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Comparative Sequence Analysis of the Mouse and Human Transferrin Promoters: Hormonal Regulation of the Transferrin Promoter in Sertoli Cells

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ABSTRACT Cell-specific expression of the iron-binding protein transferrin is in part mediated through the regulation of its promoter. Although all cells require iron from serum transferrin produced by hepatocytes, cells that create a blood barrier such as Sertoli cells in the testis and choroid plexus epithelium in the brain also express the transferrin gene to provide iron to cells sequestered within the serum-free environment. The current study provides a complete sequence of the 3-kb mouse transferrin promoter and makes a comparison with the sequence available for the human transferrin promoter. Conserved regulatory elements between these two species are identified and speculated to be potentially important response elements for the regulation of the transferrin gene. The proximal 90 bp of the mouse and human transferrin promoter was found to be 80% homologous. The previously identified protected regions in the proximal human promoter also were conserved in the mouse transferrin promoter. Our sequence analysis data revealed that an E-box response element is also conserved between mouse and human promoters. Deletion mutants of the mouse transferrin promoter were generated in CAT reporter constructs to study the regulation of the transferrin promoter in Sertoli cells. As in the case of the human transferrin promoter, the mouse 581-bp proximal transferrin promoter was sufficient to obtain basal expression. A putative cyclic AMP response element (CRE) in the minimal promoter may be needed for follicle-stimulating hormone (FSH) actions mediated via cyclic AMP. Interestingly, other regulatory agents such as the testicular paracrine factor PModS used elements in the upstream region. A repressor was identified 2.5 kb upstream from the start site of translation. Combined observations suggest for the first time that a minimal promoter is sufficient for basal transcription, but the upstream regions of the promoter are needed for the hormonal regulation of the transferrin gene in Sertoli cells. Conserved response elements between the mouse and human sequences identify potentially important regulatory elements of the promoter and are discussed. *Mol. Reprod. Dev. 50:273–283, 1998.* © 1998 Wiley-Liss, Inc.

Key Words: transferrin; Sertoli; testis; transcriptional control

INTRODUCTION

Transferrin (Tf) is an iron-binding glycoprotein involved in transporting iron to the majority of cells in the body (Aisen and Listowsky, 1980). Iron-containing molecules are necessary for cell proliferation, differentiation, and metabolism (Kahn et al., 1987). Apart from the liver, which produces the majority of serum transferrin (Morgan, 1969), several extrahepatic tissues such as Sertoli cells in the testis (Skinner and Griswold, 1980), brain oligodendrocytes (Bloch et al., 1985), and choroid plexus epithelium (Tsutsumi et al., 1989) also express transferrin. In these extrahepatic sites, transferrin is produced in an environment that is partially sequestered from access to the blood. The transferrin synthesized in these tissues appears to play a role in local iron homeostasis, which is essential for cell survival, growth, and differentiation.

Serum levels of iron regulate transferrin secretion by hepatocytes (Idzerda et al., 1986). Transferrin gene expression in the Sertoli cells is not regulated by iron but instead by hormones such as follicle-stimulating hormone (FSH) (Skinner and Griswold, 1982; Huggenvik et al., 1987; Skinner, 1987) and a testicular paracrine factor PModS (Skinner and Fritz, 1985). Sertoli cells are the epithelial cells that help form the seminiferous tubules (Fawcett, 1975). These cells are involved in maintaining the blood-testis barrier that creates a critical microenvironment for spermatogenic cell development (Setchell and Waites, 1975; Waites and Gladwell, 1982). Transferrin is a major secretory product of differentiated Sertoli cells and is involved in transporting iron to developing germ cells (Skinner and Griswold, 1980; Griswold, 1988). Transferrin mRNA has been detected in Sertoli cells in 5-day-old rats, but

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the protein itself is primarily detectable at the onset of puberty (Skinner and Griswold, 1982). This correlates with the differentiation of Sertoli cells and provides a useful marker to understand Sertoli cell differentiation (Skinner et al., 1989; Norton and Skinner, 1989). Investigation of the transcriptional control of the transferrin gene provides insight into the regulation of Sertoli cell differentiation. Expression data of the human transferrin promoter in hepatocytes and rat Sertoli cells suggested that a minimal promoter (i.e., the proximal 581 bp) is needed for basal activity of the gene (Guillou et al., 1991). Transgenic expression of a reporter gene using the mouse −3-kb promoter also suggested that a proximal 581 bp is sufficient for liver-specific expression (Idzerda et al., 1989). Through deletion analysis of the human transferrin promoter, a cell-type-specific region was identified within the proximal −175 bp (Guillou et al., 1991; Idzerda et al., 1989; Espinosa et al., 1994). In hepatocytes this proximal region binds HNF (proximal regions PRI) and c/EBP (PRII) (Theisen et al., 1993; Schaeffer et al., 1993) and imparts liver specificity. A combination of transcription factors such as the chicken ovalbumin upstream promoter transcription factor (COUPTF), C/EBP, and CRI-BP that bind to PRI, PRII, and CRI in the proximal −175 bp, respectively, may regulate brain-specific transferrin gene expression. In rat Sertoli cells, the basal transcription of the human transferrin promoter is due to the TATA box region (Guillou et al., 1991). The increase in transcription is through the combination of factors binding to the proximal sites (PRI and PRII). Interestingly, the cyclic AMP response-element binding protein (CREB) interacts with the PRII of the human transferrin promoter (Suire et al., 1995). In Sertoli cells, FSH acts primarily through the protein kinase A (PKA) pathway (Bardin et al., 1988; Kangasniemi et al., 1990). The regulation of transferrin gene expression by FSH appears to be in part due to the CREB binding at PRII; however, PRII shows no homology to a consensus cyclic AMP response element (CRE) (Suire et al., 1995). PModS, a paracrine factor secreted by peritubular cells, also has dramatic effects on Sertoli cell transferrin secretion (Skinner and Fritz, 1985; Skinner et al., 1988; Anthony et al., 1991). The mechanism by which PModS acts on Sertoli cells is different from FSH and does not involve the cyclic AMP pathway (Norton and Skinner, 1989). Our preliminary results indicate that the upstream sequences in the mouse Tf promoter are involved in PModS-induced Tf gene expression (Chaudhary and Skinner, 1995).

Transferrin is an example of a gene that is expressed in more than a single tissue or cell type and involves diverse regulatory mechanisms to control its expression. In order to understand the transcriptional regulation of transferrin gene expression, the partial sequences of the promoters of the mouse and the human transferrin genes were obtained and compared to identify potentially conserved regulatory elements. Through transfection of deletion mutants of the mouse transferrin promoter, we provide evidence that the mechanisms that regulate mouse and human transferrin promoter in Sertoli cells are different.

