be used by developing germinal cells (1). Proteases such as plasminogen activator are produced that can alter the cytoarchitecture of the cells (2). Transport proteins are produced, such as testicular transferrin involved in iron transport (3), ceruloplasmin involved in copper transport (4), and sulfated glycoproteins postulated to be involved in lipid transport (5, 6), that can deliver essential components to developing germinal cells. Sertoli cells produce an androgen-binding protein (ABP) (7–9), which is presumably involved in the localization and transport of androgens in the reproductive tract. Quantitative assays have been developed for a number of these Sertoli cell secretory products (1–4, 7–9). Therefore, these secretory products can be used as functional markers for the cell to investigate the hor-

monal regulation of Sertoli cell function.

Molecular probes have been obtained for a number of Sertoli cell secreted proteins and have provided information regarding protein structure and regulation (6, 10–12). A molecular probe for ABP has been made (10), and the gene structure deduced (11). This molecular analysis has provided detailed information on the amino acid sequence of ABP and possible hormone regulatory elements of the gene (10, 11). Testicular transferrin has also been cloned and found to be the same gene product as serum transferrin produced by the liver (12). The molecular probes for ABP and transferrin provide useful reagents to investigate the regulation of Sertoli cell function (10–12). A major objective of the current study was to analyze and compare the regulation of transferrin and ABP gene expression, as reflected in steady state levels of mRNA.

Transferrin and ABP are two secreted proteins that have previously been used to examine the regulation of Sertoli cell function and differentiation (13–15). Initial observations generally demonstrated that the regulatory agents that control transferrin production also regulate ABP production (13, 16–18). Subsequently, reports from a number of different laboratories have presented data in support (19, 20) and opposition (21, 22) of the presence of a differential regulation of these Sertoli cell functions. The absence or presence of a differential regulation of Sertoli cell function is an important physiological parameter to elucidate and a central issue in the current investigation. The current study was designed to examine the effects of hormones, regulatory agents, and culture conditions on the regulation of transferrin and ABP mRNA levels and synthesis to investigate further the regulation of Sertoli cell function and differentiation.

Materials and Methods

Cell preparation and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (22) with a modified procedure previously described (23). Decapsulated testis fragments were digested first with trypsin (2.5 mg/ml; Gibco, Grand Island, NY) to remove Leydig cells, followed by a collagenase digestion (1 mg/ml type I; Sigma, St. Louis, MO) and then a hyaluronidase digestion (1 mg/ml; Sigma). Sertoli cells were plated in 24-well (1 mg/well) Linbro plates at approximately 5×10^5 cells/well. Cells were maintained at 32 C in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco). Sertoli cultures were treated, as described in *Results*, at the time of plating and retreated after 48 h of culture when the medium was replenished. Unless otherwise stated, a 72-h medium collection on day 5 of culture was obtained for analysis, and the cells were harvested for a DNA assay. Sertoli cell cultures were treated, as outlined in *Results*, with FSH (100 ng/ml), insulin (5 μg/ml), retinol (0.35 μM), testosterone (1 μM), and $(Bu)_2$cAMP (dcAMP; 0.1 mM). Peritubular cell contamination of the Sertoli cell cultures was judged to be less than 1%, and germinal cell contamination was washed from the culture in the first medium collection and subsequently constituted less 1% of the cell population (23).

RIAs

Transferrin production by Sertoli cells was assayed by a RIA described previously (13). An aliquot of the culture medium was incubated with rabbit antirat transferrin antibody (Cooper Biomedical, Malvern, PA) and iodinated transferrin for 1 h at 37 C, followed by a 1-h incubation with goat antirabbit immunoglobulin G (IgG) antibody (Sigma). Complexed antibody was then precipitated with polyethylene glycol (Sigma) and pelleted by centrifugation, and radioactivity in the pellets was determined. This assay has a sensitivity of 5 ng transferrin and is linear up to 250 ng, with 5% and 10% intra- and interassay coefficients of variation, respectively.

ABP was assayed by a RIA described previously (24). An aliquot of the culture medium was incubated with rabbit antirat ABP (National Hormone and Pituitary Program, NIH, Bethesda, MD) and iodinated ABP at 4 C for 18 h. Goat antirabbit IgG and polyethylene glycol were then added, and the samples were incubated for an additional 18 h at 4 C. The antibody complex was pelleted by centrifugation, and the radioactivity was determined. This assay has a sensitivity of 0.5 ng ABP and is linear up to 50 ng, with 10% and 15% intra- and interassay coefficients of variation, respectively.

DNA assay

DNA was measured fluorometrically with ethidium bromide (25). At the end of the culture period the medium was removed, ethidium bromide buffer (EBB; 20 mM sodium choloride, 5 mM EDTA, and 10 mM Tris, pH 7.8; Sigma) was added to the wells, and the cells were sonicated. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide and 100 U/ml heparin in EBB), diluted 1:2 with EBB buffer, and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantitate DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 2.5 μg DNA. RNA did not have a significant effect on the DNA assay. Treatment of samples with RNAase indicated that RNA only contributed to 10–15% of the signal detected, which is within the uncertainty of the assay. An approximate estimate of the number of cells analyzed has been determined to be 8×10^5 cells/10 μg DNA.

