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Hormonal Regulation and Differential Actions of the Helix-Loop-Helix Transcriptional Inhibitors of Differentiation (Id1, Id2, Id3, and Id4) in Sertoli Cells*

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ABSTRACT

The testicular Sertoli cells support spermatogenesis by providing a microenvironment and structural support for the developing germ cells. Sertoli cell functions are regulated by the gonadotropin FSH. Sertoli cells become a terminally differentiated nongrowing cell population in the adult. In response to FSH, the Sertoli cells express a large number of differentiated gene products, such as transferrin, which transports iron to the developing germ cells. Previously, members of the basic helix-loop-helix (bHLH) family of transcription factors have been shown to influence FSH-mediated gene expression in Sertoli cells. The functions of the bHLH proteins are modulated by Id (inhibitor of differentiation) proteins, which lack the DNA-binding basic domain. The Id proteins form transcriptionally inactive dimers with bHLH proteins and thus regulate cell proliferation and differentiation. The current study investigated the expression and function of Id proteins in the postmitotic Sertoli cell. Freshly isolated and cultured Sertoli cells coexpress all four isoforms of Id (Id1, Id2, Id3, and Id4), as determined by immunoprecipitation with isoform-specific anti-Id antibodies, RT-PCR, and Northern blot analysis. Id2 and Id3 expression levels seem higher than Id1. Interestingly, the expression of Id4 in Sertoli cells is only detectable after stimulation with FSH or cAMP. The Id1 expression is down-regulated by FSH and cAMP, whereas Id2 and Id3 levels remain unchanged in response to FSH. In contrast, serum induces the expression of Id1, Id2, and Id3. Treatment of Sertoli cells with serum significantly reduces the expression of the larger 4-kb Id4 transcript and promotes the presence of a novel 1.3-kb transcript of Id4. The regulatory role of FSH in the expression of all four isoforms of Id is mimicked by a cAMP analog, suggesting that the actions of FSH are mediated through the protein kinase A pathway. An antisense approach was used to study the functional significance of Id proteins in Sertoli cells. Antisense to Id1 stimulated transferrin promoter activity in a transient transfection assay. Interestingly, an antisense to Id2 down-regulated transferrin promoter activity. Id3 and Id4 antisense oligonucleotides had no effect on FSH-mediated transferrin promoter activation. Contrary to the hypothesis that Id proteins have redundant functions, the results of the current study suggest that Id1, Id2, Id3, and Id4 are differentially regulated and may have distinct functions. Id1 may act to maintain Sertoli cell growth potential, whereas Id2 and Id4 may be involved in the differentiation and hormone regulation of Sertoli cells. (*Endocrinology* **142:** 1727–1736, 2001)

SERTOLI CELL FATE is established in the embryonic gonad at the time of testis determination (1, 2) and is followed by a phase of rapid cell proliferation and differentiation. During puberty, the final phase of Sertoli cell differentiation occurs, which is marked by irreversible changes in Sertoli cell morphology and physiology (3). These changes include the formation of the blood testis barrier, which is needed for the proper microenvironment and cytoarchitectural support for the developing germ cells (4); expression of a large number of specific gene products (5); and the development of a postmitotic cell cycle phase. In the adult, the Sertoli cell is a terminally differentiated cell population (3). An example of a Sertoli cell differentiated gene product is the iron-binding protein transferrin, which transports iron to the developing germ cells (6, 7).

The majority of the Sertoli cell functions are regulated by the gonadotropin FSH (7, 8). FSH acts on Sertoli cells via the protein kinase A pathway (7–9). The actions of FSH on Sertoli cells may also involve other signal transduction pathways, including protein kinase C and calcium mobilization (10). Together, these signal transduction pathways phosphorylate and activate a number of transcription factors, such as cAMP response element-binding protein (9), C/EBPβ (11), c-*fos* (12), c-*myc* (13), GATA-1 (14), SF-1 (15), and WIN (16). It is speculated that the activation and combinations of many of these transcription factors are responsible for the transcription of Sertoli cell-specific gene expression.

Recently, Sertoli cells have been shown to express members of the basic helix-loop-helix (bHLH) transcription factor family. The bHLH family of transcription factors are critical cell-type determinants and play important roles in cell growth and differentiation. A basic helix-loop-helix domain that is conserved from yeast to mammals characterizes members of this family (17). The bHLH domain consists of two amphipathic helixes separated by a loop that mediates homo- and heterodimerization adjacent to a DNA-binding region rich in basic amino acids (18). The bHLH dimers bind to an E Box (CANNTG) DNA consensus sequence present in a wide variety of tissue-specific promoters (19, 20). The E box domain has been shown to influence the promoters of a number of Sertoli cell-specific genes, including transferrin (21), c-*fos* (22), SF-1 (23), and FSH receptor (24).

The bHLH proteins have been classified into two distinct classes. The ubiquitously expressed class A bHLH proteins

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consist of E2–2 (25), HEB (26), and E12 and E47 [the differentially spliced products of the E2A gene (20)], which dimerize with tissue-restricted and developmentally regulated class B proteins such as MyoD and neuroD (27, 28). Previous observations suggest that the Sertoli cells express the class A proteins E47 (29) and REBα (30) (the rat isoform of human HEB). Sertoli cell-specific class B bHLH proteins are yet to be determined. Recent reports suggest that bHLH proteins regulate FSH-stimulated Sertoli cell gene expression (21, 22, 31).

The members of the Id (inhibitor of differentiation/DNA binding) family modulate the transcriptional activity of class A and B bHLH heterodimers. The four known Id proteins (Id1, Id2, Id3, and Id4) share a homologous HLH domain, but lack the basic DNA binding region (32, 33). Thus, the Id proteins act to sequester bHLH proteins by forming inactive dimers to prevent binding of bHLH proteins to the E-box sites (34–36). Therefore, Id proteins are largely considered as dominant negative regulators of differentiation pathways (37–39). Some of the activities of Id2, such as induction of apoptosis, have been shown to reside in the N-terminal domain and are independent of HLH-mediated dimerization (40). Recent studies have also demonstrated that Id2 may act as an inhibitor of proliferation and is required for the determination and maintenance of the differentiated state of alveolar epithelial cells (41). The expression of Id4, unlike other members of the Id family, is tissue-restricted and is expressed primarily in adult brain, kidney, and testis (42–44).

Interestingly, terminally differentiated Sertoli cells also express Id proteins (45). The induction of Id in various cell types has been studied in response to serum, which is known to induce cell proliferation. Because the Sertoli cell is a terminally differentiated and postmitotic cell type, the expression and function of Id proteins are unclear. The present investigation was designed: to understand whether members of the Id family are differentially expressed in response to FSH, and to determine whether these proteins influence Sertoli cell differentiated functions.

