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Mechanisms involved in the homologous down-regulation of transcription of the follicle-stimulating hormone receptor gene in Sertoli cells

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Abstract

The action of follicle-stimulating hormone (FSH) in spermatogenesis is regulated at a fundamental level by controlling the number of competent receptors present at the surface of Sertoli cells. By controlling the number of receptors, the cell is able to modulate the timing and magnitude of subsequent signal transduction in response to FSH. One mechanism of control is the down-regulation of the steady state levels of the FSH receptor gene after exposure to FSH or agents that stimulate or prolong the cAMP signal transduction cascade (homologous down-regulation) in Sertoli cells. The goals of this study were to examine possible mechanisms involved in the down-regulation of mRNA levels of this gene. Analysis of transcription and processing by a PCR-based assay showed that treatment of Sertoli cells with FSH caused at least a 50% reduction of hnRNA for the FSH receptor gene. Reporter genes controlled by 5′ flanking sequences of the FSH receptor gene that were transiently transfected into Sertoli cells were not down-regulated. In electrophoretic mobility shift assays (EMSA), cAMP-inducible nuclear protein complex containing c-Fos formed on the activator protein-1/cAMP responsive element-like site located at −216 to −210 in the promoter of the rat FSH receptor gene. We concluded from this study that there was no evidence for the putative role of ICER in the down-regulation of the FSH receptor promoter. In addition, the FSH-induced down-regulation of the transcription of the FSH receptor gene in Sertoli cells was prevented by the treatment of Sertoli cells with trichostatin A prior to the addition of FSH. This experiment coupled with other observations suggested that the down-regulation may be mediated by changes in chromatin structure.

Keywords: Follicle stimulating hormone receptor; Homologous down-regulation; Sertoli; Testis; Spermatogenesis; ICER

1. Introduction

One of the most important biochemical signaling networks involved in controlling normal spermatogenesis in mammals exists between the anterior lobe of the pituitary gland and Sertoli cells in the testis (Griswold et al., 1975, 1976, 1977; Orth, 1984; Singh and Handelsman, 1996; Kumar et al., 1997; Tapanainen et al., 1997; Dierich et al., 1998). The biological events controlled by this endocrine mechanism result from the interaction of follicle-stimulating hormone (FSH) with the FSH receptor and the subsequent transduction of molecular information across the membrane leading to the production of second messengers such as cAMP (Means et al., 1980) and Ca^{2+} (Chaudhary et al., 1996; Lalevee et al., 1999) and changes in gene expression.

Continuous stimulation of Sertoli cells with FSH leads to a desensitization of the cells to FSH (Gnanaprakasam et al., 1979). Desensitization of the FSH response in Sertoli cells involves multiple identified steps in the FSH/cAMP signal transduction pathway including the following: (1) the rapid internalization and sequestration of the ligand-bound receptor (Fletcher and Reichert, 1984; Saez and Jaillard, 1986; Shimizu et al., 1987); (2) post-translational modification of the receptor (Quintana et al., 1994; Hipkin et al., 1995); (3) reduction in adenylate cyclase activity (Le Gac et al., 1985); (4) increased phosphodiesterase activity (Conti et al., 1983, 1986); (5) direct

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inhibition of protein kinase A by protein kinase A inhibitor (Tash et al., 1979, 1981); and (6) the down-regulation of the transcription of the FSH receptor gene (Themmen et al., 1991; Monaco et al., 1995; Maguire et al., 1997). Given that the concentration of FSH in the serum of non-seasonal-breeding males is relatively constant, the regulation of the number of FSH receptors and their competency to bind FSH and transduce signal may be an important level of control on the action of FSH in males (McGuiness and Griswold, 1995).

Homologous down-regulation of transcription of the FSH receptor gene in cultured rat Sertoli cells was first reported by Themmen et al. (1991). The loss of approximately 90% of the steady-state level of FSH receptor mRNA and a parallel loss of ^{125}I FSH binding was measured 4 h after the addition of 500 ng/ml Ovine FSH (oFSH) to Sertoli cells in primary culture (Themmen et al., 1991). Themmen and coworkers suggested that down-regulation was stimulated by $(Bu)_2$cAMP, did not require de novo transcription or translation and concluded that RNA degradation was likely responsible for the homologous down-regulation of FSH receptor mRNA in Sertoli cells. Maguire and coworkers measured the relative contribution of FSH receptor mRNA decay to the process of homologous down-regulation by uncoupling transcription and mRNA decay with the transcription inhibitor actinomycin D (Maguire et al., 1997). Maguire and coworkers also demonstrated homologous down-regulation of the FSH receptor gene in vivo. The results of their work did not support the hypothesis that homologous down-regulation occurred via a cAMP-inducible mRNA decay pathway but rather suggested a role for transcription in homologous down-regulation is likely.

A model for the mechanism of homologous down-regulation of the FSH receptor in Sertoli cells that involved the inputs of de novo transcription and translation was presented by Monaco et al. (1995). They presented evidence that the inducible cAMP early repressor (ICER) bound to the rat FSH receptor promoter at a presumptive CRE-like site in vitro and repressed expression of a FSH promoter driven reporter gene. This was observed when ICER was overexpressed from the SV40 promoter on a co-transfected template in transient transfection assays (Monaco et al., 1995). In this study we have re-examined this putative CRE-like site and demonstrate that it functions as an AP-1 site. The transcription factor, c-Fos, induced by cAMP or FSH, can bind to the AP1 site in the promoter region of the FSH receptor gene in vitro and in vivo.

The restricted pattern of expression of the FSH receptor gene to Sertoli cells in males and homologous down-regulation of the gene are relaxed when non-chromatin templates are used to study the regulation of this important gene (Linder et al., 1994; Monaco et al., 1995). We were able to demonstrate that treatment of immature rat Sertoli cells in primary culture with trichostatin A, which inhibits histone deacetylase activity completely prevents the homologous down-regulation of the transcription of the FSH receptor gene.