MATERIALS AND METHODS

Cell Preparations and Culture

Sertoli cells were isolated from the testes of 20-day-old rats by sequential enzymatic digestion (Dorrington et al., 1975) with a modified procedure described by Tung et al. (1984). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Gibco-Bethesda Research Laboratories, Gaithersburg, MD) to remove the interstitial cells and then with collagenase (1 mg/ml type I; Sigma, St. Louis, MO) and hyaluronidase (1 mg/ml; Sigma). Sertoli cells were then plated under serum-free conditions in 24-well Falcon plates at 1×10^6 cells per well. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco-Bethesda Research Laboratories) with 0.01% bovine serum albumin (BSA) at 32°C. Sertoli cells were left untreated (control) or treated with either FSH (100 ng/ml; o-FSH-16, National Pituitary Agency, Rockville, MD), insulin (5 mg/ml), retinol (0.35 mm), dbcAMP (0.1 mm), or PModS (S300) (50 mg/ml). These optimal concentrations of hormones and PModS (S300) have been shown previously to dramatically stimulate cultured Sertoli cell differentiated functions (Skinner and Fritz, 1985; Skinner et al., 1988). The cells were cultured under serum-free conditions for a maximum of 5 days with a media change after 48 hr of culture. Cell number and density did not change during the culture in the absence or presence of the treatment (Skinner and Fritz, 1985; Skinner et al., 1988).

Plasmids

The CAT reporter plasmid (pUC8-CAT) containing −581 bp (−581 bp mTf-CAT) and the human growth hormone reporter plasmid containing −3.0-kb sequences of the mouse transferrin (mTf) promoter were generously provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA) (Idzerda et al., 1989). 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 (Idzerda et al., 1989). The CAT reporter plasmids containing the −3.0-kb mTf promoter and its deletions were constructed as previously described (Whaley et al., 1995).

Transfection

Sertoli cells cultured for 48 hr were transfected with a reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol), as described previously (Kingston et al., 1993). Briefly, 1.5 μg of reporter plasmid in 150 μl of transfection buffer [250 mM $CaCl_2$ mixed 1:1 v/v with 2× HEPES (28 mM NaCl, 50 mM HEPES, and 1.47 mM Na_2HPO_4, pH 7.05)] was added to each well of a 24-well plate containing 1×10^6 Sertoli cells in 1 ml of Ham's F-12 with 0.01% BSA, and incubation was performed at 32°C for 4 hr. After incubation, the cells were subjected

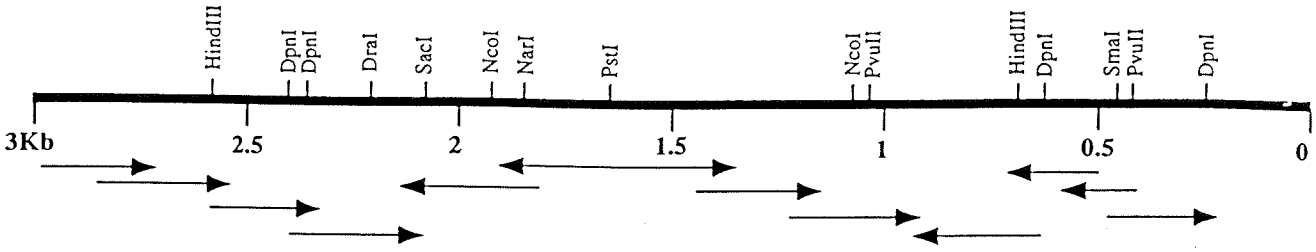


Fig. 1. Schematic and the strategy used to sequence the mouse transferrin 3-kb promoter. The arrows indicate the origin of the primer and direction of sequencing. Based on the sequence obtained, a restriction map was generated. The common restriction sites are indicated on the schematic.

to a hyperosmotic shock. The medium was aspirated, and 1 ml of 10% glycerol in Hanks' balanced salt solution (Gibco-BRL, Gaithersburg, MD) was added. The cells were incubated for 3 min, and the wells were washed twice before fresh Ham's F-12 was added. Various treatments were subsequently added, and cells were incubated for 48 hr before harvesting for CAT assays. In each experiment the transfection efficiency was monitored by transfecting the Sertoli cells by the plasmid-containing β-galactosidase gene driven by a CMV promoter. Subsequent staining and counting the cells expressing β-galactosidase (blue color) resulted in 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 phosphate-buffered saline (PBS). One hundred microliters of cell lysis buffer (Promega, Madison, WI) 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 of 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.

Gel-Shift Assays

Gel-shift assays with SE1 and SE2 fragments of mouse transferrin promoter as probes and nuclear extracts from Sertoli cells treated with either FSH or PModS (S300) were performed as described previously (Whaley et al., 1985).

Sequence Analysis

The 3-kb mouse transferrin promoter was ligated into pBluescript SK(+) (Stratagene, La Jolla, CA). Primary sequences were obtained by using M13 forward and reverse primers. Internal overlapping primers were subsequently made, and the entire 3-kb sequence was obtained using an Applied Biosystems, Foster City, CA (Model 373A) automated sequencer. The 3-kb mouse transferrin sequence can be obtained from the Genbank (Accession AF027336). Multiple sequence alignments were done using GCG software and DNASIS (Hitachi software). Identification of potential transcription factor binding sites was carried out using the updated transcription factor sites (Tfsites) database in the GCG DNA analysis package.

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 CAT activity (control CAT activity of each deletion mutant = 1), with the mean and SEM from multiple experiments determined as indicated in the figure legends. The CAT activity of the untreated controls between various deletion mutants did not change significantly and was 933 ± 110 cpm. Data were analyzed by a Student's *t* test, as indicated in the figure legends. The CAT reporter plasmids without mTf promoter were used as negative controls. In response to PModS (S300), FSH, dbcAMP (dibutryl cAMP), serum, and FIR, the relative CAT activity of the negative control plasmid was in the range of 1.5–2.0.

