The Id2 Transcriptional Repressor Is Induced by Follicle-stimulating Hormone and cAMP*

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Id (inhibitor of DNA binding/differentiation) proteins repress differentiation and promote cell division by dimerizing with and inhibiting the action of basic helix-loop-helix transcription factors including those that bind to E-box motifs. Of the four characterized Id proteins, only Id2 is found in the nucleus of Sertoli cells that support the development of spermatooza in the testis. Differential display analysis of rat primary Sertoli cell mRNA identified Id2 as being inducible by forskolin, a stimulator of cAMP production. Northern blot analysis confirmed that Id2 mRNA expression peaked in Sertoli cells 6–12 h after stimulation with forskolin or follicle-stimulating hormone (FSH), the major physiological stimulator of cAMP in Sertoli cells. Similarly, Id2 promoter activity in Sertoli cells was induced after forskolin or FSH stimulation as well as by overexpression of protein kinase A. Forskolin induction of the Id2 promoter required sequences located between positions −122 and −82. Protein(s) of 40–45 kDa were found to bind two activated transcription factor/cAMP-response element-like sites and a GATA motif within the regulatory region. The induction of the Id2 gene by FSH corresponded with a decrease in protein binding to an E-box consensus motif and decreased E-box-mediated transcription. Together, these findings raise the possibility that FSH-mediated induction of Id2 and resultant inhibition of basic helix-loop-helix transcription factor-regulated genes in Sertoli cells may contribute to the regulation of spermatogenesis.

The development of spermatooza within the testis requires specialized hormonal inputs and precise temporal control of gene expression. Follicle-stimulating hormone (FSH) and testosterone are the major hormonal regulators of spermatogenesis (reviewed in Ref. 1). Both hormones act on Sertoli cells to modulate cell processes and gene expression patterns that are required to support developing germ cells. FSH and testosterone actions are transduced through specific receptors present on the cell surface (FSH receptor) or within the cell (androgen receptor). Therefore, the proper expression of receptors for FSH and testosterone in Sertoli cells is essential for the process of spermatogenesis.

Recently, transcription of the FSH receptor and androgen receptor genes was shown to be regulated by members of the basic helix-loop-helix (bHLH) family of transcription factors (2–5). bHLH proteins are part of a larger group of HLH proteins that share an HLH structural domain (two amphipathic helices separated by a loop) that is required for dimerization with other HLH proteins (6). Adjacent to the HLH domain, bHLH proteins contain a basic region of amino acids that is required to bind DNA. bHLH proteins bind as dimers to DNA sequences in gene promoters sharing the core hexanucleotide sequence CANNNTG called an E-box (6). Two broad functional groups of bHLH proteins have been defined by their pattern of expression. Class A bHLH proteins are expressed in most tissues, whereas the expression of class B members is tissuespecific. In general, cell type-specific bHLH proteins form dimers with ubiquitously expressed bHLH proteins.

One major consequence of bHLH protein expression is the activation of genes required for the differentiation of tissues during development as well as the maintenance of normal physiological differentiation (7). For example, the differentiation processes of neurogenesis, myogenesis, and hematopoiesis as well as pancreatic development require bHLH proteins (reviewed in Ref. 7). Furthermore, bHLH proteins are thought to be required for testicular development and Sertoli cell differentiation (8, 9).