2. Materials and methods

2.1. DNA sequencing and synthesis

Automated analysis of DNA sequencing reactions derived from PCR-based cycle sequencing was performed by the Laboratory for Bioanalysis and Biotechnology (LBB) at Washington State University. Oligonucleotides were synthesized by the LBB using phosphoramidite chemistry. All oligonucleotides were gel-purified on 12% polyacrylamide gels containing 7 M urea, eluted in TEN buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.25 M NaCl), ethanol precipitated, and washed twice with 70% EtOH.

2.2. Cell culture

Sertoli cells were removed from 18- to 20-day-old male Sprague–Dawley rats as previously described (Karl and Griswold, 1990). The cells were plated in Ham's F-12 medium (Gibco BRL, Grand Island, NY) on 60-mm culture dishes for transient transfection experiments and 150-mm culture dishes for RNA extraction. To reduce the amount of contaminating germ cell nuclear protein and RNA, the number of germ cells was reduced by hypotonic shock using dilute Ham's F-12 (1:10 dilution of Ham's F-12 in dH_2O) for 2 min on the third day in culture (Toebosch et al., 1989). Fresh Ham's F-12 was added to the cultures for an additional 24 h prior to the start of all experiments.

2.3. Quantitative RT-PCR analysis of FSH receptor hnRNA

A quantitative RT-PCR-based method of measuring the level of FSH receptor hnRNA in oFSH-treated and untreated cells was performed (Elferink and Reiners, 1996). This method is based on the inclusion of a known amount of in vitro-transcribed internal standard RNA that is identical to the region of the target hnRNA that will be amplified except that a restriction endonuclease cleavage site is introduced in the standard by recombinant techniques. The internal standard RNA is reverse-transcribed and amplified with the same efficiency as the target RNA. After PCR, the internal standard is cleaved at the novel restriction endonuclease cleavage site allowing for the separation of the internal standard from the target by electrophoresis. The signal from the internal standard is then used to normalize the signal for the target.

The internal standard plasmid construct was generated by subcloning the first exon and part of the 5′ end of the first intron of the FSH receptor gene into pGEM-T (Promega). The insert was generated by PCR using subclone 54.111 of the rat FSH receptor gene as the template (Heckert et al., 1992) and the following primers: sense primer #1 (+ −80) CAG TGT GTG GAG GAG CCT (−63) and antisense primer #1 $(dT)_{20}$ GAT ATC AGT CTA ATA GGA (this primer is complementary to the FSHR hnRNA in the first intron). Antisense primer #1 adds a poly $(dT)_{20}$ tail to the antisense strand of the plasmid insert that encodes a short poly (A) tail on the in vitro-transcribed internal RNA. This poly(A) tail facilitates the purification of full-length internal standard RNA molecules from the in vitro transcription reaction by allowing oligo(dT) cellulose affinity chromatography (Elferink and Reiners, 1996). A *Nco*I cleavage site was generated in the subclone by introducing a T to G base substitution by PCR-based site-directed mutagenesis (Fig. 1A) (Higuchi, 1990). The final construct was verified by DNA sequencing and named pMDG1.

Internal standard RNA was generated by in vitro transcription using Ambion's Maxi Script protocol (Ambion). Sense FSH receptor internal standard hnRNA was generated by transcription from the SP6 promoter in *Sph*I-linearized pMDG1 DNA by SP6 RNA polymerase (Ambion) and purification by oligo(dT) cellulose affinity chromatography. The purified internal standard RNA was quantified by measuring the absorbance at 260 nm.

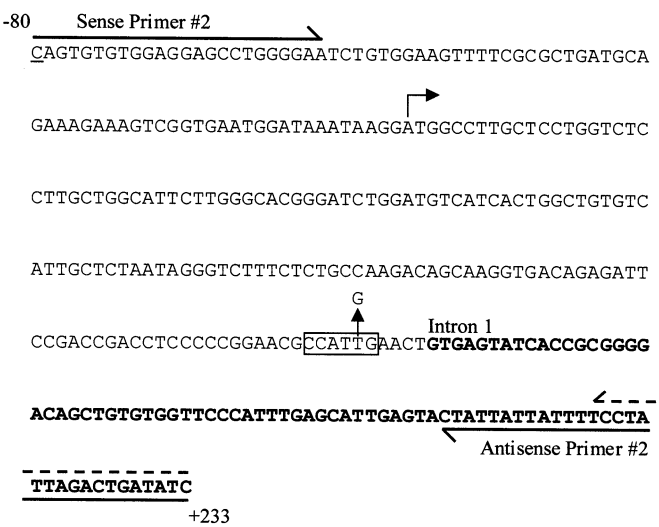

Fig. 1. Sequence of most of the first exon and 5′ end of the first intron of the rat FSH receptor gene. The 3′-most transcriptional start site identified by Heckert and coworkers is at position underlined. The translational start codon is indicated by the bent arrow. Bases that correspond to the first intron are in boldface. The position of the primer used for reverse transcription is shown by a dashed arrow above the corresponding sequence. The position of each primer used in the quantitative RT-PCR assay is indicated by a solid arrow. The *Nco*I site in the internal standard hnRNA, generated by a T-to-G base substitution, is shown in the box. The full-length PCR product generated is 315 bp in length (including the single T residues added by *Taq* DNA polymerase to 3′ ends of PCR products).

Prior to the reverse transcription reactions, rat Sertoli cells in primary culture were treated for 5 or 6 h with the following reagents depending on the experiment: 5 μg/ml actinomycin D (Sigma); 5 μg/ml actinomycin D and 2 U/ml oFSH (NIDDK-oFSH-20-SIAFP); 10 μg/ml α-amanitin (Boehringer Mannheim, Mannheim, Germany); 10 μg/ml α-amanitin and 2 U/ml oFSH; 2 U/ml oFSH only; or untreated. Total RNA, extracted from Sertoli cells as described previously (Chomczynski and Sacchi, 1987; Maguire et al., 1997), was treated with DNase I (Boehringer Mannheim) in the presence of 1 mM manganese as previously described except that reactions were scaled-up to accommodate ~30 μg of total RNA (Bauer et al., 1997).