Cyclic nucleotide assay

Cyclic nucleotide assays were performed as previously described (26). $HClO_4$ (0.5 M) was added to plated cells or collected medium samples that contained tracer amounts of [^{3}H]cAMP or [^{3}H]cGMP for estimation of cyclic nucleotide recoveries. The samples were sonicated, centrifuged to remove cell debris, and then applied to Dowex AG 50W-X8 columns. Column fractions containing cAMP or cGMP were lyophylized, dis-

solved in water, and quantitated by RIA. This assay has a sensitivity of 5 fmol and is linear up to 200 fmol cAMP.

RNA isolation and Northern analysis

RNA was obtained from Sertoli cells, extracted with 1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.5), passed through a 22-gauge needle, then digested with proteinase-K (50 μg/ml). Total RNA was obtained with an ethanol precipitation of the sample after a phenol-chloroform extraction. Polyadenylated RNA was isolated with a oligodeoxythymidine affinity column. Polyadenylated RNA was separated electrophoretically on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and analyzed with a Northern blot procedure previously described (27). The rat transferrin cRNA probe was obtained from a cDNA fragment that contained a 390-basepair coding region (12) (generously provided by Dr. M. D. Griswold, Washington State University, Pullman, WA). The rat ABP cRNA probe was obtained from a cDNA probe fragment that contained a 1400-basepair coding region (11) (generously provided by Dr. D. R. Joseph, University of North Carolina, Chapel Hill, NC). These inserts were subcloned into the plasmid SP65 in the antisense orientation with regard to the transcriptional direction of the SP6 promotor. The cRNA probes were labeled with [^{32}P]UTP, as previously described (28). A 700-basepair insert of p1B15 (29), a rat cDNA which encodes cyclophilin, was subcloned into the plasmid SP65 promotor to produce a cRNA probe. p1B15 is a gene that appears to be constitutively expressed and was used as a control probe to demonstrate intact RNA. RNA was hybridized to the various probes at 65 C for 20 h in a solution of 50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 0.1% SDS, 0.2% Ficoll, 50 μg/ml polyadenylic acid, 0.2% BSA, 0.2% polyvinylpyrrolidone, and 400 μg/ml sonicated denatured salmon sperm DNA. Posthybridization washes consisted of one 20-min wash at room temperature in 0.03 M sodium citrate, 0.3 M NaCl, and 0.1% SDS, then two 30-min washes at 68 C in 0.003 M sodium citrate, 0.03 M NaCl, and 0.1% SDS. Hybridized cRNA probes were detected with autoradiography. Scanning densitometry was performed on developed autoradiographs with an LKB 2202 UltraScan Laser Densitometer (LKB Instruments, Rockville, MD).

Solution hybridization

Solution hybridizations were carried out using modifications of a procedure previously described (12). Total RNA samples were dried and reconstituted with 20 μl reaction buffer [20 mM Tris (pH 7.5), 1.2 M NaCl, 10 mM EDTA, 0.2% (wt/vol) SDS, and 1 mg/ml yeast RNA; from Boehringer Mannheim, Indianapolis, IN) which contained 30,000 cpm [^{32}P]UTP radiolabeled cRNA probe. Samples were incubated for 18 h at 60 C. One milliliter of dilution buffer [10 mM Tris (pH 7.5), 0.3 M NaCl, and 5 mM EDTA] which contained RNAse T1 (100 U/ml; Sigma) and RNAse A (12.5 μg/ml type IIA; Sigma) was added to the samples and incubated at 37 C for 30 min. After the RNAse digestion, 100 μl 100% (wt/vol) trichloroacetic acid containing 10 μg/ml yeast RNA were added to the samples and incubated on ice for 1 h. Samples were then filtered onto GF/C filters (Whatman, Hillsboro, OR) and washed with 10% (wt/vol) trichloroacetic acid at 4 C. Filters were counted in 5 ml ACS scintillation fluid (Amersham, Arlington Heights, IL). Solution hybridizations were performed on each total RNA sample with transferrin, ABP, and 1B15 cRNA probes.

Results

Initially, the media from control and stimulated Sertoli cell cultures were analyzed for transferrin and ABP to determine the optimal time course of secretion for these two proteins. Cell cultures were stimulated with a combination of FSH, insulin, retinol, and testosterone (FIRT), which was previously shown to increase both transferrin and ABP production (13, 16). After 48-h culture periods, selected wells were terminated, the medium was removed for assay, and the cells were removed for DNA analysis. In control cultures transferrin secretion by Sertoli cells increased between days 2–4 (20 ng/μg DNA), with a gradual decline by day 10 of culture (Fig. 1). When the Sertoli cells were stimulated with FIRT, maximal transferrin secretion occurred between days 4–6 of culture and subsequently declined from days 6–10 of culture (120–40 ng/μg DNA). In control cultures ABP levels decreased from days 2–6 of culture (10 to 2 ng/μg DNA) and were maintained at low levels for the