RESULTS

A 3-kb mouse transferrin promoter was obtained from G. Stanley McKnight (University of Washington, Seattle, WA) in a growth hormone reporter construct. The promoter was subcloned into a sequencing vector, and the entire sequence was obtained as shown in Fig. 1 with overlapping sequences such that a consensus could be made. The nucleotide sequence is shown in Fig. 2. Response elements within this sequence were identified using the GCG transcription factors binding-site database and are indicated as reported in the

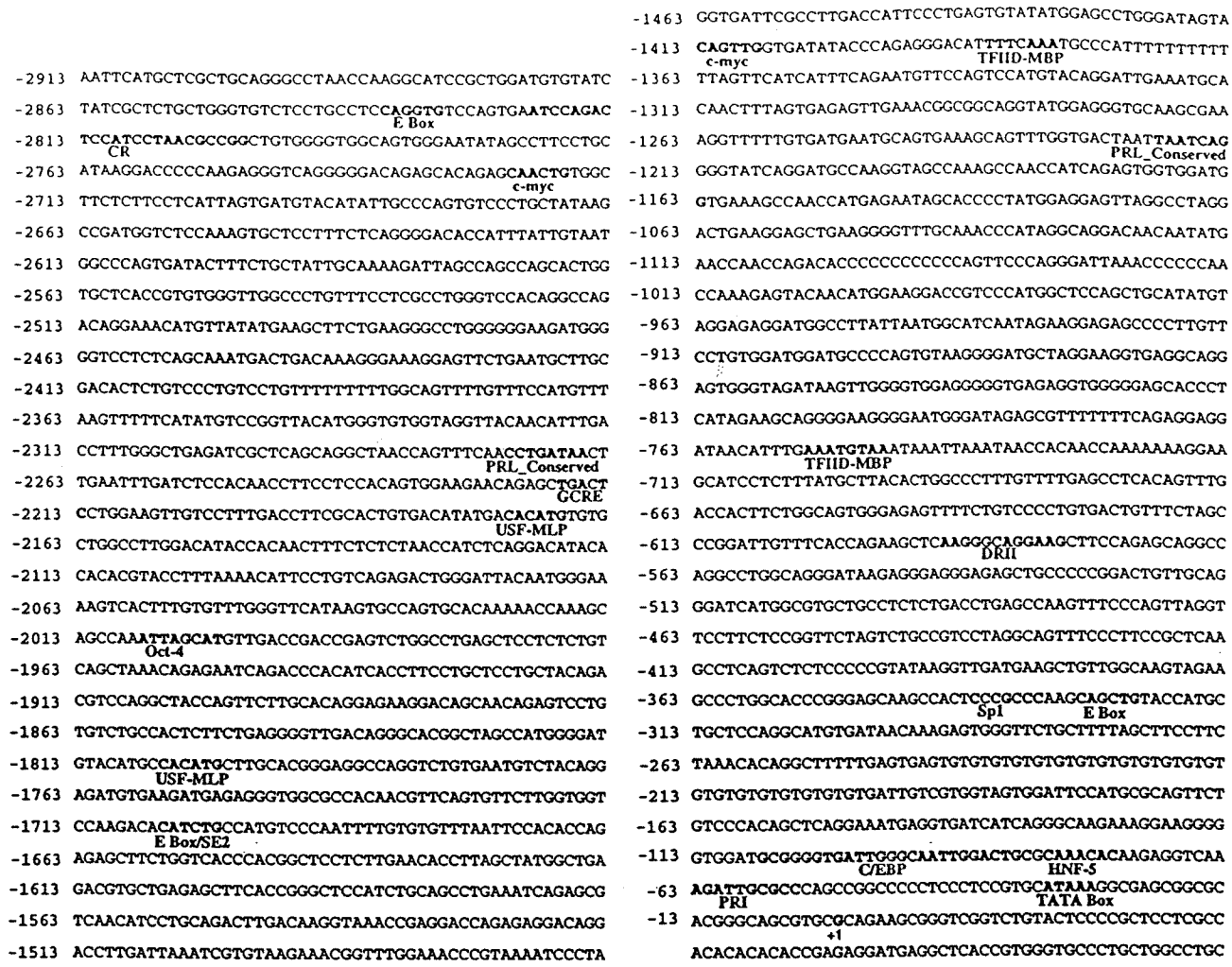
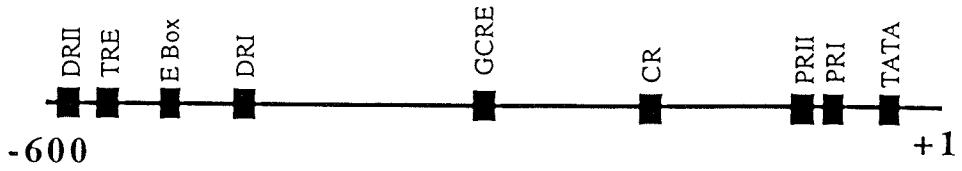


Fig. 2. Sequence of the 3-kb mouse transferrin promoter. The bold letters indicate the potential transcription factor binding sites that may be involved in regulating the transferrin promoter. Numbering is in reference to the transcriptional start site.

minimal promoter and in the new sequence information available between −3 kb and −581 bp. As a comparison, the human sequence was obtained from Genbank (Accession M15673). Response elements previously identified in the minimal 581 bp of the human promoter (Schaeffer et al., 1989) are shown (Fig. 3A). A computerized search for response elements for both the mouse and human promoter sequences reveals a number of elements that are conserved (see Fig. 3B). A comparison of the sequences that are conserved is shown in Fig. 3C. Through DNase footprinting, various protected regions were identified in the proximal human transferrin promoter. Based on their location on the promoter, these protected sites were designated as proximal region I (PRI), proximal region II (PRII), central region (CR), distal region I (DRI), and distal region II (DRII) (Schaeffer et al., 1989). A significant similarity in terms of both sequence homology and spatial distribution was observed when these regions were compared with the mouse transferrin promoter sequence. The sequence of human PRI and PRII was more than 75% conserved in the mouse transferrin promoter also. The location of PRI and PRII in the mouse transferrin promoter was highly conserved. In the mouse promoter PRI and PRII were located at 74 and 104 bp and in the human promoter PRI and PRII were at 72 and 102 bp, respectively, upstream of the start site (see Fig. 3C). The sequence homology and location were not significantly conserved in the case of DRI and DRII. In the mouse promoter two regions at −2200 and −82 bp were about 50% homologous to the human DRI sequence (see Fig. 3C). As with DRI, the human DRII was about 50% homologous at −351 and −590 bp upstream of the start site of the mouse transferrin promoter. The sequence of mouse DRII (−59 bp) is very similar to that of the human DRII sequence (−614 bp), which contains an Sp1 site. The CR (human, −190 bp) was more than 70% conserved in the mouse transferrin promoter (−2821 bp). The proximal, distal, and central regions are protected sites with more than 50% homology between