Reverse transcription reactions were performed in reactions containing a heat-denatured mixture of 2 μg of DNase I-treated total Sertoli cell RNA, 5×10^3 molecules of purified FSH receptor internal standard hnRNA, 1 nmol gene-specific RT primer (GAT ATC AGT CTA ATA GGA), and diethyl pyrocarbonate-treated dH_2O with 10 U of RNasin (Promega), AMV reverse transcriptase buffer (50 mM Tris–HCl, pH 8.3, 5 mM $MgCl_2$, 5 mM DTT, 25 mM KCl), 500 μM each dNTP, and 12.5 U of AMV reverse transcriptase (Gibco BRL) in a total of 50 μl. RT reactions were incubated for 1 h at 37°C and then stopped by heating at 94°C for 5 min. The cDNA in these reactions was diluted by adding 200 μl of dH_2O and stored at −80°C.

PCR was carried out using 5 μl of the diluted cDNA as a template in a final volume of 50 μl containing 1 × AmpliTaq DNA polymerase buffer (Perkin-Elmer), 100 μM each dNTP, 0.3 μM each primer (sense primer #1 CAG TGT GTG GAG GAG CCT; antisense primer #2 GCA TTC TAG ATG TGC GGT TTT GCT TTG T: see Fig. 1A), 5 μCi [α-^{32}P]ATP (3000 Ci/mmol, NEN LifeProducts), and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer). A master mix of all components except the cDNA templates and primers was added to individual 0.6-ml thin-walled tubes containing the appropriate cDNA template. A hot start was performed by adding the appropriate volume of primer mini-mix. Thermocycling parameters were as follows: initial hot start at 94°C; 30 cycles of 94°C for 60 s, 55°C for 10 s, 72°C for 30 s; final extension at 72°C for 10 min. After the PCR, the internal standard product was digested by adding 10 U of *Nco*I (Gibco) to the PCR mixture and incubating for 4 h at 37°C.

Radiolabeled RT-PCR products were resolved by electrophoresis on a nondenaturing 7% polyacrylamide gel in 0.5 × TBE. The gels were run at 200 V for 1.25 h and then dried by standard techniques. The data were

analyzed using a Molecular Dynamics PhosphorImager 445 SI and ImageQuant software. The integrated optical density of the target was normalized by dividing it by the integrated optical density of the signal for the large *Nco*I-digested fragment of the corresponding internal standard.

2.4. Plasmid constructs for transient transfection assays

p383/−1/Luc contains 383 bp of the 5′ flanking region (including the 5′ untranslated region) of the FSH receptor gene upstream of the luciferase reporter gene in pGL2 (Promega, Madison, WI) (Goetz et al., 1996). p383/−1/Luc was used as a template for PCR-based site-directed mutagenesis to generate mutants that contain changes in the AP1/CRE-like site (TTAGTCA) at position −216 to −210 (relative to the translation start site) in the rat FSH receptor promoter (Higuchi, 1990; Heckert et al., 1992; Goetz et al., 1996). pCRE-F-Mut/Luc (CRE in FSHR Mutant) contains the following mutation: TTAGTCA to TTTGTCA. pCRE-Pal /Luc contains a palindromic CRE promoter (Brindle and Montminy, 1992) in context with the AP1/CRE-like site within the FSH receptor promoter as follows: TTAGTCA to TGACGTCA. A mutant of the palindromic CRE (pCRE-Pal-Mut/Luc) was generated and has the following sequence at this site: TGTGGTCA. The plasmids p383/−1/Luc, pCRE-F-Mut/Luc, pCRE-Pal/Luc, and pCRE-Pal-Mut/Luc were cloned into the *Nhe*I–*Hin*dIII site of pGL2 and verified by DNA sequencing.

p2700/Luc contains approximately 2700 bp of the 5′ flanking region of the FSH receptor gene including the 5′ untranslated region (5′UTR). p2700/Luc is composed of the *Pst*I–*Eco*RV fragment of the 5′ end of the rat FSH receptor gene from λ-genomic clone 54.111 (Heckert et al., 1992) ligated to the *Eco*RV-*Hin*dIII fragment of the FSH receptor promoter in p383/−1/Luc. This ∼2700 bp fragment of the FSH receptor 5′ flanking region was cloned into the *Sac*I–*Hin*dIII site in pGL2 (Promega) after the *Pst*I and *Sac*I sticky ends were converted to blunt ends by the exonuclease activity of T4 DNA polymerase using standard techniques. This construct was verified by restriction analysis and by DNA sequencing across all junctions generated by ligation.

2.5. Transient transfection assays

Transient transfections of primary cultures of rat Sertoli cells were performed using the $CaPO_4$ coprecipitation method in 60-mm plastic culture dishes as described previously with minor modifications (Linder et al., 1994; Goetz et al., 1996). Cells were transfected on the 4th day of culture with 6 μg of reporter plasmid and 0.3 μg of pRL (Promega) coreporter plasmid. The activity of the *Renilla* luciferase encoded by pRL was used to normalize the firefly luciferase signal from pGL2- and pGL3-based constructs and control for transfection efficiency. The data from the transfection experiments reported in this manuscript were derived from at least three different experiments and two distinct preparations of plasmid DNA using the Qiagen Endotoxin-Free large-scale plasmid preparation protocol. Transfected cells were treated with 2 U/ml of oFSH or vehicle only (1 × phosphate-buffered saline, 1 mg/ml bovine serum albumen) such that all the cells in all groups were harvested at the same time and assayed for luciferase activity.

Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Cell lysates were analyzed for luciferase activity in a luminometer (MicroLumat LB 96p, EG&G Berthold, Germany). Lysates of corresponding samples (e.g. p383/−1/Luc at 4 h with oFSH and without oFSH) were placed in the same location on two different 96-well plates.

2.6. Northern blot analysis

To determine if the conditions of $CaPO_4$-mediated transfection alter homologous down-regulation of the FSH receptor mRNA, Northern analysis was performed. Sertoli cells were transfected as described above and total RNA was extracted by the method of Chomczynski and Sacchi (1987). The relative amount of FSH receptor mRNA in each sample was analyzed by Northern blot analyses as described previously (Maguire et al., 1997).