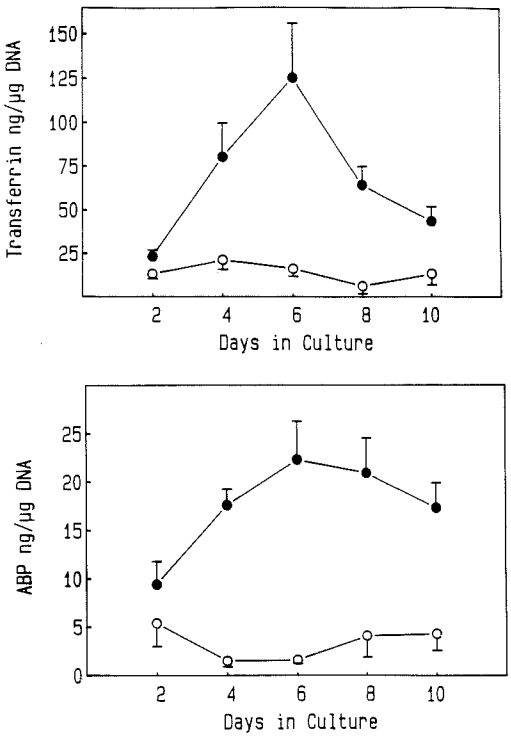

FIG. 1. Effect of increasing culture duration (days) on the responsiveness of Sertoli cells for transferrin secretion and ABP secretion. Sertoli cells were plated either with (FIRT; ●) or without (control; ○) regulatory agents and treated as described in *Results*. The *data points* are the average of duplicate wells from three experiments, with the SEM indicated.

duration of the culture period (Fig. 1). When stimulated with FIRT, maximal ABP secretion occurred between days 4–6 of culture, with a small decline by day 10 of culture. The data presented in subsequent figures were obtained from a 72-h medium collection between days 2–5 of Sertoli cell culture, which is a period of stimulation for both transferrin and ABP production (Fig. 1).

The effect of cell density on the responsiveness of Sertoli cells to regulatory agents was examined. Optimum stimulatory effects of FSH and FIRT on transferrin secretion were obtained when cells were plated at a density of 12 μg DNA/2 cm^2 (Fig. 2). When Sertoli cells were plated at densities lower or higher than 12 μg DNA/2 cm^2 the stimulatory effect of regulatory agents was reduced. When cells were plated to confluence (30 μg DNA/2 cm^2) the stimulatory effects of FSH and FIRT were negligible, and transferrin levels were the same as those found in control cultures. The culture medium was also analyzed for ABP secretion. ABP secretion appeared to be less sensitive than transferrin secretion to changes in cell density, with an optimal cell density for FSH- and FIRT-stimulated ABP secretion between 12–20 μg DNA/2 cm^2 (Fig. 2). When Sertoli cells were plated above or below the range of optimal cell density the stimulatory

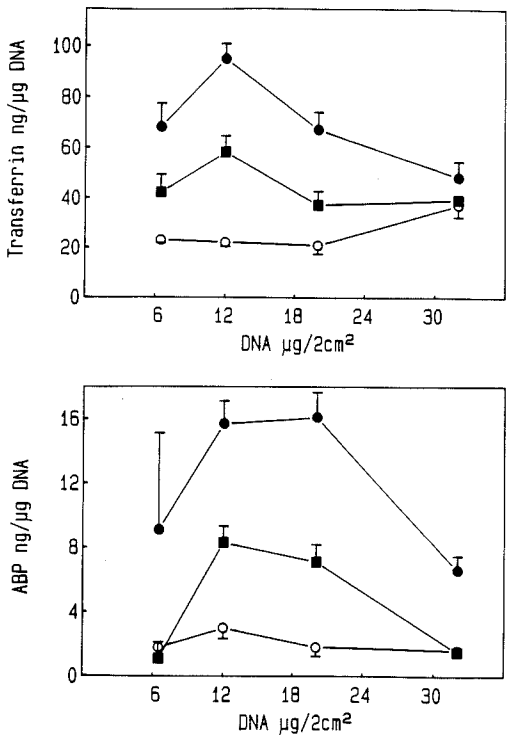

FIG. 2. The effect of cell density on the hormonal responsiveness of Sertoli cells for transferrin secretion and ABP secretion. Sertoli cells were plated at various densities (6–32 μg DNA/2 cm^2) and cultured with (■, FSH; ●, FIRT) or without (control; ○) regulatory agents. The cultures were treated as described in *Results*. The data presented are averages of replicate determinations in a minimum of three experiments, with the SEM indicated.

effects of regulatory agents were reduced and approached the levels in control cultures. The data used to determine the responsiveness of Sertoli cells to regulatory agents presented in Fig. 1 and subsequent figures were obtained from cultures where the mean (±SEM) DNA value was 10.2 ± 0.2 μg DNA/2 cm^2, well within the range necessary for an optimal response for both transferrin and ABP (Fig. 2).

The influence of culture duration on cell number was investigated to more thoroughly establish the appropriate method of data normalization. DNA values were used as an indicator of the number of cells present at the termination of the culture for each of the 48-h periods studied. DNA values declined throughout the culture period from approximately 10 μg/well on day 2 to 4–5 μg/well on day 12 of culture. When DNA values from control and FIRT-treated cultures were compared, no differences were found from days 2–10 of culture (Fig. 3). By day 12 of culture, lower DNA values were found in FIRT-treated cells than in control cultures. Observations indicate that data should be normalized with DNA values obtained at the same time the medium sample is collected. All data presented in previous and subsequent figures were normalized with DNA values obtained at the same time that a specific medium sample was collected for analysis.