A subset of HLH proteins called inhibitors of DNA binding/differentiation or Id proteins repress the actions of bHLH transcription factors. Id proteins contain the helix-loop-helix region required for dimerization but lack the basic DNA binding domain. Therefore, Id proteins act as dominant negative repressors of transcription by dimerizing with and sequestering the ubiquitously expressed class A E-box HLH proteins (10), and in some cases, class B (tissue-specific) HLH proteins (11). Four subtypes of Id proteins have been characterized in mammalian cells, Id1, Id2, Id3, and Id4 (11, 12, 14, 15). In an immunohistochemical analysis of adult mouse testis, Id1, Id2, and Id4 were observed to be expressed in germ cells at various specific stages of development (16). In this same study, Id3 was identified in the cytoplasm of Sertoli cells, whereas Id2 was the only Id protein detected in the nuclei of Sertoli cells. A more recent study detected all four Id mRNAs and proteins in primary Sertoli cells isolated from 20-day-old rats (17). Although the cellular localization of the proteins was not investigated, Id2 and Id3 expression levels were found to be higher than Id1 and Id4. Based on these available data, Id2 is likely the major Id protein regulating transcription in Sertoli cells. Overexpression of Id proteins in Sertoli cells has been shown to activate the E-box-regulated transferrin gene promoter (17) and inhibit...
The importance of Id2 for Sertoli cell function and germ cell development is highlighted by the finding that Id2 null mice display defective spermatogenesis (18) in addition to retarded growth and neonatal morbidity (19).

In this study, we present evidence demonstrating that Id2 mRNA expression is transiently induced by the cAMP-elevating agent forskolin in Sertoli and FSH-stimulated FSH cells. We also show that the Id2 promoter is induced by forskolin, cAMP, or protein kinase A through a motif located 120 to 82 bp upstream of the transcription start site. This region is shown to bind proteins of a 6% sequencing gel, and cDNAs that were reproducibly induced by forskolin (10 μM) plus IBMX or vehicle. The E-box luciferase reporter contains two consensus E-box motifs upstream of a minimal thymidine kinase promoter driving the luciferase gene. Assays for chloramphenicol acetyltransferase (CAT) and luciferase activity were performed 72 h after transfection. For CAT assays, cell lysates were incubated with fluorometric BODIPY chloramphenicol (Molecular Probes, Inc., Eugene, OR) according to the manufacturer’s instructions. The resulting products were separated by thin layer chromatography, and the levels of acetylated products were quantified using a FluorImager and ImageQuant software (Amersham Biosciences). Luciferase assays were performed as described (26). CAT and luciferase activities were normalized for total protein as determined by Bradford assay.

DNA-Protein Binding Studies—32P-radioabeled DNA probes were generated by annealing nucleotide templates containing Id2 promoter sequences to complementary 10-base primers. The overhangs were filled in with Klenow in the presence of [α-32P]dATP and mixing dCTP, dGTP, and dTTP. The probes used and their coding strand template sequences included Id2 −120 to −78 (5′-AATGGAAGGCAGGCGCTCCGTC-TCTTGTAGACGTGACACCTTCCGGA-3′), Id2 −120 to −78 ATF mt (5′-AATGGAAGGCAGGCGCTCCGTC-TCTTGTAGACGTGACACCTTCCGGA-3′), and consensus CRE (5′-GATCCGCGTGACGTAGCTAAGTTATGACG-3′). The probes were digested with restriction enzymes and allowed to hybridize to DNA-gel blots. For competitive binding, the Id2 −120 to −78 region, the consensus CRE motif, the E-box probe, and 144 to −119 region of the CREB promoter (5′-CTGGAGA-AACCGGATGTTTGGTGGT-3′), or the −152 to −120 region of the Id2 promoter (5′-GAAAGAACAAGAGGCCGGCGGGGC-3′) were co-incubated labeled Id2 −120 to −78 probe and nuclear extracts. DNA-protein complexes were resolved via 5% PAGE under non-denaturing conditions in a Tris borate/EDTA buffer. For comparing protein binding affinities of various probes, labeled probes with similar specific activities (±20%) were used in simultaneous reactions. DNA-protein cross-linking studies were performed with radio-labeled probes prepared as described above except that DNA-gel blots contained a 1:1 mixture of bromodeoxyuridine and dTTP, and binding reactions were scaled up 10-fold. The resolved DNA-protein complexes were exposed to UV light (302 nm) for 15 min in situ and then excised from PAGE gels. The gel pieces were inserted into the wells of SDS-PAGE gels and fractionated. DNA-protein complex formation was visualized by autoradiography and quantified using NIH Image 1.6 software analysis of digitized images.