2.7. Electrophoretic mobility shift assays (EMSA)

Extraction of nuclear proteins from primary cultures of Sertoli cells was performed as described by Andrews and Faller and is the same procedure used by Monaco and coworkers (Andrews and Faller, 1991; Monaco et al., 1995). Nuclear proteins were extracted from Sertoli cells that were treated with 1 mM $(Bu)_2$cAMP or 2 U/ml oFSH for 1 or 4 h and cells that were treated with vehicle only. Purified recombinant human c-Fos and Jun-B were kindly provided by Mark Nissen and Raymond Reeves at Washington State University. The c-Fos and Jun-B expression constructs were generously provided by David Tremethick at The Australian National University (Ng et al., 1997).

The following is a list of the names and sequences of the sense strand of each double-stranded oligonucleotide used in this study:

wtAP1: −225 TGACACACATTAGTCACATAT-TAAT −201

mutAP1: −225 TGACACACATTAGTTGCATAT-TAAT −201

CREpal: TGACACACATGACGTCACATATTAA-T.

The numbering is based on the translational start site. The location of the AP1/CRE-like site is underlined and base substitutions are indicated in boldface.

Single-stranded oligonucleotides were end-labeled with [α-^{32}P]ATP (3000 Ci/mmol, NEN LifeProducts) using T4 polynucleotide kinase (Gibco BRL) prior to annealing. Labeled double-stranded oligonucleotide probes were gel-purified in nondenaturing, 6% polyacrylamide gels in 0.5 × Tris Borate EDTA (TBE) and eluted in dH_2O.

Each binding reaction was performed in a total volume of 15 μl with $\sim 5.0 \times 10^4$ cpm of labeled probe (0.5 ng DNA), 3 μg Sertoli cell nuclear protein extract, 0.2 μg poly(dI)-poly(dC) (Pharmacia Biotech. Inc., Uppsala, Sweden), in a buffer composed of 12.5 mM Hepes, pH 7.9 (at 0°C), 25 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 0.05% Triton X-100, and 10% glycerol. Unlabeled competitor DNA was included in some reactions at 100-fold molar excess over the labeled oligonucleotide probe.

The identity of c-Fos in the Sertoli cell nuclear protein–oligonucleotide probe complex was determined by supershift analysis. Supershift reactions were performed with rabbit, anti-human c-Fos polyclonal antibody (Ab) SC-253, rabbit, anti-human CREB-1 polyclonal Ab SC-186, or rabbit, anti-human CREM-1 polyclonal Ab SC-440 (Santa Cruz Biotechnology, Santa Cruz, CA) by adding 1 μg of the antibody to the binding reaction after the 10-min incubation with labeled probe and incubating for one additional hour on ice. A control for nonspecific interactions between the DNA–protein complexes and antibodies was performed by adding 1 μg of rabbit, anti-human TFIID polyclonal antibody SC-273X (Santa Cruz Biotechnology) to control binding reactions and incubated on ice for 1 h.

Protein–DNA complexes were resolved by electrophoresis through a nondenaturing 4% polyacrylamide gel in 0.5 × TBE. The gels were run for 1.25 h at 200 V. After electrophoresis, the gels were dried using standard techniques. The signals were detected using a Molecular Dynamics PhosphorImager 445 SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.8. Treatment of Sertoli cells with trichostatin A

To determine if the inhibition of histone deacetylases derepressed the homologous down-regulation of the FSH receptor gene, primary cultures of Sertoli cells were prepared as described above and treated with 0.3 μM trichostatin A (TSA) (Sigma). Cells were treated with trichostatin A for 1 h prior to the addition of 2 U/ml oFSH for four additional hours. The level of FSH receptor mRNA was determined by Northern blot as described previously (Maguire et al., 1997). The Northern blot was stripped and reprobed with a fragment of the cDNA for rat clusterin (Clark and Griswold, 1997) to control for loading and transfer.

2.9. Experimental animals

All research done with animals for the work described in this paper followed university and NIH guidelines and policies.

3. Results

3.1. Determination of levels of FSH receptor hnRNA

A strategy was designed to amplify the specific cDNA that corresponds to the first exon and 5′ end of the first intron of the FSH receptor hnRNA (Fig. 1). Sample-to-sample variation in the efficiency of reverse transcription and PCR were controlled for by the inclusion of a known amount of internal standard RNA in the RT reactions. The internal standard was identical to the target in length and sequence with the exception of a single base difference that generated a *Nco*I site in the internal standard (Fig. 1). The signals generated by the target and internal standard cDNAs were in the exponential range after 30 cycles (data not shown). Given that the RT primer (antisense primer 1) was complementary to bases in the first intron (Fig. 1), rigorous controls were performed to limit the signal generated by contaminating genomic DNA. Total RNA was treated with DNase I prior to performing reverse transcription reactions and PCR was performed on all RNA samples that were placed in reverse transcription reactions devoid of reverse transcriptase (data not shown). We did not collect data for the target or internal standard if we observed significant background from genomic DNA. The identity of the target band (315 bp in Fig. 2A) and the internal standard band after cleavage with a restriction enzyme ($\sim$220 bp in Fig. 2A) was confirmed by excising these fragments from a gel, reamplifying by PCR, cloning into pGEM T (Promega) and sequencing (data not shown). FSH induced a reduction in the steady-state level of FSH receptor hnRNA by at least 50% after 5 h (Fig. 2A,B). A similar FSH-induced reduction of FSHR hnRNA was seen if the cells were also treated with either α-amanitin or actinomycin D (data not shown).