The response of Sertoli cells to a variety of regulatory agents was evaluated by monitoring the secretion of testicular transferrin and ABP. Sertoli cell cultures were treated at the time of plating and retreated after 48 h of culture. A 72-h medium collection on day 5 of culture was used for the analysis of both transferrin and ABP levels. Transferrin levels were significantly higher after the addition of FSH, insulin, or retinol to the culture medium (Fig. 4A). Testosterone had no significant effect on transferrin secretion. Maximal transferrin secretion was obtained when a combination of regulatory agents was added to the culture medium (FIRT). FSH, insulin, and retinol (FIR) was responsible for this stimulation

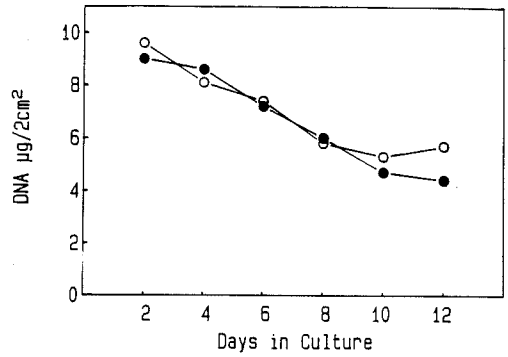

FIG. 3. Effect of increasing culture duration on the DNA levels in Sertoli cell cultures. The data are averages of DNA values obtained from plates used in Fig. 1. ○, Control; ●, FIRT.

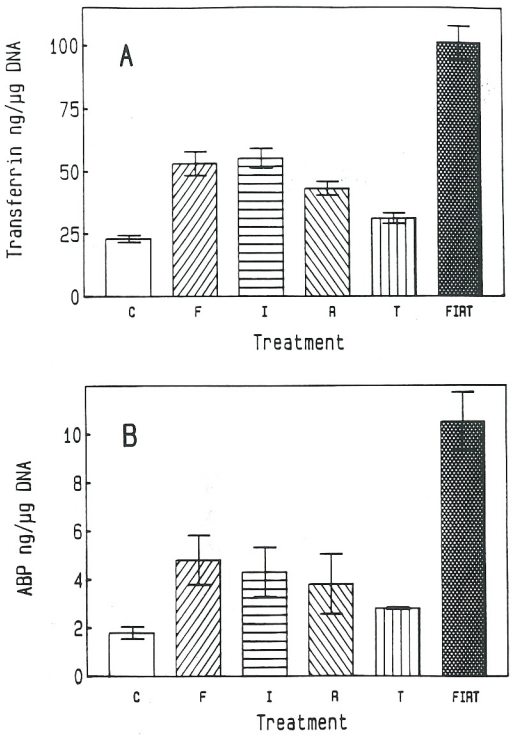

FIG. 4. The effects of regulatory agents on transferrin secretion (A) and ABP secretion (B) by cultured Sertoli cells. The cultures were treated as described in *Results* (F, FSH; I, insulin; R, retinol; T, testosterone; FIRT, a combination of agents; C, no additions, control). The data are averages of levels from triplicate wells from 8–10 experiments, except testosterone data which was from 4 experiments. The mean ± SEM is presented. The additions of FSH, insulin, retinol, and FIRT resulted in levels that were higher than control values and were statistically significant ($P < 0.01$) by Student's t test. The small increase after testosterone treatment was not statistically significant.

and had the same effect as FIRT (data not shown). Insulin and FSH resulted in a 2-fold increase in transferrin secretion, retinol in a 1.75-fold increase, and FIR or FIRT in a 4-fold increase in transferrin secretion. When ABP was measured, the pattern of Sertoli cell responsiveness to regulatory agents was similar but absolute levels were approximately one tenth of those obtained for transferrin. Addition of FSH, insulin, or retinol to Sertoli cell cultures resulted in ABP levels significantly higher than those in control cultures (Fig. 4B). Testosterone had no significant effect on ABP secretion. The combination of agents (FIRT) resulted in an apparent maximal ABP secretion. As shown with transferrin production, FIR was responsible for the stimulatory effects observed with FIRT (data not shown). Insulin, FSH, and retinol resulted in approximately a 2-fold increase in ABP secretion, and FIR or FIRT resulted in a 4- to 5-fold increase in ABP secretion. No detectable levels of transferrin or ABP were found in Sertoli cell sonicates (data not shown), indicating the absence of intracellular pools of these proteins. The data presented in Fig. 4 indicate that the regulatory agents evaluated influence the synthesis of transferrin and ABP in a similar manner.

One of the primary intracellular signal transduction mechanisms found to stimulate Sertoli cell function involves cAMP. FSH stimulated both cellular and excreted cAMP levels, but had no effect on cGMP levels (Table 1). Similar results were obtained on days 1 and 2 of culture, with a maximum response obtained on day 2 of culture and maintained through day 5 of culture (data not shown). A greater fold increase was found in excreted cAMP levels in response to FSH than in cellular levels. A pharmacological agent that can mimic the actions of a cAMP-mediated signal is dcAMP. The effects of dcAMP on Sertoli cell function were an approximately 3-fold increase in both transferrin and ABP production (Fig. 5). Therefore, the response to dcAMP was slightly greater than that to FSH alone, but did not maximally stimulate cellular function. Similar results were obtained with another cAMP analog, 8-bromo-cAMP (data not shown). A dose-response curve for dcAMP was made to determine if the concentrations required to stimulate

TABLE 1. Actions of FSH on Sertoli cell excreted and cellular cyclic nucleotide levels

Treatment	cAMP (fmol/μg DNA)		cGMP (fmol/μg DNA)	
	Cellular	Excreted	Cellular	Excreted
Control	170 ± 22	120 ± 18	16 ± 3	23 ± 3
FSH	280 ± 28	420 ± 40	16 ± 2	20 ± 3

Sertoli cells were cultured in the presence or absence (control) of FSH. Cellular levels of cAMP and cGMP were determined on day 5 of culture. Excreted levels of cAMP and cGMP were determined with a 72-h conditioned medium sample collected on day 5 of culture. Values are represented as femtomoles of cyclic nucleotide per μg Sertoli cell DNA and are presented as the mean ± SEM from three different experiments, each performed in triplicate.