Materials and Methods

Isolation of Sertoli cells

Sertoli cells were isolated from the testis of 20-day-old rats by a modified procedure described earlier (46, 47). All animal use and procedures were approved by the University Animal Care Committee. The isolated Sertoli cells were more than 98% pure and were plated under serum-free conditions. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Life Technologies, Inc., Rockville, MD) with 0.01% BSA at 32 C. Sertoli cells were treated with either FSH (100 ng/ml; o-FSH-16, National Pituitary Program, Torrance, CA), dibutryl cAMP (100 μM), 10% bovine calf serum, or vehicle alone (Ham's F-12, control). These optimal concentrations of FSH and cAMP have previously been shown to optimally stimulate cultured Sertoli cell differentiated functions (48, 49). The cells were cultured for a maximum of 5 days, with a media change and treatment after 48 h of culture. Cell number, purity, and viability did not change during the culture, in the absence or presence of treatment.

Western blotting and immunoprecipitation

Sertoli cells were cultured in 150-mm plates and were treated with FSH and cAMP as above. After 72 h of treatment, the cells were washed twice with HBSS and lysed with 1 ml M-PER lysis buffer (Pierce Chemical Co., Rockford IL) supplemented with miniprotein protease inhibitor cocktail (Boehringer Ingelheim GmbH, Indianapolis, IN) at 4 C for 30 min. Lysates were centrifuged at 10,000 $\times$ *g* for 30 min at 4 C, and supernatants were collected. The protein concentration in the supernatants was estimated using Bradford's assay (Bio-Rad Laboratories, Inc., Hercules, CA). Approximately 50–150 μg protein in SDS sample loading buffer was boiled for 5 min and electrophoresed on a 4–20% gradient mini-SDS gel (Bio-Rad Laboratories, Inc.). The protein was subsequently transferred onto nitrocellulose membrane and probed with specific antibodies to Id1, Id2, Id3, and Id4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specific antigen-antibody complex was visualized using an alkaline phosphatase chemiluminescent detection kit (Bio-Rad Laboratories, Inc.).

For immunoprecipitation, the Sertoli cells were cultured in 150-mm plates and were treated with FSH and cAMP as above. After 72 h of treatment, the cells were rinsed three times with HBSS and labeled with 50 μl/0.5 ml [^{35}S]methionine for 4 h at 32 C in methionine free DMEM (Life Technologies, Inc.). The cells were then washed with PBS and lysed at 4 C for 30 min with 1 ml M-PER lysis buffer (Pierce Chemical Co.) supplemented with miniprotein protease inhibitor cocktail (Boehringer Ingelheim GmbH). Lysates were centrifuged at 10,000 $\times$ *g* for 30 min at 4 C, and supernatants were collected. Each supernatant was incubated with 5 μl normal rabbit IgG and 50 μl packed protein A-agarose beads (Santa Cruz Biotechnology, Inc.) at 4 C for 1 h. The complex formed was removed by centrifugation at 500 $\times$ *g* for 5 min at 4 C. The supernatant was then incubated with 5 μl of either antirabbit Id1, Id2, Id3, or Id4 antibodies for 4 h at 4 C. Immunoprecipitates were collected on protein A-agarose beads and washed four times with M-PER lysis buffer and two times in TSA buffer (10 mM Tris pH8.0, 140 mM NaCl). The precipitate was dissolved in 30 μl SDS sample buffer subjected to electrophoresis on a 4–20% gradient mini SDS gel. The ^{14}C-methylated protein markers (Amersham Pharmacia Biotech, Piscataway, NJ) was used to determine approximate sizes of the fractionated immune complexes. The gels were fluorographed according the procedure of Skinner and Griswold (50).

RNA preparation

Freshly isolated or cultured Sertoli cells were lysed directly using TRI Reagent (Sigma, St. Louis, MO). The cell lysate was then passed several times through a Pasteur pipette to form homogenous lysate. To avoid any possible contamination of extracellular material and high-molecular-weight DNA in the final RNA samples, the homogenate was centrifuged at 12,000 $\times$ *g* for 10 min at 4 C. Total RNA was then isolated from the cell lysate, following the manufacturer's protocol for RNA isolation using TRI Reagent. The final RNA pellet was dissolved in distilled water at a concentration of 1 mg/ml.

PCR

Total RNA (2 μg) was reverse transcribed in a final vol of 20 μl containing 20 U RNasin (Promega Corp., Madison, WI); 200 μM each of deoxy-ATP, deoxycycidine triphosphate, thymidine 5′-triphosphate, and deoxy-GTP; 1 μg oligo dT (Pharmacia, Peapack, NJ), 10 μM dithiothreitol, and 200 U MMLV reverse transcriptase (Life Technologies, Inc.) in the MMLV first-strand synthesis buffer supplied by the manufacturer (Life Technologies, Inc.). The RNA and oligo dT primer in the buffer were first denatured for 5 min at 65 C, then cooled on ice before addition of nucleotides and enzyme. The reverse transcriptase reaction was carried out at 37 C for 1 h. PCR was performed using the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT) with 30 cycles as follows: 94 C, 1 min (denaturation); 58C, 2 min (primer annealing); and 72 C, 1 min (primer extension). Each PCR reaction contained 250 pg reverse-transcribed DNA, 1 μM of each 5′ and 3′ oligonucleotide primers; 2.5 U *Taq* polymerase (AmpliTaq, Perkin-Elmer Cetus), 200 μM of each deoxy-ATP, deoxycycidine triphosphate, deoxy-GTP, and thymidine 5′-triphosphate.

The primer pair sequences used were obtained from published sequences of Id1, 2, 3, and 4 (Table 1) and synthesized from commercial sources. The possible contamination of RNA with DNA was distinguished by performing the RT reaction without MMLV reverse transcriptase. The absence of any product in the amplification reaction using such a reverse-transcribed preparation indicated the absence of any contaminating DNA in our RNA samples.

Each RT reaction was performed using three different samples. The

TABLE 1. Sequences of isoform and species specific Id primers, antisense and scrambled oligonucleotides

Id isoform	Species	Accession no.	PCR primers/ antisense/scrambled	5′ Primer (start bp); 3′ primer (end bp) antisense—end bp
Id1	Rat	D10862	PCR	5 primer (190 bp) 5′-tgg acg aac agc agg tga acg-3′ 3′ Primer (433 bp) 5′-gca ctg atc tcg ccg ttc agg-3′
			Antisense	50 bp 5′-gac ctt cat gat cct-3′
			Scrambled	5′-ggt tct acc taa cct-3′
Id2	Rat	D10863	PCR	5′ primer (69 bp) 5′-agc ctt cag tcc ggt gag gtc c-3′ 3′ Primer (419 bp) 5′-tca gac gcc tgc aag gac agg-3′
			Antisense	71 bp 5′-gct ttc atg ctg ctc-3′
			Scrambled	ggt tct acc taa cct
Id3	Rat	D10864	PCR	5′ primer (42 bp) 5′-tgc tac gag gcg gtg tgc tg-3′ 3′ Primer (328 bp) 5′-agt gag ctc agc tgt ctg gat cgg-3′
			Antisense	23 bp 5′-cgc ctt cat gtt gga-3′
			Scrambled	5′-acg tat gct gcg tct-3′
Id4	Mouse	X75018	PCR	5′ primer (268 bp) 5′-gcg ata tga acg act gct ac-3′ 3′ Primer (538 bp) 5′-tca ccc tgc ttg ttc acg gc-3′
			Antisense	83 bp 5′-cac cgc ctt cat cgc gcg-3′
			Scrambled	5′-cgc tac ctt ctc cgc-3′

PCR-based amplification reactions were carried out in duplicate on each reverse-transcribed RNA sample. Simultaneous PCR reactions were also carried out using primers designed to rat cyclophilin to monitor the efficiency of the PCR. Cyclophilin was faithfully amplified in all the PCR reactions, indicating consistency in the quality of RT and PCR reactions. The data presented is therefore a representative of three different RT-PCR reactions carried out in duplicate.