A



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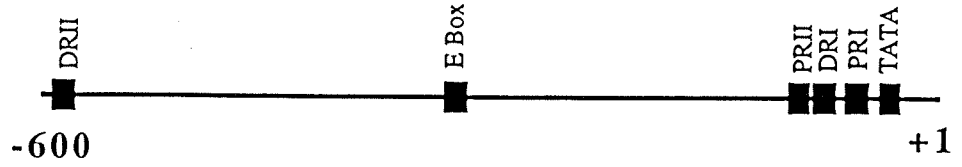

C

Distal Region I (DRI) -474 tctttgaccttgagcccagct -454 -2200 CTTTGACCTTCGCACTGTGA -2181	**Proximal Region I (PRI)** -72 cgggaggtcaaagattgcgc -53 -74 CAAGAGGTCAAAGATTGCGC -54
DRI -454 agctgg.gctcaaggtcaaaga -474 -82 CGCAAACACAAGAGGTCAAAGA -61	**Proximal Region II (PRII)** -102 gggcgattgggcaacccggctgcac -78 -104 GGGTGATTGGGCAATTGGACTGCGC -80
Distal Region II (DRII) -591 tgggctggaaaacttcccgccctc -614 -351 GGGAGCAAGCCACTCCCGCCCAA -329	**Central Region (CR)** -190 tgctggactccttccactcgcggg -167 -2821 ATCCAGACTCCATCCTAACGCCGG -2798
DRII -614 gagggcgggaagttttccagccca -591 -590 AAGGGCAGGAAGCTTCCAGAGCAG -567	**E-Box** -506 cacctg -501 -327 CAGCTG -322

Fig. 3. The spatial location of the conserved regions in the proximal promoter of (A) human and (B) mouse. C. Comparison of the human and mouse transferrin promoters. The previously identified regions (protected fragments by DNase footprinting) of the proximal 600 bp of the human transferrin promoter (lowercase) were compared with the whole 3 kb of the mouse transferrin promoter (uppercase). Regions with significant similarity are shown. Since the regions of the human promoter are protected fragments, the actual binding site may be only a part of the region. The E-box response element is also conserved between the human and the mouse proximal promoter sequences.

these regions (see Fig. 3C). Transcription factor recognition domains may be within this protected region. Another observation was the presence of an E-box (CANNTG) response element. The human (−506 bp, CACCTG) and mouse (−327 bp, CAGCTG) elements are located in the proximal −581 bp of the promoter sequence. As expected, the HNF transcription factor binding site, which is important for liver-specific expres-

sion of transferrin gene (Schaeffer et al., 1993), also was found conserved in the mouse transferrin promoter. With the availability of the upstream sequence of the mouse transferrin promoter, several elements were identified upstream that include multiple E-boxes, USF-MLP, and cMyc (see Fig. 2). These and other response elements may be potential candidates in regulating the mouse transferrin promoter in a cell-specific manner.

Functional analysis of the promoter utilized deletion mutants in CAT reporter constructs as described previously (Chaudhary and Skinner, 1995; Whaley et al., 1995). These constructs were transiently transfected into cultured rat Sertoli cells isolated from 20-day-old rats testis. After transfection, the cells were left untreated (control) or were treated with either PModS (S300), FSH, dbcAMP, serum, or a combination of FSH, insulin, and retinol (FIR) for 48 hr before analysis of the cell extracts for CAT activity. The 581-bp minimal promoter under control conditions was found to be sufficient for basal levels of gene expression. This also confirms previous results on the human transferrin promoter (Kahn et al., 1987; Kangasniemi et al., 1990). PModS (S300) activated the −2.6-kb construct to a greater degree ($P < 0.001$) than the 581-bp minimal promoter (Fig. 4B, superscript b). The 1-kb construct between −1.6 and −2.6 kb also was stimulated (see Fig. 4B, indicated by superscript d; $P < 0.001$, as compared with the activity of this plasmid in response to FSH, cAMP) by PModS (S300). The −2.6/−1.6-kb CAT plasmid was constructed by ligating the 1-kb fragment between −2.6 and −1.6 kb to the CAT reporter plasmid containing Tk minimal promoter. This plasmid was stimulated 1.5- to 2-fold by dbcAMP and FSH (superscript c in Fig. 4D and F). The effects of serum on the various deletion mutants of the mTf -CAT reporter construct did not show any significant difference as compared with the activity of the −581-bp promoter. As in the case of PModS (S300), the −1.6/−2.6-kb Tf-Tk-CAT construct was stimulated with serum (see Fig. 4C, 4-fold, superscript d; $P < 0.001$) and FIR (see Fig. 4E, 3-fold, superscript d; $P < 0.001$). Based on gel-shift assays, two regions, SE1 and SE2, in the upstream 1-kb fragment were identified previously (Chaudhary and Skinner, 1995). SE1 gave a retarded band with serum- and PModS-treated Sertoli cell nuclear extracts, whereas with SE2 a retarded band was observed only after PModS treatment. Therefore, the increase in the CAT activity of the −1.6/−2.6-kb Tf -CAT construct is postulated to be due to the presence of SE1 and SE2 fragments.

In response to FSH, the −581-bp minimal promoter was maximally stimulated and was significantly higher than either the −3-, −2.6-, or −1.6-kb reporter construct (see Fig. 4D). These data suggest that the proximal −581-bp sequence contains the elements that bind transcription factors stimulated by FSH and that this activation may be negatively regulated by the presence of upstream sequences. Another interesting observation was the lack of significant CAT activity of the −1.6/−2.6-kb construct in response to FSH. This is consistent with the gel-shift data. A band is not observed with either SE1 or SE2 fragment when incubated in the presence of FSH-stimulated Sertoli cell nuclear proteins (Fig. 5).

The mechanism by which FSH acts on Sertoli cells is primarily through the cyclic AMP pathway. Other signaling mechanisms possibly involving c-*fos* also have been reported (Whaley et al., 1995; Hall et al., 1988; Norton and Skinner, 1992). To determine whether the FSH induced repression of the −3, −2.6, or −1.6 kb was due to the cyclic AMP pathway, the Sertoli cells were treated with the cyclic AMP analogue dbcAMP. As shown in Fig. 4F, the CAT activity of the −3, −2.6, and −1.6 kb was similar to the minimal −581-bp promoter construct. The repression observed in response to FSH by the presence of sequences upstream of −581 bp was not present when the cells were treated with dbcAMP. This result is similar to the data obtained with serum treatment. Similar response also was observed when cells were maximally stimulated with FIR (see Fig. 4E). No repression was observed with additional sequences present upstream of the −581-bp minimal promoter. Therefore, the ability of insulin and retinol to enhance the actions of FSH on transferrin gene expression appear distinct from the promoter activation by FSH alone. An interesting observation was the absence of any effect on the −1.6/−2.6-kb promoter with either FIR or dbcAMP, indicating that regulation of the activity of the transferrin promoter involves a combination of multiple transcription factors. This is also supported by the observations that a gel-shift band was not observed when nuclear extracts from Sertoli cells treated with cyclic AMP were incubated with SE1 or SE2 fragments of the mouse transferrin promoter (data not shown).