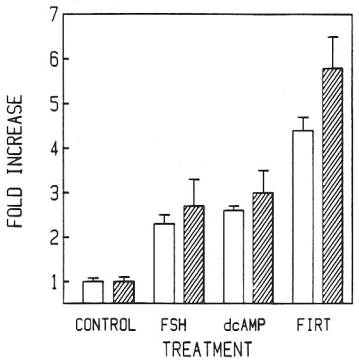

FIG. 5. The effects of regulatory agents on the production of ABP (▨) and transferrin (□). Sertoli cells were cultured in the absence (control) or presence of FSH, dcAMP, and FIRT. Data are presented as the fold increase above control levels for nanograms per μg DNA and represent the mean ± SEM of a minimum of three different experiments, each performed in triplicate.

ABP production are similar to those required for transferrin production. Similar curves were found for both ABP and transferrin production, with a minimum effective concentration of 1 μM and a maximum effective concentration of 10 μM (Fig. 6). This indicates that the concentrations of dcAMP required to stimulate transferrin and ABP production are the same.

Investigation of transferrin and ABP steady state levels of mRNA initially used a Northern analysis of polyadenylated RNA isolated from Sertoli cells. Hybridization with cRNA probes demonstrated a single 2.6-kilobase transcript for transferrin and a single 1.7-kilobase transcript for ABP (Fig. 7). Northern analysis of RNA isolated from Sertoli cells cultured in the absence or presence of FIRT for 5 days indicated a higher level of hybridization in stimulated cultures for both transferrin and ABP (Fig. 7). Scanning densitometry of the Northern blots indicated a 1.9-fold increase in transferrin hybridization and a 2.5-fold increase in ABP hybridization with FIRT treatment. A cRNA probe (1B15) was obtained to a constitutively produced protein, cyclophilin, to assess the integrity of the RNA analyzed. Analysis of the Northern blots shown in Fig. 7 with the 1B15 probe demonstrated no apparent degradation of the RNA, through the detection of a single 1-kilobase cyclophilin transcript, and no effect of FIRT on the level of hybridization (data not shown).

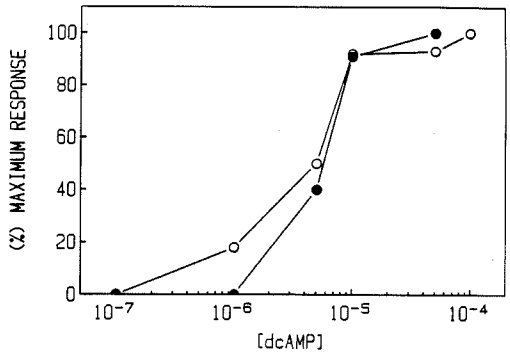

FIG. 6. Effects of dcAMP on transferrin (●) and ABP (○) production. Sertoli cells were cultured in the presence of varying concentrations of dcAMP. The percent maximal response of stimulation is presented for both ABP and transferrin production. The data presented are the mean of a triplicate determination and are from a representative experiment of three different experiments.

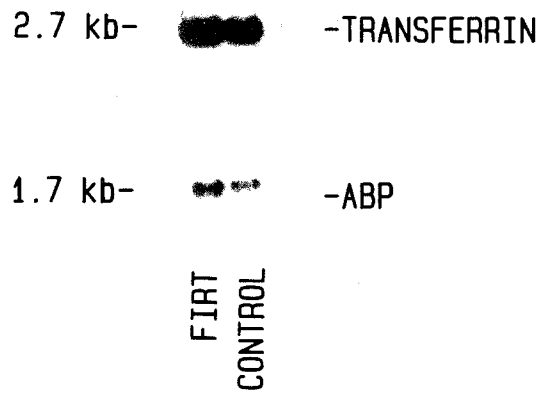

FIG. 7. Northern blot analysis of Sertoli cell poly(A) RNA for transferrin and ABP expression. Sertoli cells were cultured for 5 days in the absence (CONTROL) or presence of FIRT, followed by the isolation of poly(A) RNA. The size (kilobases) is designated for both transferrin and ABP transcripts. Data are representative of six different experiments.

To quantitatively assess the effects of regulatory agents on mRNA levels, a solution hybridization assay was established for both transferrin and ABP. Increasing amounts of total RNA isolated from Sertoli cells resulted in an increase in hybridization of the respective cRNA probes to transferrin and ABP (Fig. 8). The levels of total RNA required to obtain equivalent amounts of hybridization were approximately 10-fold higher for ABP than for transferrin. This implies that the abundance of the transferrin mRNA is approximately 10-fold higher than that of ABP. Data presented in Fig. 8 also demonstrate the linear range of the solution hybridization assays for transferrin (10–50 μg RNA) and ABP (10–100 μg RNA). Detailed analysis of the actions of regulatory agents used Sertoli cells cultured for 5 days, followed by isolation of total RNA. Data obtained with the solution hybridization are expressed as fold increase above control values. Solution hybridizations were also carried out with a 1B15 cRNA probe to the constitutively produced protein cyclophilin. Regulatory agents had negligible effects on cyclophilin expression, 1B15 hybridization, and were expressed as a ratio of hybridization from treated/nontreated control cells (data not shown). All data obtained for transferrin or ABP hybridization were normalized with 1B15 hybridization. This normalization corrected for alterations in cell number and variable yields in the RNA isolation procedure. Similar results were obtained when data were normalized with micrograms of DNA or milligrams of total protein (data not shown). Both transferrin and ABP steady state levels of mRNA were increased by FSH, insulin, retinol, dcAMP, and FIR. Testosterone had no significant effect on either transferrin or ABP gene expression (Fig. 9). Maximum stimulation was obtained with a combination of FIR, which was similar to the stimulation observed with FIRT. FSH had a significant effect on ABP mRNA levels, which was similar to that of FIR and greater than that of dcAMP. In contrast, FSH alone had only a small effect on transferrin mRNA levels which was less than that observed with dcAMP (Fig. 9A). Other than these differences in FSH actions the regulation of transferrin and ABP gene expression was similar.