Subcloning and sequencing

After amplification, the PCR products of each reaction were subjected to electrophoresis through 1.5% agarose gel in buffer, and the products were visualized by ethidium bromide staining. The bands were then dissected out, and the DNA was isolated from the gel using Glass MAX DNA isolation system (Life Technologies, Inc.). The purified DNA fragments were subcloned into pGEM-TEZ (Promega Corp.) plasmid. The cloned DNA fragments were sequenced using standard M13 forward and reverse primers in an automated fluorescence-based sequencer (PE Applied Biosystems, Norwalk ,CT). All the sequences reported are consensus of two different experiments. The sequence alignments [Genetics Computer Group (Madison, WI) DNA analysis software] were carried out using the available sequences of rat Id1, Id2, and Id3 and mouse Id4.

Northern blot analysis

Total RNA was extracted from Sertoli cells cultured in 6-well plates and treated with FSH, cAMP, serum, or vehicle alone (control), as above, using TRI-Reagent (Sigma). Approximately 10 μg total RNA was fractionated on a 1% formaldehyde-agarose gel. After fractionation, the RNA in the gel was transferred onto nylon membrane (Hybond$^+$ N, Amersham Pharmacia Biotech) in 10 $\times$ SSC buffer and UV-cross-linked as described previously (51). The membranes were then prehybridized in Quick Hybridization buffer (Stratagene, La Jolla, CA) for 30 min at 60 C. The hybridization was carried out at 60 C for 1 h with ^{32}P-labeled Id probes. The pGEM-tEZ plasmids containing the Id complementary DNA (cDNA) fragments obtained by RT-PCR of Sertoli cell RNA were used as templates to generate random primed (Stratagene) probes for Northern blotting. The membrane was subsequently stripped and rehybridized with the constitutively expressed rat cyclophilin. All the probes were labeled using prime-it II kit from Stratagene. X-OMAT AR Film (Eastman Kodak Co., Rochester, NY) was exposed to the membranes overnight at −80 C, and densitometry values were obtained by scanning with Imagequant Digital Image analysis system (Molecular Dynamics, Inc., Sunnyvale, CA). The densitometric values obtained for Id blots were normalized to the values of rat cyclophilin so as to control for variation in loading and determining the magnitude of expression.

Plasmids and antisense oligonucleotides

The CAT reporter plasmid (pUC8-CAT) containing −581 bp (−581 bp mTf-CAT) was generously provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA). The mouse transferrin promoter used in the present study included the transcriptional initiation site of the transferrin gene, which is 54 bp upstream of the start site of translation (52).

The antisense oligonucleotide to rat Id1, 2, and 3 and mouse Id4 was designed to incorporate 15 bases around and including the translational initiation site (Table 1). The scrambled oligonucleotides were generated by Genetics Computer Group software analysis package using the respective Id antisense oligonucleotides. The antisense and the scrambled oligonucleotides (showing no substantial homology to any known genes, as determined through a BLASTn search) were synthesized from commercial sources using phosphorothioate modification. The expression plasmid pCI-neo-Id2 was constructed. The human Id2 (GenBank Accession No. M97796) PCR primers were designed to amplify the human Id2 coding sequence (99–468 bp). The 369-bp Id2 PCR fragment obtained through RT-PCR of human SKOV3 cell line RNA was first subcloned in pGEM-T-EZ (Promega Corp.) plasmid. The pGEM-T-EZ plasmid containing the Id2 fragment was then digested with *Eco*RI, and the resulting fragment was ligated into *Eco*RI-digested pCIneo expression plasmid (Promega Corp.).

Transfections

Sertoli cells, cultured in 24-well plates at the density of 10^6 cells for 48 h, were transfected with a reporter gene construct by the calcium

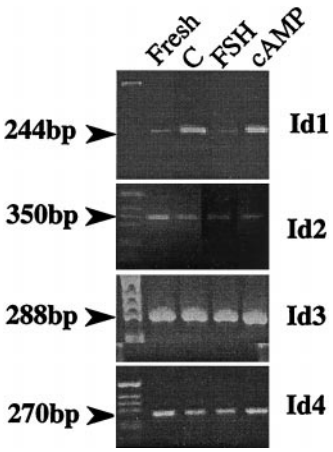

FIG. 1. Id1, Id2, Id3, and Id4 gene expression in cultured Sertoli cells, by RT- PCR. RT-PCR was performed on the RNA isolated from Sertoli cells cultured for 72 h in the presence of vehicle alone [control (C)], FSH, or dibutryl cAMP. The RT-PCR was performed using isoform-specific Id primers shown in Table 1. The approximate size of the transcript amplified by the PCR primers used is indicated. The data are representative of three separate PCRs carried out on at least three different RT-mRNA samples.

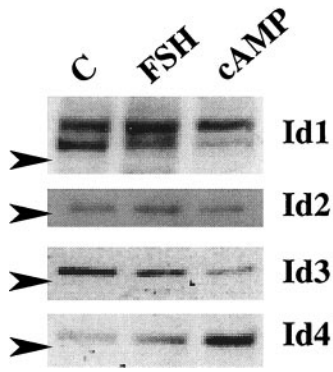

FIG. 2. Id1, Id2, Id3, and Id4 protein expression in cultured Sertoli cells. The Sertoli cells were cultured for 72 h in the presence of vehicle alone (C), FSH, or dibutryl cAMP. The cells were washed and metabolically labeled with ^{35}S-methionine. After cell lysis, the Id1, Id2, Id3, and Id4 proteins were immunoprecipitated with isoform-specific Id antibodies, electrophoresed on 4–20% SDS polycarylamide gels, and fluorographed. A *solid arrow* shows the location of the 14.3-kDa molecular mass marker. Two Id1 antibody immunoreactive bands, at approximately 16 and 20 kDa, were observed. Single immunoreactive bands with Id2, Id3, and Id4 antibodies at approximately 15, 20, and 18 kDa, respectively, were observed. The data are representative of three separate immunoprecipitation reactions carried out on at least three different Sertoli cell preparations.