3.2. Transient transfection analysis of the 2.7 kb promoter

The possibility that the cis-element(s) required for homologous down-regulation are localized in the pro-

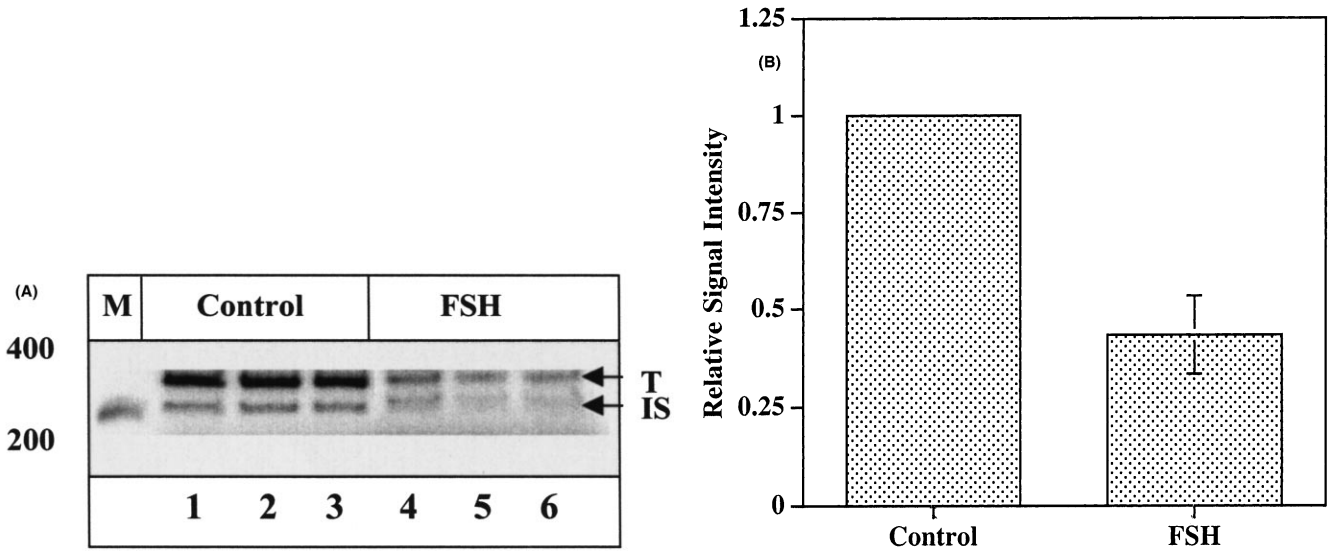

Fig. 2. Quantitative RT-PCR analysis of FSH receptor hnRNA in Sertoli cells. (A) PCR products from Sertoli cells that were not treated (lanes 1–3), or treated with FSH (lanes 3–6) were digested with *Nco*I and resolved by polyacrylamide gel electrophoresis. The low molecular weight DNA mass ladder (Gibco) was radiolabeled and used as a marker (M). The identity of each band is given on the right as follows, T (315 bp target), IS (internal standard *Nco*I-digestion products). (B) The integrated optical densities of the target bands were divided by the integrated optical densities of the large (~ 220 bp) internal standard *Nco*I-digestion products and normalized to control samples. Data are the means from three separate experiments ± S.E.

moter at a site in the extended (~ 2700 bp) 5′ flanking region was tested (Fig. 3A). Lysates from Sertoli cells that were transiently transfected with p2700/Luc and treated with 2 U/ml oFSH for 4, 8, 12, and 24 h were assayed for luciferase activity. The 2700 bp promoter was not sufficient to direct homologous down-regulation in transient transfection assays (Fig. 3A).

3.3. Transient transfection analysis of the AP1/CRE-like site

A two- to three–fold increase in luciferase activity was observed in Sertoli cells transiently transfected with the luciferase reporter gene driven by the proximal 383-bp fragment of the wild-type FSH receptor promoter (p383/ − 1/Luc) 8 h after the addition of oFSH (Fig. 3B). The increase in luciferase activity is abolished when a single base mutation (− 214 A to T) is made in the AP1/CRE-like site contained within the proximal 383 bp of the FSH receptor promoter (pCRE-F-Mut/Luc in Fig. 3B). The − 116 A to T base substitution mutates this site such that it is less like either the AP1 or CRE consensus sequences (Faisst and Meyer, 1992). Altering the AP1/CRE-like site in the FSH receptor gene promoter to match the palindromic CRE consensus sequence (pCRE-Pal/Luc) did not alter the magnitude or kinetics of the FSH-induced signal compared to p383/ − 1/Luc (Fig. 3B,C). None of the constructs used in Fig. 3B,C directed down-regulation in this transient transfection system even when transfected cells were lysed and assayed 48 h after the addition of oFSH (data not shown). The decrease in signal at the longer time points was probably due to the instability of the luciferase or the induction signal.

Northern blot analysis was performed to determine if the FSH-induced signal transduction pathway responsible for the down-regulation of the FSH receptor gene was functioning under conditions of transient transfection. The steady-state amount of FSH receptor mRNA was measured by Northern blot in total RNA from cells that were transfected with p383/ − 1/Luc and left untreated (Control) or treated with oFSH (Fig. 4). Homologous down-regulation occurred with the same kinetics as reported by Themmen et al. (1991). Therefore, the FSH-induced signal transduction pathway responsible for down-regulation of the endogenous gene is apparently not disrupted by the experimental conditions of transient transfection used in this study.

3.4. Inducible c-Fos complex formation on the AP1/CRE-like site

A specific complex forms between Sertoli cell nuclear proteins and an oligonucleotide probe (wtAP1) that contains the wild-type AP1/CRE-like site (Fig. 5A). This result was expected because of the contribution of this site to the modest enhancement of expression from transiently transfected templates in FSH-treated Sertoli cells as shown in Fig. 5A,B. There was a marked increase in the specific complex (SC) after 4 h of treatment with $(Bu)_2$cAMP and oFSH (Fig. 5A, lanes 9 and 10). A nonspecific complex (NS) is present in

nearly all experiments performed with this oligonucleotide probe (Fig. 5A,B). This nonspecific complex is not inducible with $(Bu)_2$cAMP or oFSH and is not removed by competition with any oligonucleotide used in this study (Fig. 5A,B). Addition of 100-fold excess of unlabeled wtAP1 probe abolished the signal for the specific complex (SC).