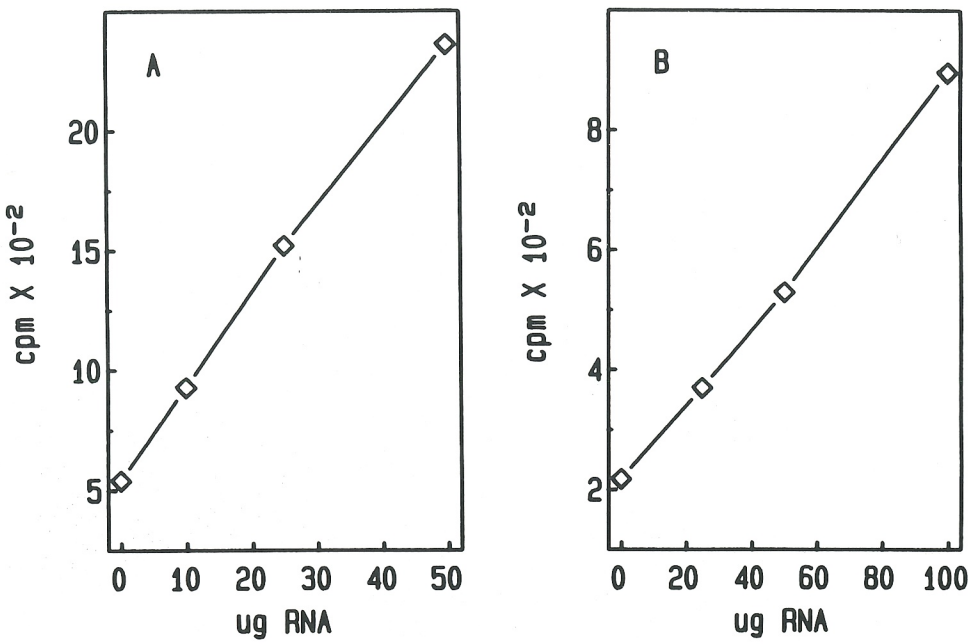

FIG. 8. Solution hybridization for transferrin (A) and ABP (B) expression with total RNA. The amount of hybridization is expressed as counts per min and related to total RNA concentration, expressed as micrograms of RNA. Data shown are the mean of duplicate samples and are representative of three separate experiments.

Discussion

The regulation of Sertoli cell function has been investigated by a number of laboratories with the use of several biochemical parameters, including transferrin and ABP production. Observations have previously been presented that both support (19, 20) and oppose (12, 13, 21, 24) a potential differential regulation of ABP and transferrin production by Sertoli cells. Elucidation of the absence or presence of a differential regulation of Sertoli cell function is required to understand the physiological aspects of both ABP and transferrin production, as well as provide insight into the hormonal regulation of Sertoli cell function. Before a detailed analysis of the regulation of transferrin and ABP mRNA levels and synthesis, several potential variables of cell culture that can alter data interpretation were investigated. These cell culture variables investigated were culture duration, cell density, and the method of data normalization. The time course of secretion for transferrin and ABP demonstrate maximum stimulation by regulatory agents between days 4–6 of culture for both proteins. ABP secretion was maintained at high levels for up to 10 days of culture, while transferrin secretion declined. Therefore, data obtained at later periods of culture do not reflect the ability of regulatory agents to stimulate the production of both proteins. For example, results obtained on day 10 of culture imply that ABP synthesis is responsive to hormones while transferrin production has a minimal response. Therefore, the duration of cell culture needs to be considered in evaluating the effects of regulatory agents on different cellular functions. The reason for the ability of regulatory agents to maintain elevated ABP secretion for a longer culture duration while transferrin stimulation declines is not known at present, but may be related to the relative abundance of these two proteins. Another cell culture parameter considered is cell density. Optimal cell densities were found for the ability of regulatory agents to stimulate both transferrin and ABP production. The use of confluent cultures of Sertoli cells resulted in regulatory agents having minimal effects on either transferrin or ABP production. However, the reduction in ABP stimulation was not as severe as that found for transferrin production. Basal levels of transferrin produced per cell remained constant, with only a small increase in confluent cultures while the basal levels of ABP produced per cell did not change with cell density. Therefore, the reduced ability of hormones to stimulate transferrin and ABP production at higher cell densities was not due solely to a rise in the basal level of synthesis. The reason for the reduced ability of hormones to stimulate both transferrin and ABP production at either high or low cell densities remains to be investigated, but may be related to altered cell viability, altered protein synthesis, reutilization of proteins by the cell, and altered ability of hormones to act on the cell. Observations indicate that cell density is an important parameter to consider in an analysis of the hormonal regulation of Sertoli cell function. Results presented also indicate a 50% reduction in cell number between days 2 and 10 of Sertoli cell culture. For this reason normalization of data with DNA values obtained from cells harvested at the time that a specific medium sample was collected is critical. If data are normalized with DNA values obtained from cells harvested at a later or earlier time point the results may not be representative. Determination of cell number with