phosphate method coupled with hyper osmotic shock (10% glycerol) as previously described (21, 53). Briefly, 1.5 μg reporter plasmid in 150 μl transfection buffer [250 mM $CaCl_2$, mixed 1:1 vol/vol with 2 × Hebes (28 mM NaCl, 50 mM HEPES, and 1.47 mM Na_2HPO_4, pH 7.05)[rsqb] was added to each well of a 24-well plate containing 1×10^6 Sertoli cells in 1 ml Ham's F-12 with 0.01% BSA, and incubation was performed at 32 C for 4 h. After incubation, the cells were subjected to a hyper osmotic shock. The medium was aspirated, and 1 ml 10% glycerol in HBSS (Life Technologies, Inc.) was added. The cells were incubated for 3 min, and the wells were washed twice before fresh Ham's F-12 was added. The transfected Sertoli cells were treated with 4 μM of either the antisense or scrambled oligonucleotide immediately after transfection. Various treatments were added to the cells 2 h after the addition of antisense oligonucleotides. The cells were retreated with the oligonucleotide every 12 h, for a total of 72 h. In each experiment the transfection efficiency was monitored by transfecting the Sertoli cells with a plasmid containing the β-galactosidase gene driven by a CMV promoter. Subsequent staining and counting the cells expressing β-galactosidase (blue color) resulted in approximately 25% transfection efficiency.

CAT assay

Assay of CAT activity was performed as follows: medium was removed from the wells, and the cells were washed once with PBS. One hundred microliters of the cell lysis buffer (Promega Corp.) was added to each well, and incubation was carried out for 15 min at room temperature. The wells were then scraped, and buffer was collected in 1.5-ml microfuge tubes. Tubes were heated to 65 C for 10 min to inactivate endogenous acetylases and then centrifuged at 12,000 × *g* for 10 min at 4 C to remove cell debris. An aliquot of cell extract (54 μl) was mixed with 65 μl 0.25-M Tris (pH 8.0), 25 μg *N*-butyryl coenzyme A (5 mg/ml; Sigma), and 0.1 μCi(1 μl) of ^{14}C-chloramphenicol (ICN, Costa Mesa, CA) and incubated overnight at 37 C. The mixture was extracted once with 300 μl mixed xylenes and back-extracted with 100 μl 0.25-M Tris (pH 8.0). A 200-μl aliquot of the organic phase was counted in a scintillation counter to determine the relative amount of CAT activity. The average conversion of CAT substrate for treated cells ranged between 20 and 30%. This assay was found to be linear with the protein concentration used. The CAT reporter plasmid without the mTf promoter was used as negative control. In response to FSH and dbcAMP, the relative CAT activity of the negative control plasmid was in the range of 1.5–2.

Statistical analysis

All transfection data were obtained from a minimum of three different experiments unless otherwise stated. Each data point (from treatments) was converted to a relative value, with the mean and SEM from multiple experiments determined as indicated in the figure legends. Data were analyzed by an ANOVA with the SAS Institute, Inc. (Durham, NC) statistical package as indicated in the figure legends. When stated, data were also analyzed with a Student's *t* test, with a comparison between the control and treatment group.

Results

Primary cultures of Sertoli cells, prepared from 20-day-old rat testis, were analyzed during culture for the expression of Id1, Id2, Id3, and Id 4 gene expression, by RT-PCR using specific Id primers (Table 1). As shown in Fig. 1, all four members of the Id family are expressed in the Sertoli cells. Cloning and sequencing of the PCR products confirmed the identity of the Id1, Id2, Id3, and Id4 transcripts. The four Id transcripts were observed when the cultured Sertoli cells were untreated or treated with FSH or the cAMP analog dbcAMP for 72 h. This PCR procedure demonstrates the absence or presence of the Id expression, whereas the Northern blot procedure below was used to quantitate messenger RNA (mRNA) levels. Expression of the Id transcripts was also observed in freshly isolated Sertoli cells, whole 20-day-old testis, and embryonic day-15 testis (data not shown). To extend this analysis, the expression of the Id proteins was determined. Western blotting of 50–150 μg total Sertoli cell protein, extracted after 72 h of culture, was performed with isoform specific Id antibodies. The immunoreactivity alone was low and inconsistent, presumably because Id proteins are expressed at low levels and have a short half-life (54). To circumvent this problem, immunoprecipitation was performed on total Sertoli cell proteins metabolically labeled with ^{35}S-methionine. The labeled proteins were immunoprecipitated with isoform-specific Id antibodies, electrophoretically separated on SDS polyacrylamide gels and fluorographed. As shown in Fig. 2, Id1, Id2, Id3, and Id4 proteins were detectable in Sertoli cells. These observations demon-

strate that all four members of the Id family are expressed in Sertoli cells. Single bands were observed for Id2 (14 kDa), Id3 (20 kDa), and Id4 (18 kDa), whereas two bands at 16 and 20 kDa were observed for Id1.

Northern blot analysis was performed to explore the potential regulatory role of FSH on Id gene expression in Sertoli cells. Cultured Sertoli cells were treated with FSH for 72 h, and the RNA was examined by Northern blot analysis using specific Id1, Id2, Id3, and Id4 cDNA probes. The cDNA used for probe was the isoform-specific Id RT-PCR subcloned product (Fig. 1). A single 1.2-kb Id1 (Fig. 3A) and 1.6-kb Id2 (Fig. 4A) transcript was observed after exposure of the Northern blot. Two Id3 transcripts (Fig. 5A) of approximately 1.8 and 1.4 kb were observed in Sertoli cells after exposure of the Northern blots. The presence of two Id3 mRNA transcript sizes in Sertoli cells (Fig. 5A) is a novel observation (55) and may be attributable to modifications in the 3′ untranslated region. The 3′ modification is presumably a result of differential splicing or alternatively used poly adenylation sites within the 3′ untranslated region. Such a modification at the 3′ end has also been reported previously for Id4, which results in multiple mRNA transcripts (approximately 1.7, 2.8, and 4 kb) (56, 57). Multiple transcripts of Id4 were also observed when RNA from Sertoli cells treated with FSH was hybridized with Id4 cDNA (Fig. 6A). The Northern blot analysis confirms the RT-PCR (Fig. 1) and immunoprecipitation (Fig. 2) data, indicating that all four isoforms of Id are expressed in Sertoli cells. Based on the normalization of the Northern blots with a constitutively expressed cyclophilin gene, it is suggested that the Id genes are expressed at different levels. Id2 and Id3 genes show the highest levels of expression, followed by Id1, and the expression of Id4 is the lowest in control untreated Sertoli cells (data not shown). Previous studies have shown cyclophilin to be constitutively expressed and not affected by hormone treatment of Sertoli cells (16, 21, 31).