DISCUSSION

Transferrin is an iron-binding protein involved in transporting iron to cells and is required for cell proliferation, differentiation, and metabolism (Kahn et al., 1987). The liver is the primary source of transferrin in serum. Several other tissues also produce transferrin, including Sertoli cells (Skinner and Griswold, 1980), brain oligodendrocytes (Bloch et al., 1985), choroid plexus (Tsutsumi et al., 1989), and mammary gland (Chen and Bissel, 1987). In the liver, the expression of transferrin is regulated primarily by iron in the serum (Idzerda et al., 1986). In other tissues, transferrin expression is independent of serum iron levels and is under the control of various hormones and other stimuli. Therefore, transferrin is an example of a gene that is expressed in more than one cell type and involves diverse transcriptional control mechanisms regulating its expression. In the current study, the elements involved in regulating Sertoli cell–specific expression of this gene are investigated. Transferrin is secreted by differentiated Sertoli cells, and understanding the factors involved in regulating transferrin gene expression will provide insight into Sertoli cell differentiation.

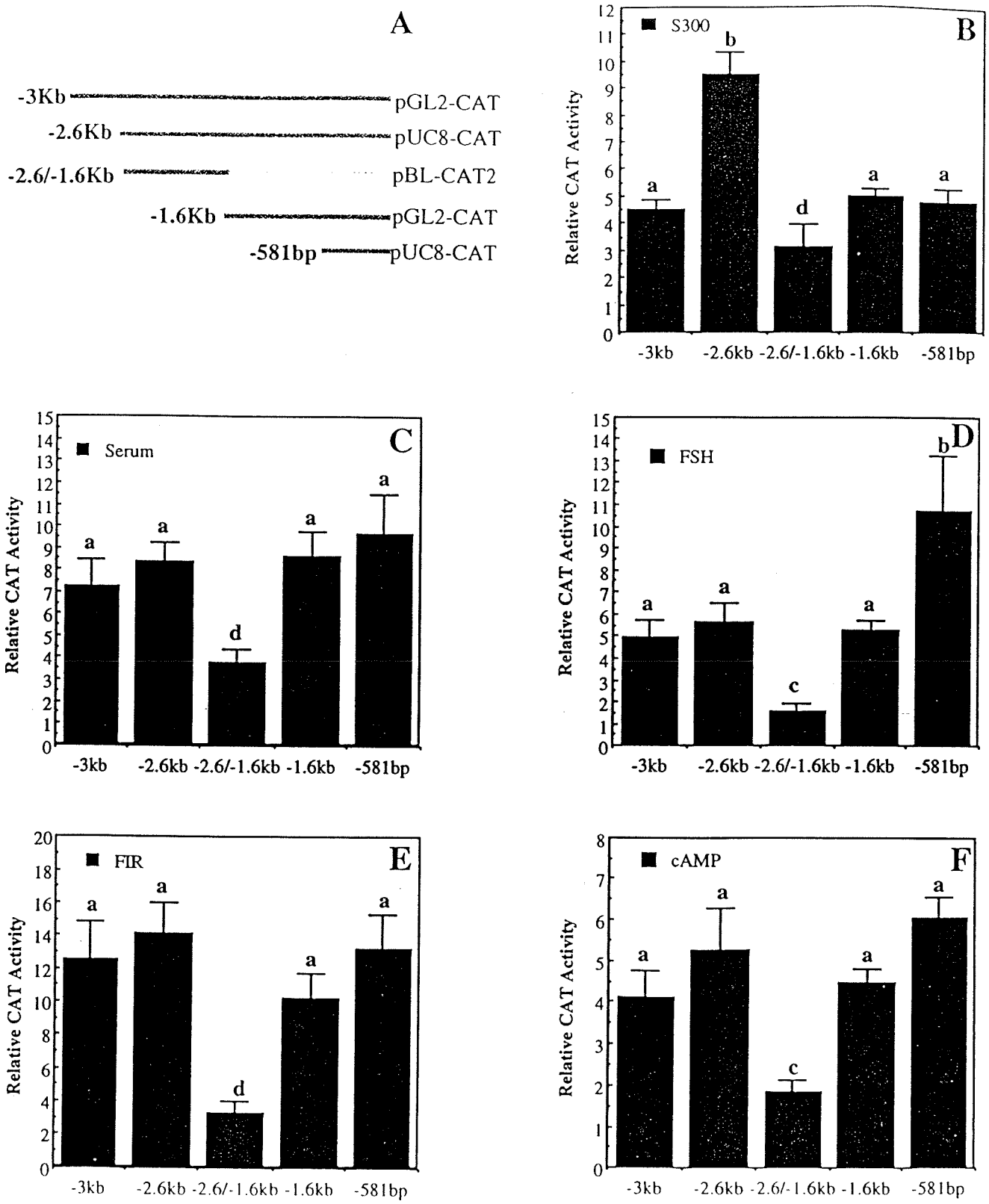

Fig. 4. CAT activity of various transferrin deletion mutants (A) in Sertoli cells. Following transfection the cells were treated with (B) S300, (C) serum, (D) FSH, (E) FIR, and (F) cAMP. Data are represented as mean ± SEM of at least three experiments done in triplicate. Within each treatment group, the CAT activity of the control untreated group is set to 1, and the change in activity following treatment is adjusted accordingly and is represented as fold stimulation over the control data. Different superscript letters indicate statistically significant differences: (a) significantly higher than controls but not a significant difference between various treatments; (b) significantly higher than controls and from deletion mutants represented with the superscript a; (c) nonsignificant difference as compared with untreated controls; (d) a significant difference ($P < 0.001$) from untreated controls (−2.6/−1.6 kb) and not compared with any other treatment.