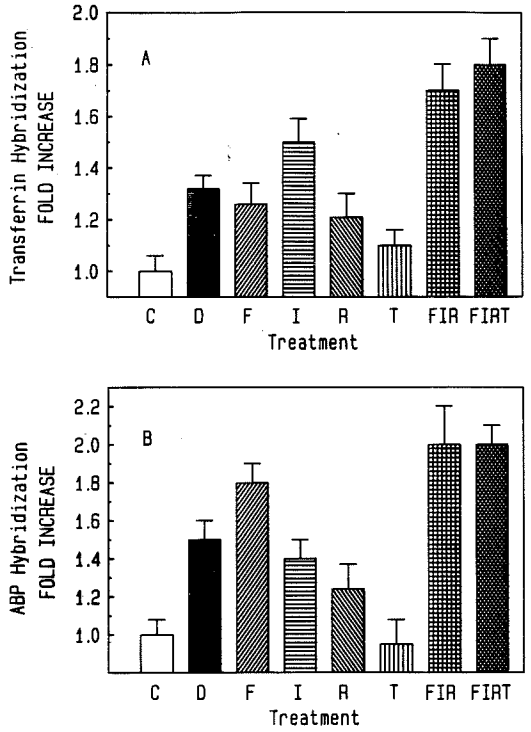

FIG. 9. Solution hybridization of transferrin (A) and ABP (B) expression, as influenced by various regulatory agents. Sertoli cells were cultured for 5 days in the absence [control (C)] or presence of FSH (F), insulin (I), retinol (R), testosterone (T), dcAMP (D), or a combination of these agents. Results are expressed as the fold increase above control and normalized for 1B15 expression. Data are presented as the mean ± SEM from 12 different experiments, each performed in replicate. The error associated with the control (C) is derived from the coefficient of variation of the assay. All but the testosterone (T) treatment were statistically different from the control value (C) by Student's t test ($P < 0.01$).

an automated cell counter can result in a artificially low cell count due to the ability of Sertoli cells to form junctional complexes and the counting of cell aggregates. This is a particular problem at high cell densities. Therefore, quantitation of DNA values was used in the current study to eliminate this variable. Determination of the number of cells associated with DNA is difficult due to tight junctions between Sertoli cells, but has been estimated at 8×10^5 cells/10 μg DNA. Combined observations indicate that further investigation of the hormonal regulation of Sertoli cell function must use optimum cell densities, a culture period during which both transferrin and ABP are maximally stimulated, and normalization of data with DNA analysis on cells harvested at the time of medium collection. Optimization of these culture parameters is essential in determining the actions of regulatory agents of transferrin and ABP production.

To further investigate the regulation of Sertoli cell function the synthesis of transferrin and ABP by Sertoli cells and steady state levels of mRNA were evaluated. Due to the absence of intracellular pools of these proteins, synthesis of transferrin and ABP was determined by the level of protein produced and secreted. Previous studies have shown that an increase in protein secretion by Sertoli cells correlates with an increase in the incorporation of radioactive amino acid precursors (13). Gene expression was assessed by determination of steady state levels of mRNA which could be influenced through either transcription or translation of the messages. Therefore, the current study examines only overall gene expression of transferrin and ABP and does not address at which level this expression is regulated. To determine whether the effects observed on mRNA levels were not due to a nonspecific increase in the expression of total mRNA, a constitutively produced protein, cyclophilin (29), was investigated. The regulatory agents evaluated were not found to influence cyclophilin gene expression in either a Northern blot analysis or a solution hybridization assay. Therefore, the effects on transferrin and ABP mRNA levels observed do not appear to be the result of a nonspecific effect on total message expression. The use of a solution hybridization procedure provides the most quantitative method to assess steady state levels of mRNA. The concentrations of regulatory agents used were maximal, as determined from dose-response curves previously reported (13).

FSH has been shown to be an important regulator of Sertoli cell function and was found to stimulate both transferrin and ABP synthesis 2-fold. This confirms previous observations that FSH can stimulate both ABP (7–9, 15, 16, 18, 19, 22, 24) and transferrin (12, 13, 15, 17, 21) production. In general, most Sertoli cell functional parameters examined are stimulated by treatment of the cells with FSH (15, 30, 31). Previous reports concerning a lack of transferrin stimulation by FSH may not have used optimal culture conditions, particularly cell density (19). FSH has also been shown to stimulate both transferrin and ABP mRNA levels (10–12). Interestingly, the magnitude of the FSH stimulation of transferrin mRNA levels was reduced (only a 1.25 fold-increase) from that expected from the effects on protein synthesis previously described (12). In contrast, FSH stimulation of ABP mRNA levels was greater than expected from the protein synthesis data (a 2-fold increase) and similar to the maximal stimulation observed. Therefore, while FSH stimulated transferrin and ABP synthesis in a similar manner (a 2-fold increase), effects on mRNA levels were different, with the magnitude of transferrin stimulation being low and that of ABP stimulation being high. These altered effects of FSH on the magnitude of the stimulation of mRNA levels were the only major differences detected in the current study regarding the regulation of transferrin and ABP gene expression and synthesis. The reason for this difference may be related to the relative abundance of the two

proteins and/or to the translational *vs.* transcriptional control of transferrin *vs.* ABP gene expression. Although the magnitude of effects on mRNA levels were different, observations presented indicate that FSH stimulates both transferrin and ABP gene expression and synthesis.