RESULTS

Identification of Forskolin-stimulated mRNAs in Sertoli Cells—To identify candidate genes that are induced by FSH and cAMP in the testis, primary rat Sertoli cells were stimulated with forskolin, an inducer of adenylate cyclase, in conjunction with the phosphodiesterase inhibitor IBMX. A limited differential display analysis of gene expression was performed on RNA collected after 12 h of stimulation. From three exper-

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ments, a total of 20 mRNAs were found to be induced, and 6 were sequenced. Two of the partial transcripts did not match sequences present in the GenBank™, European Molecular Biology Laboratory (EMBL), or Swiss-Protein data bases. The unique cDNAs are the subject of another study. Four other transcripts were identified by DNA sequence analysis. These included 1) ATP synthase lipid-binding protein, 2) S-100b (an EF-hand calcium-binding protein), 3) non-muscle myosin alkali light chain, and 4) Id2. Of the identified forskolin-induced cDNAs, the HLH protein Id2 was chosen for further study because of the possibility that Id2 may be a regulator of FSH-mediated gene transcription in Sertoli cells.

Forskolin or FSH Enhance Id2 Gene Expression—To confirm that Id2 gene expression was stimulated by forskolin, Northern blotting experiments were performed using mRNA isolated from primary rat Sertoli cells stimulated with forskolin and IBMX. In agreement with earlier studies demonstrating that Id2 probes do not cross-hybridize with other Id mRNAs (17, 29), only a single Id2 transcript was detected. In primary Sertoli cells, Id2 levels rose 2 h after forskolin stimulation, peaked at 6–12 h, and fell to near basal levels by 24 h (Fig. 1). To determine whether the major physiological inducer of cAMP in Sertoli cells, FSH, could also induce the Id2 gene, FSH and IBMX were added to primary Sertoli cells for 2, 6, 12, and 24 h. Although the peak of Id2 mRNA induction was delayed, the pattern of Id2 induction by FSH was similar to that seen for forskolin.

The Id2 Promoter Is Induced by Forskolin—Transient transfection studies were performed to determine whether the forskolin-mediated induction of Id2 mRNA was due to increased transcription initiation from the Id2 promoter. Primary Sertoli cells were transfected with the -834Id2CAT (30) reporter plasmid containing 834 bp of the Id2 promoter linked to the CAT reporter gene. The -834Id2 promoter was induced 3.3- and 2.8-fold by forskolin and FSH, respectively (Fig. 2A). Cotransfection of a vector expressing the catalytic subunit of PKA also induced the -834Id2CAT promoter (4.4-fold). In studies employing the MSC-1 mouse Sertoli cell line that does not express FSH receptors (21), overexpression of the catalytic subunit of PKA also induced the -834Id2 promoter (19.0-fold). Transcription from a control reporter plasmid (κBTATACAT) having the CAT gene driven by a TATA box and two κB enhancer motifs (24) was not altered by forskolin, FSH, or PKA. Together, these data suggest that the induction of Id2 mRNA by forskolin and FSH is due to stimulation of the Id2 promoter via PKA.

The -122 to -82 Region of the Id2 Promoter Contains a cAMP-inducible Element—To localize the regions of the Id2 promoter that are required for basal and forskolin-inducible activity, primary Sertoli cells and the MSC-1 Sertoli cell line were transfected with CAT reporter plasmids containing vari-

![Fig. 1. Id2 mRNA induction by forskolin or FSH.](image1)

![Fig. 2. The Id2 promoter is induced by forskolin (forsk), FSH, or PKA.](image2)
FSH Induces Id2

The Id2 promoter contains a cAMP responsive element between –120 and –82. Primary Sertoli cells (A) and MSC-1 Sertoli cells (B) were transfected with CAT reporter plasmids containing either the –834 Id2 promoter or Id2 promoter 5′ deletion mutants as indicated. Cells were stimulated 48 h after transfection with vehicle (stippled bars) or with forskolin (forsk, 10 μM) plus IBMX (0.5 mm) (black bars) and recovered 24 h later. CAT activity was adjusted for protein concentrations and expressed relative to –834Id2CAT plus vehicle (+ S.E.). The mean -fold induction relative to vehicle treated -834Id2CAT activity, but the competition observed using the consensus CRE probe and the 120 to 78 or 82 probe but that the CRE motif was a less effective competitor (Fig. 4B).