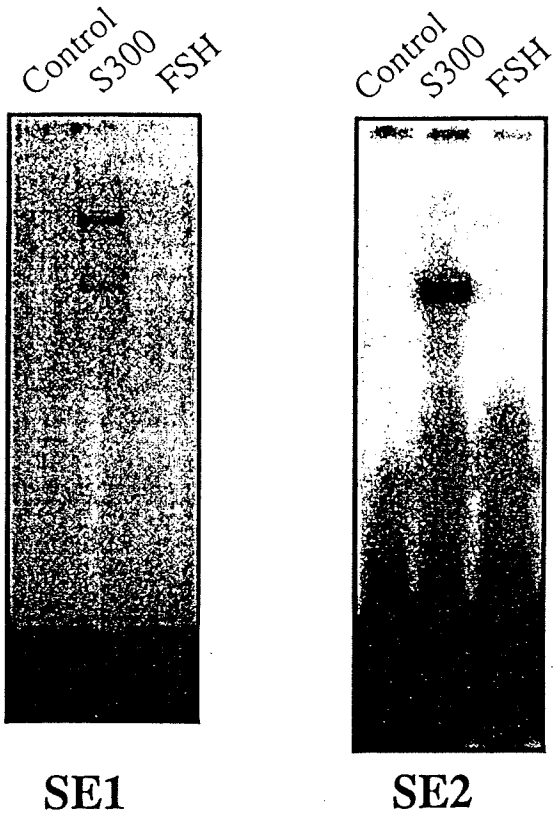

Fig. 5. Gel mobility shift assay with ^{32}P-radiolabeled SE1 and SE2 regions of the mouse Tf promoter. Nuclear extracts from Sertoli cells cultured in the absence (c) or presence of PModS (S300) or FSH were used. The DNA protein complexes were electrophoretically separated on 5% polyacrylamide gels and then dried and autoradiographed. The data are representative of three different experiments.

To understand how the mouse transferrin (mTf) promoter is regulated in a cell-specific manner, the proximal 3-kb mouse transferrin promoter was sequenced and compared with the available 1-kb sequence of the human transferrin promoter. Interestingly, the major response elements involved in regulating the human transferrin promoter activity also were found conserved in the proximal mouse transferrin promoter. In particular, the proximal human transferrin proximal region (PRI), proximal region II (PRII), C/EBP, distal region (DRI), and distal region II (DRII) sequences (Guillou et al., 1991; Schaeffer et al., 1989, 1993) were conserved in the mouse transferrin promoter. Another interesting observation was the presence of an HNF binding site 3' to the C/EBP binding domain. The transcription factor HNF is involved in liver-specific expression (Schaeffer et al., 1993), and in the human transferrin promoter it binds at the PRI site, which is also 3' to the C/EBP site. Brain-specific transferrin gene expression is achieved through a combination of at least three different transcription factors. Unlike liver, the PRI region in oligodendrocytes binds COUPTF, whereas PRII and CRI regions bind C/EBP and CRI-BP. The upstream region between −872 and −1140 bp also acts as a positive region in oligodendrocytes and may have a role in cell-specific expression of the transferrin gene. In addition to the sites listed above, a number of other transcription factor binding sites such as TFIID-MBP, USF-MLP, Oct 4, and cMyc also were found. It remains to be determined if these sites identified upstream of the 581-bp proximal promoter are involved in Sertoli cell–specific transcriptional regulation.

Our data from transient transfection of cultured rat Sertoli cells with various deletion mutants of the 3-kb mouse transferrin promoter suggest that the proximal 581 bp of the promoter was sufficient for basal level induction. This region of the mouse promoter consists of the PRI (−74), PRII (−104), DRI (−82), and DRII (−590) and the highly conserved (as compared with the human transferrin promoter) proximal −52 bp of the sequence previously shown to be important for the Sertoli cell–specific expression of human transferrin promoter (Schaeffer et al., 1993). Deletions of the PRI/PRII and DRI/DRII of the human promoter have been shown to significantly reduce the activity of the transferrin promoter (Guillou et al., 1991; Schaeffer et al., 1993). The significance of the proximal 581 bp of the mouse transferrin promoter also was apparent from the mouse transgenic model using the 3-kb mTf-hGH reporter construct, although the authors did not report any data on the expression in testes (Idzerda et al., 1989). The deletion construct containing the −581- to +50-bp region of the mouse transferrin promoter was sufficient to direct a high level of liver-specific expression in transgenic mice.

Sertoli cell function is regulated primarily by the gonadotropin FSH and involves a cyclic AMP–mediated protein kinase A (PKA) pathway (Bardin et al., 1988; Kangasniemi et al., 1990). The cyclic AMP–PKA pathway regulates transcription through the cyclic AMP response-element binding protein (CREB) that binds to the CRE site present in the promoters of a number of genes (Karin, 1992; Lalli and Sassone-Corsi, 1994). Interestingly, the PRII site in the human transferrin promoter binds CREB, although this site is not a CRE consensus site (Suire et al., 1995). Observations indicate that mutations in the PRII of human transferrin downregulate FSH- or cyclic AMP–induced stimulation (Suire et al., 1995). The presence of a conserved PRII site in the mouse transferrin promoter suggests that PRII is involved in FSH- and cyclic AMP–induced transferrin gene expression. In addition to CREB, the PRII of the human transferrin promoter also binds two additional proteins, SP-A and SP-D, in rat Sertoli cells (Schaeffer et al., 1993). The identity of these two putative transcription factors remains to be determined. It is highly possible that transcription factors similar to SP-A and SP-D also may bind PRII of the mouse transferrin promoter used in the present study and impart tissue specificity. Another interesting observation worth consideration is the presence of a large CpG island in the proximal human transferrin pro-

moter (unpublished observation). Such a potential CpG island, however, is not apparent in the mouse transferrin promoter. The cytosine at the CpG palindrome is a potential methylation candidate. Hypermethylation of cytosines within the CpG island results in transcriptional inactivation, whereas hypomethylation leads to the potential for gene activity (Cedar, 1988). Further studies on the state of methylation in the human and identification of a potential CpG island in the mouse transferrin promoter will provide important clues to the mechanism by which transferrin promoter is regulated in Sertoli cells.

Interestingly, the differences in the response to FSH and cyclic AMP were observed when sequences upstream of 581 bp were present in the mouse transferrin promoter. The activity of the 3-, 2.6-, and −1.6-kb deletion mutants decreased significantly as compared with the basal 581-bp minimal promoter in response to FSH. In response to cyclic AMP, however, the activity of the deletion mutants remained unchanged. This observation implies that a repressor responsive to FSH is present from −3 kb to −581 bp. Sequences upstream of 3 kb may be needed for complete transcriptional regulation of the promoter in response to FSH. These upstream sequences may not be regulated through the PKA pathway and may involve different signaling mechanisms that are activated by FSH. Previously it has been shown that the actions of FSH on Sertoli cells may involve multiple signaling pathways possibly involving the serum response element of the c-*fos* promoter (Whaley et al., 1995; Hall et al., 1988; Jia et al., 1996).