The specificity for the AP1/CRE-like site (TTAGTCA) within the wtAP1 probe was rigorously demonstrated in competition experiments with 100-fold excess of an oligonucleotide that has a mutation in the AP1/CRE-like site (mutAP1) (Fig. 5B). The complex that forms between Sertoli cell nuclear proteins and the wtAP1 probe forms at the AP1/CRE-like site within the

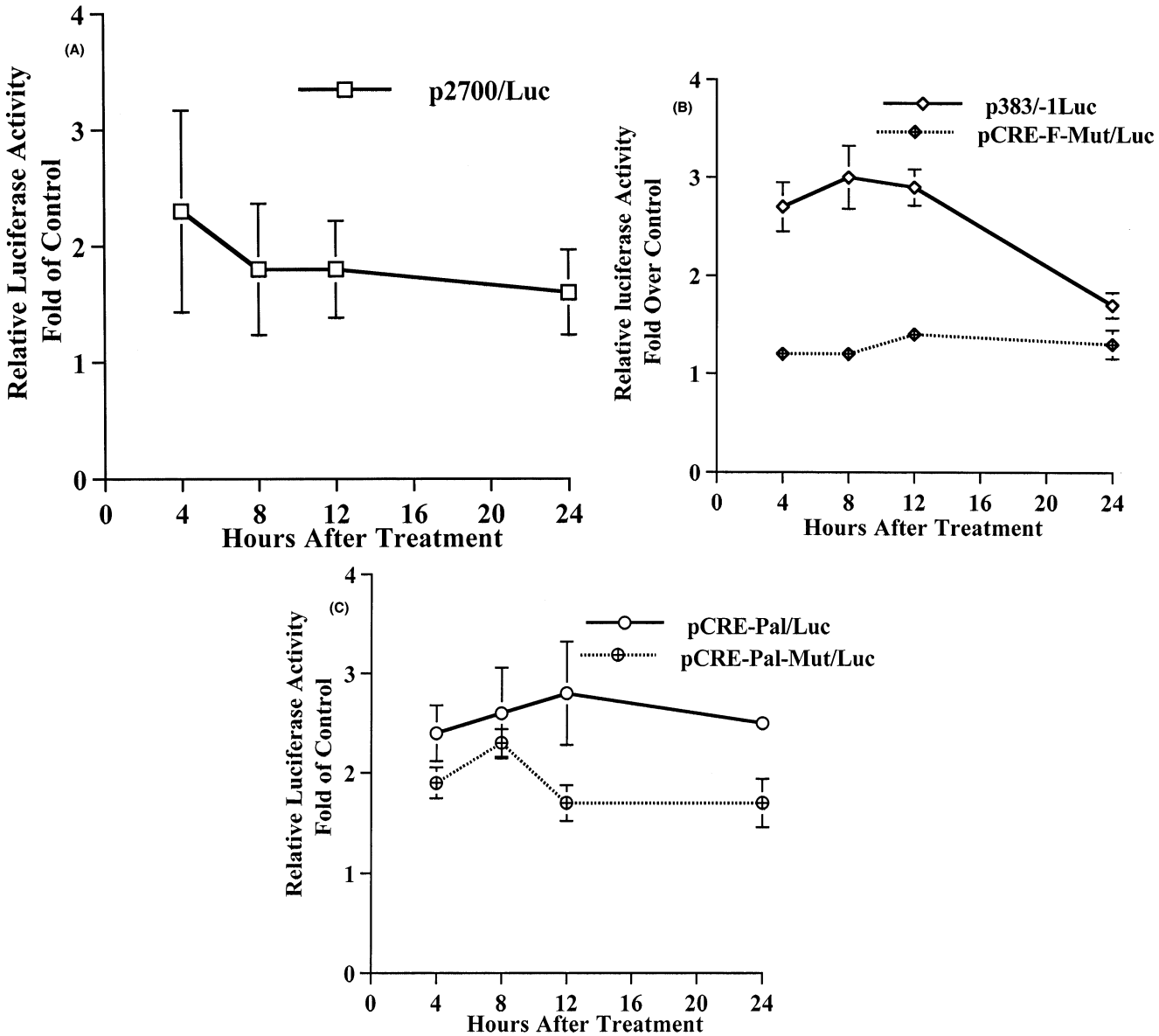

Fig. 3. Regulation by oFSH of transiently transfected luciferase reporter genes driven by the proximal FSH receptor promoter. (A) Transient transfection analysis of p2700/Luc in rat Sertoli cells. Sertoli cells were transfected with p2700/Luc and then treated, or not, for 4, 8, 12, and 24 h with 2 U/ml oFSH. The luciferase activity in the lysates of treated cells was normalized to control (untreated). The data shown are the means ± SEM for two experiments (different preparations of Sertoli cells) run in triplicate. (B) Constructs containing wild type (TTAGTCA: p383/ − 1/Luc) or mutant (TTTGTCA: pCRE-F-Mut/Luc) AP1/CRE-like site were transfected into rat Sertoli cells. Luciferase activity was measured in treated and untreated cells 4, 8, 12, and 24 h after addition of 2 U/ml oFSH to the treatment groups. To determine the effect of oFSH on reporter expression, data for the oFSH-treated cells were placed on a control (untreated) basis. The relative luciferase activities shown were derived by dividing the means of the oFSH-treated cells by the means of the untreated (Control) cells at each time point. (C) Relative luciferase activity in lysates from Sertoli cells transfected with a construct containing a palindromic CRE (pCRE-Pal/Luc) and in lysates from cells transfected with a mutant CRE site (pCRE-Pal-Mut/Luc). The data shown are normalized to control as in A. The data are the means from two separate experiments (different preparations of Sertoli cells) run in triplicate ± S.E.