Forskin-induced region in the Id2 promoter downstream of position –177 (31).

The –120 to –78 Region of the Id2 Promoter Binds Proteins of 41–45 kDa—We next investigated potential DNA-binding proteins that might be responsible for forskolin induction of the Id2 promoter through the –122 to –82 region. EMSA analysis of Sertoli cell nuclear extracts identified a triplet of DNA-protein complexes that were formed with a probe extending from positions –120 to –78 probe or a probe containing a CRE motif. Equal levels of protein (ranging from 2 to 5 μg) were used for each assay. DNA-protein complexes were visualized by autoradiography. The unbound radiolabeled probe is not shown. The data displayed are representative of at least three independent experiments.

Previous studies of the Id2 promoter described two ATF/CRE-like motifs within the –120 to –78 region that contribute to promoter activity in C2 myoblasts but not HeLa cells (30). EMSA studies were performed to compare Sertoli nuclear extract binding to a probe containing wild-type –120 to –78 sequences and a probe in which the ATF/CRE-like sequences were mutated. These studies demonstrated that the ATF/CRE-like sites are required to bind Sertoli nuclear proteins (Fig. 5A). The competition observed using the consensus CRE probe and the dependence on an ATF/CRE-like motif for protein interactions raised the possibility that the binding complex contained a member of the CREB-ATF family of proteins. However, in-

FIG. 3. The –120 to –78 region specifically binds nuclear proteins in Sertoli cells. A, EMSA studies were performed using a 32P-labeled oligonucleotide containing the –120 to –78 region of the Id2 promoter and nuclear extracts from primary Sertoli cells. Binding reactions were performed in the absence and presence of a 50-fold molar excess of competitor oligonucleotides containing the same sequences as the probe (Id2 –120 to –78), a CRE, an E-box, the –144 to –119 region of the CREB promoter, or the –152 to –120 region of the Id2 promoter as indicated. B, competition EMSA assays were performed with the Id2 –120 to –78 probe and Sertoli cell nuclear extracts in the absence or presence of a 10-, 25-, or 50-fold excess of either unlabeled Id2 –120 to –78 probe or a probe containing a CRE motif. Equal levels of protein (ranging from 2 to 5 μg) were used for each assay. DNA-protein complexes were visualized by autoradiography. The unbound radiolabeled probe is not shown. The data displayed are representative of at least three independent experiments.

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Fig. 5. The −120 to −78 region specifically binds 41- and 45-kDa proteins in Sertoli cells. A, EMSA analysis was performed with Sertoli nuclear extracts incubated with either the Id2 −120 to −78 probe (wt) or a similar probe having the ATF sites mutated (mt). B, Sertoli nuclear extracts as well as NF-xB p50 and CREB produced by in vitro translation were incubated with the Id2 −120 to −78 or consensus CRE probes, and EMSA analysis was performed as in A. C, a photocross-linked DNA-protein adducts resolved by SDSPAGE are shown. The relative mobility of molecular size markers are indicated to the left. D, the wild-type Id2 −120 to −78 probe (lane 1) and mutant probes containing 2 bp changes as indicated (lanes 2–14) were incubated with Sertoli nuclear extracts, and EMSA analysis was performed. Lane 8 was inserted from a separate, identical experiment. The unbound probe was run off the gel for all studies. The sequence of the −108 to −83 region is displayed above, and the 2 bp mutations corresponding to each lane are shown immediately below the wild-type sequence. DNA-protein complexes were visualized by autoradiography. Data shown are representative of at least three independent experiments.