The effect of serum and FIR on the activity of the 3-kb mouse transferrin deletion mutant was similar to that observed with cyclic AMP. Both serum and FIR are known to maximally stimulate Sertoli cells. Our data on the effect of serum on Sertoli cells transfected with various deletion mutants of the mouse transferrin promoter are different from those observed with transfecting deletion mutants of the human transferrin promoter in rat Sertoli cells. In response to serum, a repressor was observed between −581 bp and −3 kb of the human transferrin promoter (Guillou et al., 1991). The presence of an additional 300 bp upstream of −3 kb restored full promoter activity (Guillou et al., 1991). In the mouse transferrin promoter, no repression in CAT activity was observed when sequences between −581 bp and −3 kb were present. Taken together, these results suggest that a different combination of transcription factors may be responsible for the regulation of mouse and human transferrin promoters.

The effect of the paracrine factor PModS (S300) on the mouse transferrin promoter was different from the effects of FSH, cyclic AMP, FIR, or serum. PModS did not maximally stimulate the 581-bp minimal promoter. The additional 2-kb sequence was required for maximum stimulation. A decrease in activity of the 3-kb promoter construct implied the presence of a repressor upstream of 2.6 kb. To identify the region(s) responsible for PModS activation, gel shifts were carried out using 200-bp restriction fragments of the 3-kb promoter. Two fragments named SE1 and SE2 located within 1 kb of the sequence between −2.6 and −1.6 kb were identified (Chaudhary and Skinner, 1995). Transient transfection of this 1-kb fragment in a CAT reporter plasmid gave a stimulation suggesting that the PModS- but not FSH-responsive elements are present in SE1 and SE2 (Whaley et al., 1995). Consistent with the gel-shift data, the 1-kb (−2.6/−1.6 kb) CAT reporter plasmid also was stimulated in response to serum but not either FSH or dbcAMP. The differences in the response of various mouse transferrin promoter deletion mutants to hormones and PModS suggests that a combination of multiple transcription factors may regulate the activity of the mouse transferrin promoter. Analysis of SE1 and SE2 fragments for potential transcription factor binding sites indicated the presence of an E-box (−1705 bp, CATCTG). Apart from this E-box, no other transcription factor binding site with a potential of regulating mouse transferrin promoter in rat Sertoli cells was present. A gel shift with the SE2 fragment as a probe was displaced with the E-box (CATCTG and CACCTG), suggesting that E-box binding proteins bind to SE2 and are responsive to PModS (Chaudhary et al., 1997). E-box (consensus CANNTG) elements are a motif to which the bHLH class of transcription factors bind (Murre et al., 1989a, 1989b). These proteins are involved in cell-specific transcriptional control in a number of tissues, including muscle and brain (Weintraub et al., 1991; Ishibashi et al., 1994; Jan and Jan, 1993; Lee et al., 1995). The bHLH proteins have a conserved helix-loop-helix domain essential for dimerization of different bHLH proteins, as well as a basic domain that mediates binding to an E-box (Murre et al., 1989a, 1989b).

An E-box is also present in the proximal human transferrin promoter (−506 bp, CACCTG). A similar E-box (−327 bp, CAGCTG) also was observed in the mouse transferrin promoter, suggesting that bHLH proteins may regulate the transcriptional activity of mouse and human promoters. We have shown previously that overexpression of Id (a negatively acting HLH protein lacking basic DNA binding domain) downregulates the −2.6-kb mTf-CAT reporter construct in response to FSH and PModS (Chaudhary et al., 1997). This downregulation by Id implies that an E-box is involved in the regulation of transferrin CAT activity. In response to FSH, the 581-bp mTf-CAT reporter construct also was downregulated by the overexpression of Id (unpublished observations). Whether this inhibition in the presence of Id is directly due to the binding of bHLH proteins to the E-boxes remains to be determined. Experiments are currently in progress to mutate these E-boxes to get a better understanding on how bHLH proteins regulate the mouse transferrin promoter.

Taken together, the transfection data, sequence analysis, and comparison of the mouse transferrin promoter

with the human promoter suggest that the transcription factors necessary for the basal activity of these promoters may be similar. The regulation of transcription in response to various stimuli appears to be more complex and may require upstream sequences and a combination of different transcription factors. In addition to the DNA-protein interaction, protein-protein interactions between transcription factors also may provide additional cell-specific transcriptional control. Our preliminary observations indicate that E-box sequences have an important role in regulating Sertoli cell transferrin gene expression. The cell specificity of the bHLH proteins binding to E-boxes may provide cell-specific expression.

REFERENCES

Aisen P, Listowsky I (1980): Iron transport and storage proteins. Annu Rev Biochem 49:357.

Anthony CJ, Rosselli M, Skinner MK (1991): Actions of the testicular paracrine factor (P-Mod-S) on Sertoli cell transferrin secretion throughout pubertal development. Endocrinology 129:353–360.

Bardin CW, Cheng CY, Musto NA, Gunsalus GL (1988): The Sertoli cell. In E Knobil, JD Neal (eds): "The Physiology of Reproduction," Vol 1. New York: Raven Press, pp 933–974.

Bloch B, Popovici T, Levin MJ, Tuil D, Kahn A (1985): Transferrin gene expression visualized in oligodendrocytes of the rat brain by using in situ hybridization and immunohistochemistry. Proc Natl Acad Sci USA 82:6706–6710.

Cedar H (1988): DNA methylation and gene activity. Cell 53:3–4.

Chaudhary J, Skinner MK (1995): Transcriptional regulation of Sertoli cell differentiation (transferrin promoter activation) during testicular development. Dev Genet 16:114–118.

Chaudhary J, Cupp AC, Skinner MK (1997): Role of basic helix loop helix transcription factors in Sertoli cell differentiation: Identification of an E-box response element in the transferrin promoter. Endocrinology 138:667–675.

Chen L, Bissel MJ (1987): Transferrin mRNA level in the mouse mammary gland is regulated by pregnancy and extracellular malice. J Biol Chem 262:17247–17250.

Dorrington JH, Roller NF, Fritz IB (1975): Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. Mol Cell Endocrinol 3:57–70.

Espinosa de Los Monteros A, Sawaya BE, Guillou F, Zakin MM, de Vellis J, Schaeffer E (1994): Brain-specific expression of the human transferrin gene. J Biol Chem 269:24505–24510.