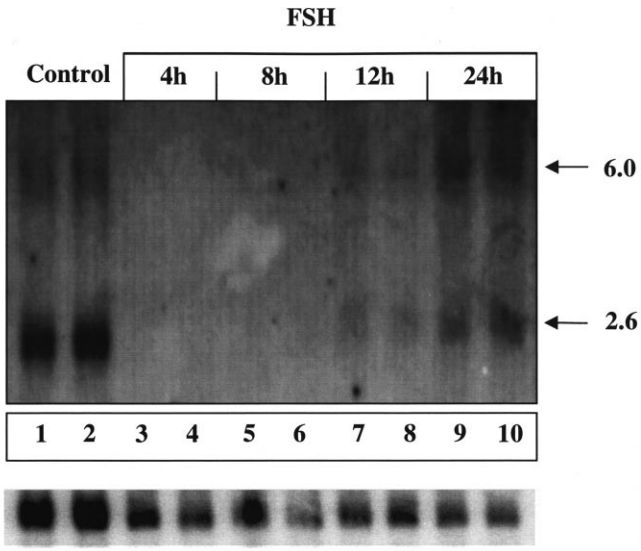

Fig. 4. Northern analysis of FSH receptor mRNA from transfected Sertoli cells. Total RNA was extracted from Sertoli cells after transfection with p383/−1/Luc by the $CaPO_4$ coprecipitation method and subsequent treatment with 2 U/ml oFSH for 4, 8, 12, or 24 h and run on a denaturing agarose gel. Samples were run in duplicates (20 μg total RNA/lane) and hybridized to a cDNA probe for the rat FSH receptor. Control samples were transfected with p383/−1/Luc but were not treated with oFSH. Control samples were harvested at 24 h after the start of the treatments (at the same time that the 24-h samples were harvested: lanes 9 and 10). Treatment of the 4-, 8-, and 12-h samples was staggered such that all samples were harvested at the same time. The approximate sizes of the two FSH receptor mRNA transcripts normally detected in Sertoli cells are indicated in kilobases. Lower panel: the same blot as in the upper panel that has been stripped and reprobed with CHO B cDNA for a loading control (Harpold et al., 1979).

oligonucleotide (Fig. 5B). A 100-fold excess of unlabeled mutAP1 was unable to compete with the complex (Fig. 5B, lanes 10–13) whereas, competition with the unlabeled wtAP1 oligonucleotide significantly reduced specific complex formation (Fig. 5B, lanes 6–9). Importantly, the induced Sertoli cell nuclear protein–wtAP1 complex is also specific for the AP1/CRE-like site within the wtAP1 probe. Recombinant human c-Fos/Jun-B heterodimers also formed a specific complex within the AP1/CRE-like site in the wtAP1 oligonucleotide probe (Fig. 5A). In a separate experiment, Jun-B homodimers were tested for their ability to bind to the wtAP1 probe. No binding was observed by Jun-B homodimers to the wtAP1 probe (data not shown).

Direct evidence for the presence of c-Fos in the specific and inducible complexes shown in Fig. 5A,B was provided by supershift analysis using rabbit, anti-human c-Fos polyclonal antibody (Fig. 6A,B). To ensure that the supershifts observed in Fig. 6A were not a result of nonspecific binding of the specific complex to any antibody, a control experiment was performed using a heterologous antibody (rabbit-anti-human TFIID polyclonal antibody) (Fig. 6B). Rabbit, anti-human TFIID antibody was unable to supershift the specific, nor the nonspecific complexes (Fig. 6B).

3.5. Control for ICER in nuclear extracts

Complexes of high mobility on non-denaturing, non-reducing PAGE that could correspond to ICER-containing complexes were not detected by EMSA. To determine if ICER was present in the protein extracts, a control experiment was performed using an oligonucleotide probe that contained a palindromic CRE site in context with the endogenous FSH receptor gene promoter sequence. Nuclear proteins from Sertoli cells treated with oFSH formed several inducible complexes with the CREPAL probe (Fig. 7). The pattern of the inducible ICER complex migration through the gel is similar to that shown by Walker et al. (1998) when using CRE-containing probes of the CREB gene. Supershift analysis was performed to determine if the inducible high mobility complexes contained ICER. Given that the polyclonal anti-CREB and anti-CREM antibodies react with many members of the ATF family of transcription factors, the supershift of the ICER complex and the heterodimer (Walker et al., 1998) complexes was expected (Fig. 7, Lanes 4 and 5). The anti-c-Fos antibody did not shift the high mobility complexes containing ICER as expected (Fig. 7, Lane 3).

3.6. Inhibition of histone deacetylases

The involvement of histone deacetylation and chromatin structure in the homologous repression of the FSH receptor gene was examined with the histone deacetylase inhibitor trichostatin A (TSA). Sertoli cells in primary culture were treated with 0.3 μM TSA for 1 h prior to the addition of oFSH. After four more hours the mRNA was isolated and the level of FSH receptor mRNA was determined by Northern blot analyses. The TSA treatment completely eliminated the homologous down-regulation of the gene (Fig. 8, compare lanes 3 and 4).

4. Discussion

The results of this study do not support the previously proposed role of ICER in the down-regulation of transcription of the FSHR gene (Monaco et al., 1995). In this proposed role, ICER down-regulated the FSH receptor gene by binding to the AP1 site in the FSH receptor promoter and displacing or competing for an activating transcription factor. Monaco and coworkers reported that the AP-1 site was actually a CRE-like site and that inducible cAMP early repressor (ICER) bound to the rat FSH receptor promoter at this site in vitro and repressed expression of a reporter gene (Monaco et