Id genes are primarily expressed in undifferentiated and growing cells and represent a part of the primary genomic response to stimulation with serum and growth factors (58). Id genes are down-regulated in quiescent cells during differentiation (38). The gonadotropin FSH is known to regulate

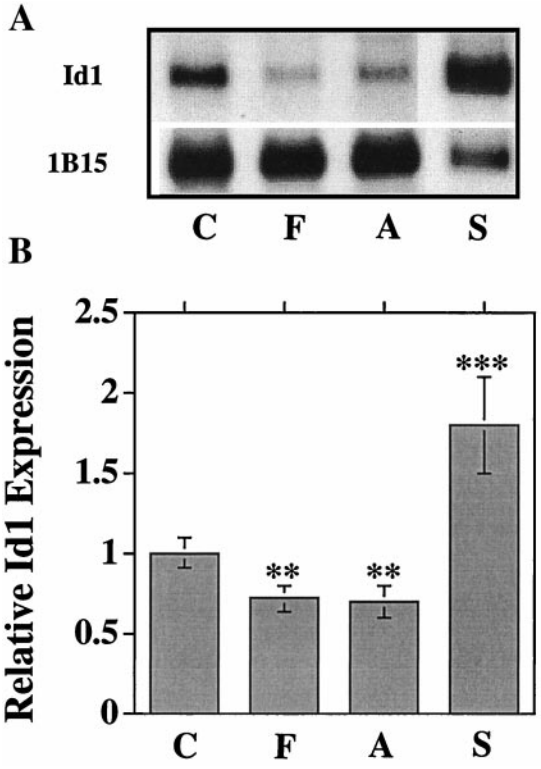

FIG. 3. Northern blot analysis of Id1. A, The predicted Id1 transcript size of approximately 1.2 kb was detected when approximately 10 μg total RNA from cultured Sertoli cells, treated with either vehicle alone (C), FSH (F), cAMP (A), or serum (S), was probed with random primed 244-bp Id1 cDNA. Also, *at the bottom*, is the blot for the constitutively expressed cyclophilin gene (1B15). The data are representative of three different Northern blots performed on separate RNA samples collected at different times. B, Scanning densitometry of the blots was used to quantitate the bands with the mean ± SEM presented for three different experiments. Data were normalized for cyclophilin expression previously shown to be unaffected by hormones. The data are presented as relative expression (mean ± SEM), in relation to the expression of Id1 from cultured Sertoli cells treated with vehicle alone (Control) set to 1. **, $P < 0.01$; ***, $P < 0.001$.

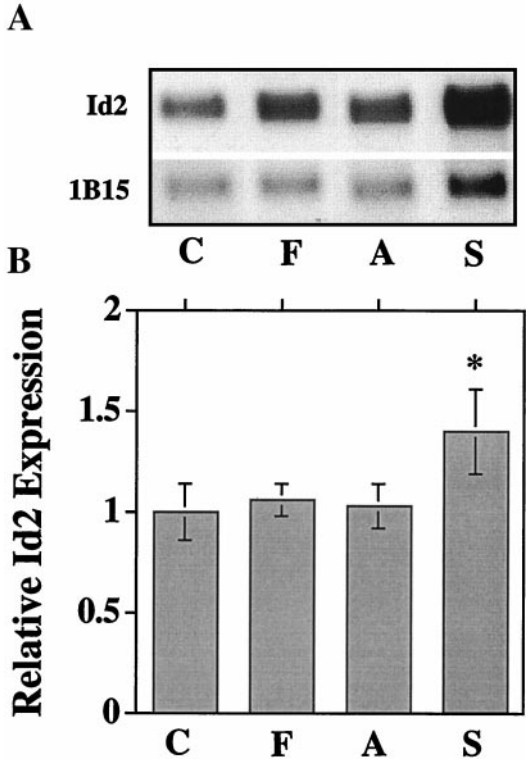

FIG. 4. Northern blot analysis of Id2. A, The predicted Id2 transcript size of approximately 1.6 kb was detected when approximately 10 μg total RNA from cultured Sertoli cells, treated with either vehicle alone (C), FSH (F), cAMP (A), or serum (S), was probed with random primed 350-bp Id2 cDNA. Also, *at the bottom*, is the blot for the constitutively expressed cyclophilin gene (1B15). The data are representative of three different Northern blots performed on separate RNA samples collected at different times. B, Scanning densitometry of the blots was used to quantitate the bands with the mean ± SEM presented for three different experiments. Data were normalized for cyclophilin expression previously shown to be unaffected by hormones. The data are presented as relative expression (mean ± SEM), in relation to the expression of Id2 from cultured Sertoli cells treated with vehicle alone (Control) set to 1. *, $P < 0.05$.

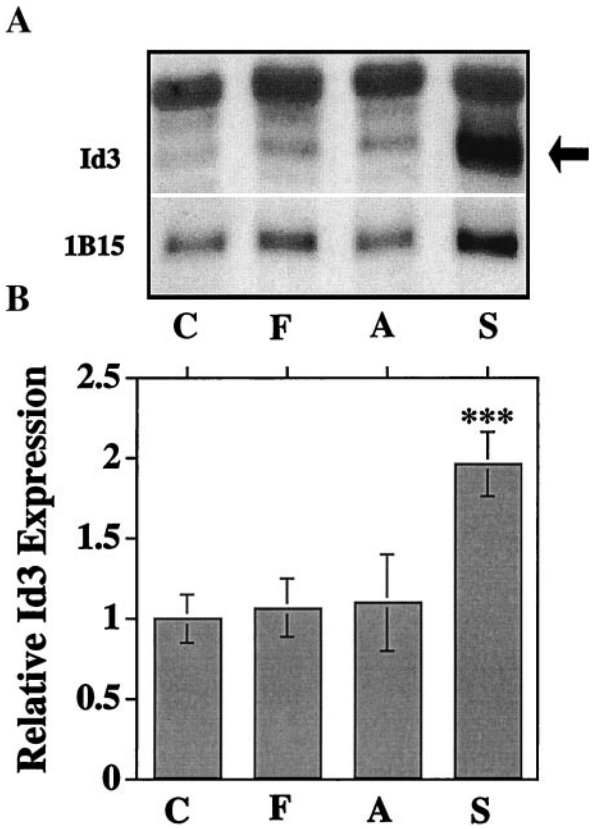

FIG. 5. Northern blot analysis of Id3. A, The predicted Id3 transcript size of approximately 1.5 kb was detected when approximately 10 μg total RNA from cultured Sertoli cells, treated with either vehicle alone (C), FSH (F), cAMP (A), or serum (S), was probed with random primed 288-bp Id3 cDNA. In addition to the 1.4-kb transcript, a larger 1.8-kb Id3 transcript was also observed. Also, *at the bottom*, is the blot for the constitutively expressed cyclophilin gene (1B15). The data are representative of three different Northern blots performed on separate RNA samples collected at different times. B, Scanning densitometry of the blots was used to quantitate the 1.4-kb band with the mean ± SEM presented for three different experiments. Data were normalized for cyclophilin expression previously shown to be unaffected by hormones. The data are presented as relative expression (mean ± SEM), in relation to the expression of Id1 from cultured Sertoli cells treated with vehicle alone (Control) set to 1. ***, $P < 0.001$.