Fawcett DW (1975): The ultrastructure and functions of the Sertoli cell. In RO Greep, EW Hamilton (eds): "Handbook of Physiology," Vol 5, Sec 7. Washington: American Physiological Society, pp 22–55.

Griswold MD (1988): Protein secretions of Sertoli cells. Int Rev Cytol 110:133–156.

Guillou F, Zakin MM, Part D, Biossier Schaeffer E (1991): Sertoli cell specific expression of the human transferrin gene. J Biol Chem 266:9876–9884.

Hall SH, Joseph DR, French FS, Conti M (1988): Follicle-stimulating hormone induces transient expression of the protooncogene c-*fos* in primary Sertoli cell cultures. Mol Endocrinol 2:55–61.

Huggenvik JT, Idzerda RL, Haywood L, Lee DC, McKnight GS, Griswold MD (1987): Transferrin messenger ribonucleic acid: Molecular cloning and hormonal regulation in rat Sertoli cells. Endocrinology 120:332–340.

Idzerda RL, Huebers H, Finch CA, McKnight GS (1986): Rat transferrin gene expression: Tissue-specific regulation by iron deficiency. Proc Natl Acad Sci USA 83:3723–3727.

Idzerda RL, Behringer RR, Theisen M, Huggenvik JI, McKnight GS, Brinster RL (1989): Expression from the transferrin gene promoter in transgenic mice. Mol Cell Biol 9:5154–5462.

Ishibash M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R (1994): Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J 8:1799–1805.

Jan YN, Jan LY (1993): HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell 75:827–830.

Jia MC, Ravindranath N, Papadopoulos V, Dym M (1996): Regulation of c-fos mRNA expression in Sertoli cells by cyclic AMP, calcium and protein kinase C mediated pathways. Mol Cell Biochem 156:43–49.

Kahn A, Levin MJ, Zakin MM, Bloch B (1987): The transferrin gene. In G Guroff (ed): "Oncogenes, Genes, and Growth Factors." New York: John Wiley & Sons. pp 277–309.

Kangasniemi M, Kaipia A, Mali P, Toppari J, Huhtaniemi I, Parvinen M (1990): Modulation of basal and FSH-dependent cyclic AMP production in rat seminiferous tubules staged by an improved transillumination technique. Anat Rec 227:62–76.

Karin M (1992): Signal transduction from cell surface to nucleus in development and disease. FASEB J 6:2581–2590.

Kingston RE, Chen CA, Okayama H (1993): Calcium phosphate transfection. In FA Ausabel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl (eds): "Current Protocols in Molecular Biology." New York: Greene and Wiley-Interscience, pp 9.1.1–9.1.3.

Lalli E, Sassone-Corsi P (1994): Signal transduction and gene regulation: The nuclear response to cAMP. J Biol Chem 269:17359–17362.

Lee JL, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H (1995): Conversion of *Xenopus ectopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Nature 268:836–844.

Morgan EH (1969): Factors affecting the synthesis of transferrin by rat tissue slices. J Biol Chem 244:4193.

Murre C, McCaw PS, Baltimore D (1989a):. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56:777–783.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB (1989b): Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537–544.

Norton JN, Skinner MK (1989): Regulation of Sertoli cell function and differentiation through the actions of a testicular paracrine factor PModS. Endocrinology 124:2711–2719.

Norton JN, Skinner MK (1992): Regulation of Sertoli cell differentiation by the testicular paracrine factor PModS: Potential role of immediate-early genes. Mol Endocrinol 6:2018–2026.

Schaeffer E, Boissier F, Py MC, Cohen GN, Zakin MM (1989): Cell type specific expression of the human transferrin gene. J Biol Chem 264:7153–7160.

Schaeffer E, Guillou F, Part D, Zakin M (1993): A different combination of transcription factors modulates the expression of the human transferrin promoter in liver and Sertoli cells. J Biol Chem 268: 23399–23408.

Setchell BP, Waites GMH (1975): The blood testis barrier. In RO Greep, EW Hamilton (eds): "Handbook of Physiology," Vol 5, Sec 7. Washington: American Physiological Society, pp 143–172.

Skinner MK (1987): Cell-cell interactions in the testis. Ann NY Acad Sci 513:158–171.

Skinner MK, Fritz IB (1985): Testicular cells secrete a protein under androgen control that modulates Sertoli cell function. Proc Natl Acad Sci USA 82:114–118.

Skinner MK, Griswold MD (1980): Sertoli cells synthesize and secrete transferrin-like protein. J Biol Chem 255:9523.

Skinner MK, Griswold MD (1982): Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biol Reprod 27:211–221.

Skinner MK, Fetterolf PM, Anthony CT (1988): Purification of the paracrine factor, P-Mod-S, produced by testicular peritubular cells that modulates Sertoli cell function. J Biol Chem 263:2884–2890.

Skinner MK, Schiltz SM, Anthony CT (1989): Regulation of Sertoli cell differentiated function: Testicular transferrin and androgen binding protein expression. Endocrinology 124:3015–3024.

Suire S, Fontaine I, Guillou F (1995): Follicle-stimulating hormone (FSH) stimulates transferrin gene transcription in rat Sertoli cells: Cis and trans-acting elements involved in FSH action via cyclic 3', 5'-monophosphate on the transferrin gene. Mol Endocrinol 9:756–766.

Theisen M, Behringer RR, Cadd GG, Brinster RL, McKnight GS (1993): A c/EBP-binding site the transferrin promoter is essential for expression in the liver, but not the brain of transgenic mice. Mol Cell Biol 13:7666–7676.

Tsutsumi M, Skinner MK, Sanders-Bush E (1989): Transferrin gene expression and syntheses by cultured choroid plexus epithelial cells. J Biol Chem 264:9626–9631.

Tung PS, Skinner MK, Fritz IB (1984): Fibronectin synthesis is a marker for peritubular cell contaminants in Sertoli cell enriched cultures. Biol Reprod 30:199–211.

Waites GMH, Gladwell RT (1982): Physiological significance of fluid secretion in the testis and blood-testis barrier. Physiol Rev 62:624–671.

Weintraub H, Dwarki VJ, Verma I, Davis R, Hollenberg S, Snider L, Lassar A, Tapscott SJ (1991): Muscle-specific transcriptional activation by MyoD. Genes Dev 5:1377–1386.

Whaley PD, Chaudhary J, Cupp A, Skinner MK (1995): Role of specific response elements of the c-*fos* promoter and involvement of intermediate transcription factor(s) in the induction of Sertoli cell differentiation (transferrin promoter activation) by the testicular paracrine factor PModS. Endocrinology 136:3046–3053.