These observations regarding FSH actions were extended by an analysis of the actions of an intracellular second messenger, cAMP. FSH actions on Sertoli cell function and differentiation have been shown to be mediated by an increase in cAMP (32) and confirmed by data, presented in Table 1, for both cellular and excreted cAMP levels. This observation suggests that pharmacological agents that mimic the actions of cAMP would also mimic the actions of FSH. Sertoli cells treated with a cAMP analog, dcAMP, had an increase in the production of both transferrin and ABP. The magnitude of the stimulation obtained with dcAMP was slightly higher than that found with FSH alone, but did not result in a maximal stimulation of the cell. Therefore, additional signal transduction events are required to maximally stimulate transferrin and ABP production. The concentrations of dcAMP required to stimulate transferrin and ABP production were the same. This observation implies that the levels of cellular cAMP required to stimulate transferrin and ABP production are the same and could not differentially regulate production of the two proteins. Combined results indicate that regulatory agents that act through a cAMP-mediated signal transduction mechanism will probably regulate transferrin and ABP production by Sertoli cells in a similar manner.

Insulin was also found to stimulate both transferrin and ABP mRNA levels and synthesis in a similar manner. Increased transferrin and ABP production by insulin treatment of Sertoli cell cultures has previously been reported (13, 16). The actions of insulin on Sertoli cells requires a high concentration (5 μg/ml), which has previously been shown to most likely act through the insulin growth factor (IGF) receptors (33). Sertoli cells contain IGF receptors (34), and IGF is postulated to be the physiologically important regulator of Sertoli cell function (33, 34). Retinol also stimulated both transferrin and ABP mRNA levels and synthesis, as previously reported (12, 13, 16). Retinoids are important in the maintenance of spermatogenesis (35) and have been shown to maintain Sertoli cell function *in vivo* using molecular probes to testicular transferrin (12). Both retinol and retinoic acid can stimulate transferrin production (13), and which retinoid metabolite is physiologically important is under investigation. Although individually retinol, insulin, and FSH can stimulate transferrin and ABP gene expression and synthesis, a combination of these regulatory agents is required to obtain maximal stimulation. Previously, FIRT has been shown to stimulate both transferrin (13, 17) and ABP (16, 18) production maximally. In the current study FIR was found to have the same effects as FIRT. FIR stimulated transferrin and ABP gene expression 2-fold and synthesis greater than 4-fold. Observations indicate that testosterone had no detectable contribution to the stimulatory activity of FIR. The actions of insulin and retinol indicate that transferrin and ABP mRNA levels and synthesis can be regulated by cAMP-independent mechanisms. Therefore, the maximal stimulation of transferrin and ABP gene expression and synthesis appears to require the combined actions of cAMP and alternate signal transduction pathways.

Testosterone had no detectable effect on either transferrin or ABP mRNA levels or synthesis. The lack of a testosterone response with Sertoli cell cultures has previously been reported for both transferrin (13) and ABP (11, 24) synthesis. Peritubular cell contaminates in Sertoli cell cultures have been shown to indirectly mediate the testosterone stimulation of Sertoli cell function (24). A paracrine factor, P-Mod-S, produced by peritubular cells under androgen control can have significant effects on Sertoli cell functions (17, 36). For these reasons peritubular cell contaminates in Sertoli cell cultures will need to be taken into consideration in an analysis of testosterone effects on Sertoli cells. Direct effects of testosterone on Sertoli cell function have been reported (31), but now need to be evaluated, taking into consideration cell-cell interactions in the testis. Observations presented in the current study imply that testosterone does not play a major role in regulating transferrin or ABP mRNA levels or synthesis *in vitro* and support the postulate that peritubular cell-Sertoli cell interactions mediated via P-Mod-S may provide a primary mode of androgen action on the Sertoli cell.

With the use of optimal cell culture conditions, the agents evaluated regulated transferrin and ABP production in a similar manner. Although *in vitro* conditions can be altered to obtain an apparent differential regulation, the physiological significance of these *in vitro* conditions to the regulation of Sertoli cell function remains to be investigated. Observations presented in the current study demonstrate that transferrin and ABP mRNA levels and synthesis can be stimulated and regulated in a coordinate manner. The regulation observed probably occurs at several levels, such as transcription, translation, processing, and secretion. The ability of FSH to have dramatic effects on ABP gene expression while having small effects on transferrin gene expression support this speculation. Although no evidence of a differential regulation of transferrin and ABP production was observed in the current study, other regulatory agents may potentially influence ABP and transferrin expression differentially. Cellular functions that may be differentially regulated will either not be responsive to a

specific signal transduction pathway or be suppressed by that pathway. Alternatively, a unique regulatory agent may have a very specific transduction mechanism, such that selected cellular functions can be regulated. Therefore, differential regulation of Sertoli cell function will require the presence of specific cellular functions that are nonresponsive or suppressed by general second messengers, such as cAMP, or the presence of highly specific regulatory agents. These possibilities clearly exist because of the large number of unknown Sertoli cell functions and *in vivo* regulatory agents.

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