FSH stimulation transiently represses E-box binding activity—The stimulation of Id2 expression by inducers of cAMP would be expected to enhance Id2 dimerization with E-box-binding proteins and inhibit E-box-protein interactions with target DNA sequences. To test this idea, EMSA studies were performed with a probe containing a consensus E-box motif and nuclear extracts from Sertoli cells treated with FSH. E-box binding activity was high in extracts from untreated and 6-h stimulated Sertoli cells but decreased 85% after 12 h and then recovered to 45% of basal levels after 24 h of FSH stimulation (Fig. 6, A and C). In contrast, CREB binding to a consensus CRE motif increased after 12–24 h of FSH stimulation, perhaps reflecting elevated CREB protein levels as a result of previously described FSH induction of the CREB gene (33) (Fig. 6, B and C). Together, these data suggest that the transient induction of Id2 detected 6–12 h after FSH stimulation corresponds with the down-regulation of E-box binding activity in Sertoli cells.

FSH regulation of E-box-mediated transcription in primary Sertoli cells was then investigated in transient transfection assays using a luciferase reporter gene driven by two E-box motifs upstream of a minimal thymidine kinase promoter. These studies revealed that luciferase activity increased 81% within 6 h of FSH stimulation but then decreased to 38% above basal levels by 12 h (Fig. 6D). Luciferase activity was then allowed to increase more significantly to 347% over basal levels by 24 h. This pattern of expression suggests that FSH can initially stimulate transcription of the reporter but that stimulation is transiently interrupted during the period when Id2 levels are elevated and heterodimerization of Id2 and E-box proteins is favored. The later induction of E-box-mediated transcription coincides with decreased Id2 expression and increased E-box-binding activity.

DISCUSSION

Initial differential display analyses suggested that the cAMP-elevating agent forskolin increased Id2 mRNA levels within 12 h of stimulating primary Sertoli cell cultures. Subsequently, we confirmed that forskolin- or FSH-induced Id2 mRNA expression peaks after 6–12 h and falls to near basal levels after 24 h. Stimulation of the Id2 gene is apparently independent of the effects of serum, a known inducer of Id2 (34,
the variable contributions to promoter activity exhibited by the
−152 to −132 region in primary Sertoli cells and the MSC-1
Sertoli cell line may be reflective of the differentiation state or
proliferation capabilities of the cells (primary Sertoli cells iso-
lated from 16-day-old rats are nearly fully differentiated and at
the end of their proliferation period, whereas MSC-1 cells prolif-
erate continuously).

In contrast to the differential basal regulation of the Id2
promoter in primary Sertoli cells versus the MSC-1 cell line, the
induction of mRNA accumulation and promoter activity is sim-
lar for both types of Sertoli cells. Transient transfection stu-
dies of primary Sertoli cells demonstrated that the 894-bp Id2
promoter can be induced by forskolin, FSH, or the catalytic
subunit of PKA, suggesting that FSH-induced increases in Id2
mRNA are due to increased transcription efficiency that is
mediated via a cAMP/PKA-mediated pathway. In MSC-1 and
primary Sertoli cells, promoter deletion analyses revealed that
forskolin-inducible Id2 promoter activity required sequences
located between −122 and −82. Previously, two binding motifs
were identified between −110 and −90 of the Id2 promoter that
are potential binding sites for the ATF and CREB transcription
factors. These motifs were shown to confer doxorubicin-induced
expression from the promoter and specifically bind nuclear
proteins; however, neither of the major cAMP-responsive pro-
teins, ATF-1 and CREB, was found to bind to the region (30).

In our studies of Sertoli cell nuclear protein binding to the
−120 to −78 region, we detected a triplet of sequence-specific
DNA-protein complexes. The competition for protein binding by
a consensus CRE probe and the lack of DNA-protein complexes
formed using a probe having mutated ATF/CRE-like motifs
raised the possibility that CREB or ATF proteins might bind to
the −120 to −78 region. However, binding studies employing
recombinant CREB protein suggest that CREB is not the major
protein binding to this region of the Id2 promoter and is not
responsible for induction via the PKA pathway. Consistent
with this idea is our inability to supershift the DNA-protein
complexes with CREB antisera (data not shown). Furthermore,
it is not likely that an ATF protein binds to the ATF/CRE-like
sequences of the Id2 promoter sequence because of the known
ATF proteins, ATF-1 is the most similar in size (35 kDa) to the
proteins that could be cross-linked to the probe, but ATF-1 is at
least 5 kDa smaller than the protein(s) that we identified.