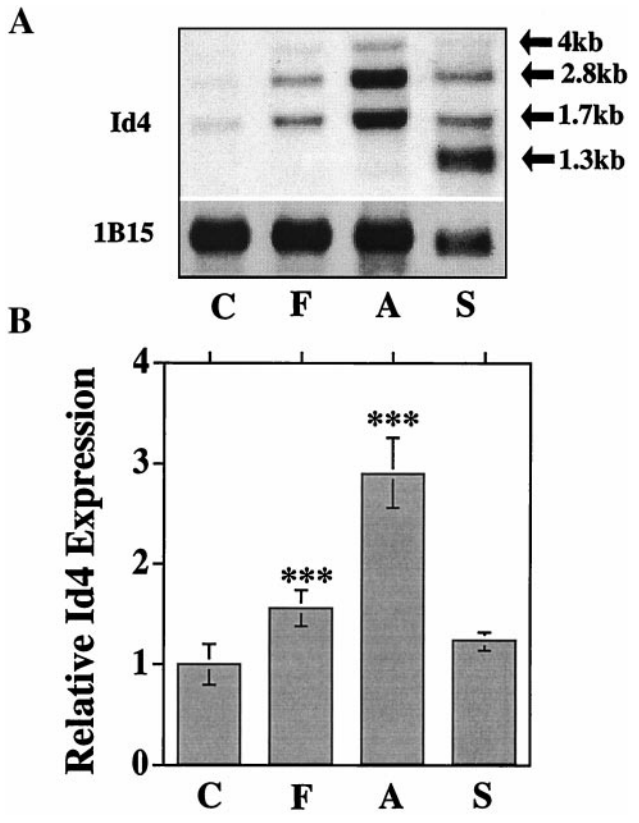

FIG. 6. Northern blot analysis of Id4. A, The three predicted Id4 transcript sizes of approximately 1.7 kb, 2.8 kb, and 4 kb were detected when approximately 10 μg total RNA from cultured Sertoli cells, treated with either vehicle alone (Control, C), FSH (F), cAMP (A), or serum (S), was probed with random primed 270-bp Id4 cDNA. After treatment of Sertoli cells with serum, the larger 4-kb transcript was undetectable; and instead, a smaller 1.3-kb transcript was observed in addition to the 1.7-kb and 2.8-kb transcripts. Also, *at the bottom*, is the blot for the constitutively expressed cyclophilin gene (1B15). The data are representative of three different Northern blots performed on separate RNA samples collected at different times. B, Scanning densitometry of the blots was used to quantitate the 1.7-kb band with the mean ± SEM presented for three different experiments. Data were normalized for cyclophilin expression previously shown to be unaffected by hormones. The data are presented as relative expression (mean ± SEM), in relation to the expression of Id1 from cultured Sertoli cells treated with vehicle alone (Control) set to 1. ***, $P < 0.001$.

Sertoli cell differentiated functions (59). To evaluate the effect of FSH on the expression levels of various isoforms of Id in Sertoli cell, autoradiograms of the Northern blots of all the four isoforms of Id and the constitutively expressed gene cyclophilin were subjected to densitometric scans. Results indicated that FSH differentially regulates the expression of Id genes in Sertoli cells. The Id1 mRNA levels reduced by 30% (Fig. 3B), whereas the steady-state levels of Id2 (Fig. 4B) and Id3 (Fig. 5B) mRNA remained essentially unchanged in response to FSH. Interestingly, FSH stimulated a significant increase (>50%) in the levels of all three Id4 transcripts (Fig. 6B). The quantitative data presented were performed on the 1.7-kb transcript of Id4.

The actions of FSH on Sertoli cells are primarily through the cAMP protein kinase A pathway (9). To explore the potential regulatory role of the cAMP pathway on Id gene expression in Sertoli cells, a cell permeable cAMP analog known to promote cell differentiation was added to the cells, and the expression of various isoforms of Id was evaluated. Similar to the effect of FSH, cAMP significantly reduced (30%) the expression of Id1 (Fig. 3). Treatment of Sertoli cells with cAMP had no effect on the levels of Id2 (Fig. 4) and Id3 (Fig. 5). The effect of cAMP on Id4 expression (all three transcripts) was more dramatic than FSH, which resulted in more than a 3-fold increase in the levels of Id4 over the controls (Fig. 6). Taken together, these observations suggest that the expression of Id1 and Id4 in Sertoli cells is influenced by FSH through the cAMP-protein kinase A pathway.

Previous literature suggests that the expression of Id is inducible by serum (58). To assess the effect of serum on Sertoli cell Id expression, the cells were treated with 10% bovine calf serum. Treatment of Sertoli cells with serum significantly stimulated Id1 and Id2 expression (Figs. 3 and 4). The expression of the 1.4-kb Id3 transcript was signifi-

cantly increased in response to serum, whereas the expression of the larger 1.8-kb transcript remained unchanged. In contrast, the expression of Id4 (1.7 kb) transcript was significantly decreased (Fig. 6). Surprisingly, the 4-kb Id4 transcript was virtually undetectable in the Northern blot analysis after serum treatment (Fig. 6A). However, a novel 1.3-kb Id4 transcript was observed after serum treatment (Fig. 6A). This novel 1.3-kb Id4 transcript was expressed at relatively high levels, compared with the 2.8-kb and 1.7-kb Id4 transcripts (Fig. 6A). Observations demonstrate opposing effects of FSH and serum on expression of the Id genes. The expression of Id1 (Fig. 3) and Id4 (Fig. 6), in response to these agents, is particularly interesting and suggests that both these isoforms may have opposing functions in Sertoli cells.

An antisense approach was used to better understand the role of Id1, Id2, Id3, and Id4 in regulating Sertoli cell differentiated functions. Sertoli cells from 20-day-old rats, cultured under serum-free conditions, were transfected with a proximal transferrin-promoter CAT construct (Tf-CAT) as a marker of Sertoli cell differentiation (6, 60). The DNA phosphorothioate modified antisense oligonucleotides covering the region around the ATG initiation codons of rat Id1, Id2, and Id3 and mouse Id4 (Table 1) were added to the Sertoli cells and were subsequently treated with either FSH, cAMP, or serum. As expected, FSH, cAMP, and serum stimulated the Tf-CAT activity, reflecting their ability to promote Sertoli cell differentiation (Fig. 7). In the presence of Id1 antisense oligonucleotide, the Tf-CAT activity in response to FSH and cAMP increased over 2-fold and 1.2-fold, respectively, compared with the corresponding Tf-CAT activity observed in the absence of antisense oligonucleotide (Fig. 7). The antisense oligonucleotides have been successfully used to target and reduce individual Id mRNA levels (37). In contrast to the effects of Id1 antisense oligonucleotide on transferrin promoter activity, addition of Id2 antisense oligonucleotide to the Sertoli cells transfected with Tf-CAT reporter plasmid resulted in an inhibition of CAT activity in response to FSH and cAMP (Fig. 8). The down-regulation of transferrin promoter activity in the presence of Id2 antisense oligonucleotide suggests that, in Sertoli cells, Id2 expression may be required to maintain a differentiated response. To address this hypothesis, Sertoli cells were cotransfected with the transferrin promoter reporter construct and with an Id2 expression plasmid. The ectopic expression of Id2 resulted in a small, but significant, increase in transferrin promoter activity (Fig. 9). Taken together, the Id2 antisense and overexpression data suggest that Id 2 may be involved in the regulation of transferrin promoter activity in Sertoli cells. Surprisingly, the addition of Id3 and Id4 antisense oligonucleotides had no effect on the transferrin promoter activity (data not shown). However, the lack of an effect of Id4 antisense oligonucleotide may be because the Id4 antisense oligonucleotide was designed using the 5′ end of the mouse Id4 sequence. The 5′ end of the mouse Id4 sequence may not be homologous to the 5′ region of the rat Id4 sequence that is currently not known. The potential function of Id4 requires