The −106 to −89 region was found to be required for the
formation of all three DNA-protein complexes observed with the
Id2 −120 to −78 probe. The fastest migrating complex was
interrupted by mutation of any 2 bp in the −106 to −101 region
corresponding to the core of the upstream ATF/CRE-like se-
quence. With one exception, probes having mutations within
the −98 to −89 region eliminated the formation of the two
slower migrating complexes. We noted that the 5′ end of the
−98 to −89 region contains a consensus GATA-protein binding
site. Of the six well characterized GATA proteins, the major
forms expressed in Sertoli cells are GATA-1 and GATA-4 (37).
Supershift EMSA assays and immunoprecipitation of UV
cross-linked DNA-protein complexes using Sertoli cell nuclear
extracts did not identify GATA-1 or GATA-4 bound to the
proteins that could be cross-linked to the probe, but ATF-1 is at
least 5 kDa smaller than the protein(s) that we identified.

Examination of Id2 promoter deletion mutants in MSC-1 and
primary Sertoli cells revealed differences in the regions respon-
sible for maintaining basal promoter activity. Although the −122
to −82 region of the promoter was required for basal activity in
primary Sertoli cells, the removal of the −152 to −132 region
resulted in a marked decline in basal Id2 promoter activity in
MSC-1 cells. This difference in the control of basal activity is
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−152 to −132 region was required for Id2 promoter activity in C2
myoblasts but was less important in HeLa cells. It is possible that

35), because Id2 is induced in primary Sertoli cells cultured in
serum-free medium as well as in MSC-1 cells maintained in
10% serum. The transient Id2 mRNA induction explains the
results of a previous study in which no Id2 stimulation was
observed 72 hr after treatment with FSH or a cAMP analogue
(17). It is likely that the induction of Id2 mRNA is terminated
due to a negative feedback mechanism mediated by Id2 inter-
fering with the binding of bHLH proteins to E-box motifs
within the Id2 promoter. This negative feedback hypothesis is
supported by earlier studies showing that the Id2 promoter is
inducible through E-box motifs after the overexpression of
bHLH proteins but that the co-expression of Id2 blocks the
stimulation of the Id2 promoter (31, 35, 36).

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myoblasts but was less important in HeLa cells. It is possible that
the factors responsible for FSH, forskolin, and PKA induction of the Id2 promoter remain to be determined.

Our transfection studies demonstrated that after an initial FSH-mediated induction of E-box-regulated transcription, further transcription was repressed until 12–24 h after FSH administration. This pattern of FSH-regulated transcription corresponded to the increased Id2 expression and decreased E-box binding activity that we observed in response to FSH. In previous studies of Sertoli cells, overexpression of Id2 was found to repress E-box-mediated transcription from the promoters of the FSH receptor (2, 8). Together, these observations suggest that FSH-induced sequestration of E-box factors by Id2 contributes to the decreases in endogenous FSH receptor RNA observed within 6–8 h of FSH stimulation prior to the later restoration of FSH receptor mRNA to normal levels that has been reported previously (39, 40). In vivo, the levels of FSH receptor on the Sertoli cell surface rise and fall in a cyclical manner, causing related changes in cAMP levels (13, 41). It would be expected that Id2 expression would respond to the increased Id2 expression and decreased E-box binding activity that we observed in response to FSH. Together, the temporal induction of Id2 by FSH and Id2 down-regulation of FSH receptors on Sertoli cells in the testis. Together, the cyclical changes in cAMP concentration. The induction of Id2 by FSH and Id2 down-regulation of E-box-mediated transcription implicate Id2 as an important transducer of hormonal signals that are required to support sperm development.

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