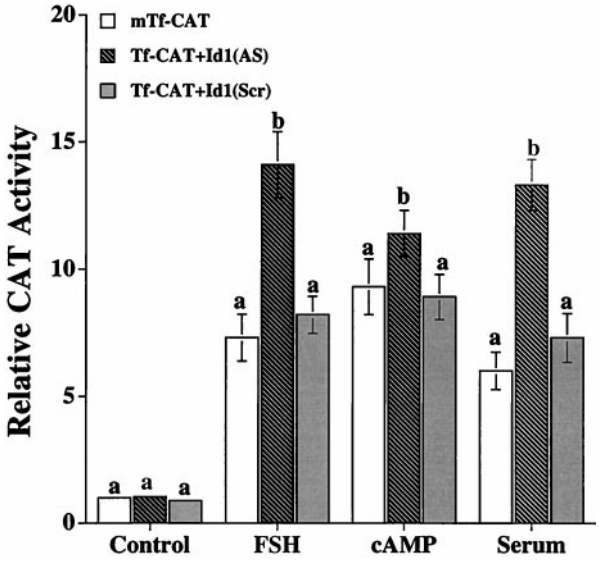

FIG. 7. Antisense oligonucleotide to Id1 effects on the transferrin promoter-CAT activity. The cultured Sertoli cells were transfected with the proximal 600-bp mouse transferrin promoter-CAT construct (mTf-CAT). Immediately after the transfection, Id1 antisense (AS) or scrambled (Scr) phosphorothioate-modified oligonucleotides (4 μM) were added. The cells were challenged with FSH, dibutryl cAMP, or 10% serum, 2 h after the addition of oligonucleotides. The oligonucleotides were subsequently added every 12 h until the cells were harvested for CAT assay (72 h). The data are presented as relative CAT activity of mTf-CAT control (without any treatment) set to 1 and are the mean ± SEM of triplicate samples in three separate experiments. *Different superscript letters above the error bars* represent a statistically significant difference ($P < 0.001$) with ANOVA analysis within each treatment group.

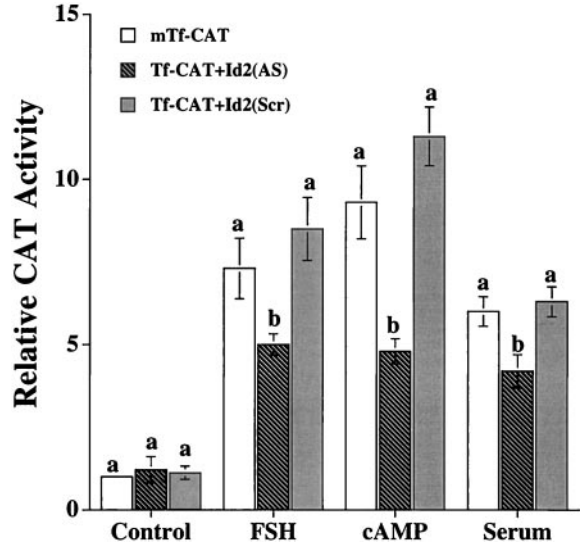

FIG. 8. Antisense oligonucleotide to Id2 effects on the transferrin promoter-CAT activity. The cultured Sertoli cells were transfected with the proximal 600-bp mouse transferrin promoter-CAT construct (mTf-CAT). Immediately after the transfection, Id2 antisense (AS) or scrambled (Scr) phosphorothioate-modified oligonucleotides (4 μM) were added. The cells were challenged with FSH, dibutryl cAMP, or 10% serum, 2 h after the addition of oligonucleotides. The oligonucleotides were subsequently added every 12 h until the cells were harvested for CAT assay (72 h). The data are presented as relative CAT activity of mTf-CAT control (without any treatment) set to 1 and are the mean ± SEM of triplicate samples in three separate experiments. *Different superscript letters above the error bars* represent a statistically significant difference ($P < 0.001$) with ANOVA analysis within each treatment group.

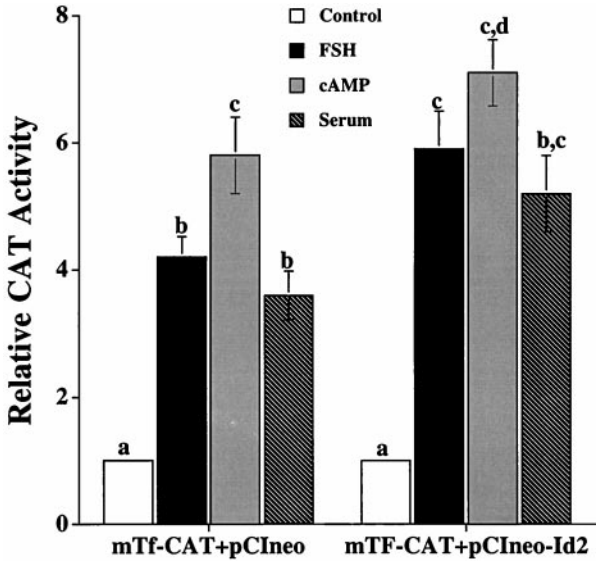

FIG. 9. Effects of overexpression of Id2 on the activity of proximal 600-bp mouse transferrin promoter-CAT activity. The cultured Sertoli cells were transfected with transferrin promoter-CAT construct or cotransfected along with pCIneo or the Id2 overexpression plasmid pCIneo-Id2. After transfection, the cells were left either untreated (Control) or treated with FSH, dibutryl-cAMP (cAMP), or 10% serum, as indicated. The data are presented as relative CAT activity of mTf-CAT control (without any treatment and in the presence of pCI-neo plasmid) set to 1 and are the mean ± SEM of triplicate samples in three separate experiments. *Different superscript letters above the error bars* represent a statistically significant difference ($P < 0.001$) with ANOVA analysis within each treatment group.

further investigation. The change in the activity of the transferrin promoter activity in the presence of antisense oligonucleotide is not attributable to nonspecific effects of the oligonucleotides, because control-scrambled oligonucleotides (Figs. 7 and 8) and Id3 and Id 4 antisense oligonucleotides (data not shown) had no effect on transferrin promoter activity.

Discussion

In several epithelial cell types, the expression of Id1, Id2, Id3, and Id4 has been positively correlated with proliferation (37, 58). These genes are often up-regulated in proliferating, undifferentiated cells, and down-regulated upon the induction of cellular differentiation (58). Considerable evidence exists that Id proteins are either expressed at very low levels or not expressed at all in quiescent and differentiated cells (38). Moreover, ectopic expression of Id proteins has been shown to block differentiated functions of a number of cell types by sequestering cell-specific gene expression of bHLH transcription factors (55, 61, 62).

The experiments presented in the current study show that all four Id-family members are expressed in postmitotic and differentiated Sertoli cells. Distinct differences in steady-state expression levels of the Id isoforms under various physiological conditions were observed. Interestingly, Id1 expression was down-regulated by FSH, whereas Id4 expression was up-regulated by FSH. In contrast, serum up-regulated the expression of Id1, 2, and 3, but down-regulated Id4 expression in Sertoli cells. Therefore, the expression of Id isoform genes is differentially regulated in Sertoli cells.

The decrease in the expression of Id1 observed after the treatment of cultured Sertoli cells with FSH was anticipated, because FSH is required to maintain differentiated functions (59). Further evidence that Id1 negatively influences Sertoli cell differentiated function is furnished by the observation that the ectopic expression of Id1 in Sertoli cells blocks FSH-mediated transferrin (21) and c-*fos* (22) promoter activation. Ectopic expression of Id1 was also shown to strongly inhibit the myelin promoter activity in Schwann cells (63). Conversely, Id1 antisense oligonucleotide, which selectively targets the Id1 mRNA, increases the transferrin promoter activation in response to FSH. The reduction in the levels of Id1 mRNA by an Id1 antisense oligonucleotide can be manifested, in terms of decrease in the Id1 protein levels (37). A decrease in Id1 protein may allow the formation of functional dimers, which can then bind to the E- Box in the proximal transferrin promoter. In contrast, ectopic expression of Id1 in Sertoli cells inhibits Tf-CAT activity, presumably because Id inhibits the binding of bHLH proteins to the E-Box in the transferrin promoter by forming nonfunctional dimers (21). The effect of FSH on Id1 gene expression is mediated primarily through the cAMP-protein kinase A system, because cAMP treatment also reduced Id1 gene expression. A similar decrease in the Id1 mRNA level was also observed in Schwann cells treated with cAMP (63). This experiment supports the role of Id1 as a negative regulator of Sertoli cell differentiation and implicates the HLH family of transcription factors as key regulators of Sertoli cell differentiation.

In general, the Id proteins are expected to have an overlapping function because of their ability to form nonfunctional dimers with differentiation-inducing bHLH proteins. Recent studies suggest that this may not be true, and some Id proteins (*i.e.* Id2 and Id 4) may, in fact, be required to induce and maintain the differentiated state of a particular cell (41, 64–67). Id2 is required for the determination and maintenance of the differentiated alveolar epithelial cells (41). The constitutive expressions of Id2 and Id3 mRNA in Sertoli cells suggests that both these proteins may have a significant role in maintaining Sertoli cell function. This hypothesis is supported by the observation that an antisense oligonucleotide to Id2 inhibits transferrin promoter activation, which is in contrast to the effect of Id1 antisense oligonucleotide. The exact mechanism by which Id2 regulates the promoter activity is not known. The speculation is presented that Id2 may inhibit a specific bHLH transcription factor complex that may normally repress Sertoli cell differentiated functions. Id2 was recently shown to homodimerize and inhibit cyclin A promoter activity in alveolar cells (41). The expression of Id3 and the lack of an antisense effect in Sertoli cells is intriguing. It is likely that Id3 may be involved in transcriptional events not directly involved in transferrin promoter activation.

The dynamics of Id4 expression in Sertoli cells is of particular interest. Id4 mRNA expression is up-regulated in response to FSH and cAMP. This observation contrasts sharply with the long-standing view that high levels of Id mRNA in proliferative and undifferentiated cells decrease as they are induced to differentiate (58). The specificity of this

Id4 regulation is distinct, because the levels of mRNA encoding Id2 and Id3 remained unchanged, and those of Id1 decreased after treatment of Sertoli cells with FSH or cAMP. The elevated expression of Id4 in response to FSH implicates its role in Sertoli cell differentiation. The mouse Id4 antisense oligonucleotide had no effect on the transferrin promoter activation, possibly because it may not have targeted the rat Id4 mRNA in Sertoli cells. The mechanism by which Id4 influences cellular function remains to be elucidated. Id4 expression is differentially regulated in various cell types. Id4 expression is up-regulated in differentiated adipocytes by hormones such as insulin, dexamethasone, and methylisobutyllxanthine (64) but is down-regulated in astrocytes by cAMP (56). These observations suggest that Id4 may selectively dimerize with stage- and cell-specific bHLH proteins in the various cell types. The significance of Id4 in regulating Sertoli cell differentiated functions requires further investigation.

The expression of all four Id isoforms in Sertoli cells in response to serum is entirely opposite to that observed after FSH and cAMP treatment. The effect of serum, a mixture of growth factors, hormones, and mitogens on cells at the concentration used in the present study is generally considered a proliferative signal. The increase in the expression of Id1, Id2, and Id3 in response to serum was anticipated and confirms previous studies that Id proteins are serum-inducible (38, 68, 69). The serum-mediated decrease in the expression of Id4 is intriguing. The observed increase in the expression of Id4 in response to differentiating signals like FSH and a decrease in response to proliferative signals, such as serum, supports the hypothesis that Id4 may be required to maintain or regulate Sertoli cell differentiated functions.

All four Id proteins, when fused to the heterologous GAL4 DNA-binding domain, can activate GAL4-dependent transcription, which required an intact HLH activity (70). Cotransfection with exogenous class A bHLH protein (E-proteins) can greatly potentiate the *trans*-activation, which is abolished upon cotransfection with class B bHLH proteins (70). The Sertoli cells express high levels of E47 (29) and REBα (30) class A bHLH protein. It is likely that the Id proteins may differentially bind to Sertoli cell bHLH proteins and influence promoter activation. Although the HLH domains of all four Id proteins are largely conserved, the highly divergent C- and N-terminal domains (58) may have selective binding preferences and *trans*-activation potential in Sertoli cells. These transcriptional actions of the Id isoforms will be of interest to elucidate.

In summary, the postmitotic and differentiated Sertoli cells express high levels of Id. The expression of Id1 and Id4 is regulated by signals that induce differentiation and proliferation, such as FSH and serum, respectively. Contrary to the hypothesis that Id proteins have redundant functions, the observations presented in the current study suggest that Id1 may act to maintain growth potential, whereas Id2 and Id4 may be involved in the differentiation of Sertoli cells. Based on the expression of Id2 and its effect on transferrin promoter, it is speculated that Id2 may have a dual function in Sertoli cells. Id2 may be required to maintain the growth potential of Sertoli cells in response to mitogenic stimuli such as serum, but may also promote differentiation in response to FSH. Therefore, the Id proteins seem to have functions other than simply the inhibition of differentiation. These differential functions of Id may involve molecular mechanisms, such as transcriptional activation, sequestering of bHLH proteins, and other isoform specific functions that remain to be fully understood.

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