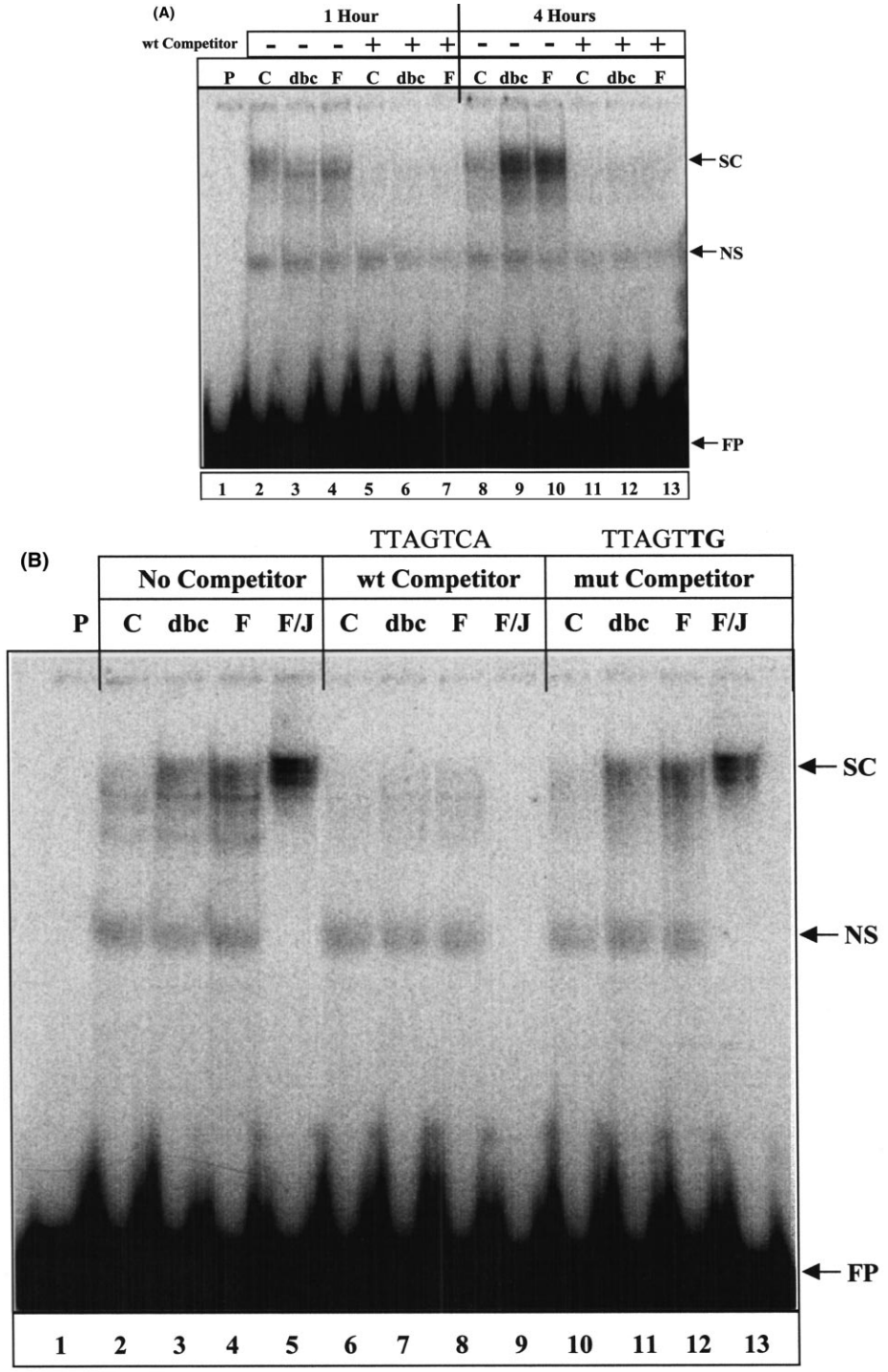

Fig. 5. (A) Demonstration by EMSA that an inducible Sertoli cell nuclear protein–DNA complex forms on the wtAP1 oligonucleotide probe. Nuclear extracts from rat Sertoli cells that were untreated, treated with 1 mM $(Bu)_2$cAMP (dbc), or treated with 2 U/ml oFSH (F) for 1 h (lanes 2–7) or 4 h (lanes 8–13) were used in binding reactions with radiolabeled wtAP1 oligonucleotide probe. To determine if the interaction between the nuclear proteins and wtAP1 was specific, a 100-fold excess of unlabeled wtAP1 competitor was added to some reactions. A nonspecific (NS) complex that was not removed upon the addition of unlabeled competitor was formed in all lanes with nuclear protein. Lane 1 is a probe-only (P) control and the migration of unbound, free probe is indicated (FP). (B) EMSA analysis of the AP1/CRE-like site within the wtAP1 oligonucleotide probe. Sertoli cell nuclear proteins from cells that were untreated, treated with 1 mM $(Bu)_2$cAMP (dbc), or treated with 2 U/ml oFSH (F) for 4 h were added to labeled wtAP1 oligonucleotide probe (lanes 2, 3, 4, 6, 7, 8, 10, 11, and 12). Recombinant human cFos/Jun-B heterodimer was added to labeled wtAP1 in some reactions (lanes 5, 9, and 13). A probe-only control is shown in lane 1. To determine specificity for the wtAP1 probe, a 100-fold excess of unlabeled wtAP1 was added as a competitor to the reactions shown in lanes 6–9. Specificity for the AP1/CRE-like site within the wtAP1 probe was determined by including a 100-fold excess of unlabeled competitor oligonucleotide that contains a mutation in the AP1/CRE-like site (mutAP1). Positions of the specific complex (SC), nonspecific complex (NS), and free probe (FP) are shown.

al., 1995). The results of EMSA analyses in our study using the wtAP1 oligonucleotide probe clearly demonstrate the preferential binding of c-Fos, and likely Jun-B, to this site relative to any other protein in the extracts. The c-Fos and Jun-B transcription factors are members of the AP1 family of transcription factors, can function as transcriptional activators or repressors and are rapidly induced by cAMP in rat Sertoli cells (Hall et al., 1988; Hamil et al., 1994). Members of the ATF and AP1 families of transcription factors can bind to a wide variety of CRE and AP1 sites, including non-consensus sites, and in a variety of homodimer and heterodimer combinations (Habener, 1990; Drust et al., 1991).

FSH stimulated the luciferase activity in Sertoli cells transfected with p383/−1/Luc (Fig. 3B). This increase was apparently due to the AP1 site since an inactivating mutation suppressed the stimulation. The AP1 site or putative ICER binding site is not a consensus CRE and is not conserved in the mouse and human promoters in the FSH receptor gene (Roesler et al., 1988; Brindle and Montminy, 1992; Faisst and Meyer, 1992; Maguire et al., 1997). Conversion of the site to an authentic palindromic CRE site still resulted in a FSH-stimulated increased luciferase activity. None of the transfected constructs were down-regulated as a result of treatment of the cells with FSH. Clearly, ICER was not active on these transfected transcripts. It is possible that the number of templates present in a transfected cell overcomes the down-regulation machinery but the observation that reporter constructs driven by the TSH receptor promoter are down-regulated by the endogenous machinery in FRTL-5 cells counters that possibility (Ikuyama et al., 1992). Also, ICER has been reported to be a powerful repressor, able to repress transcription at substoichiometric concentrations (Lalli et al., 1996). In the studies reported by Monaco et al. (1995), endogenous ICER was not able to down-regulate the expression of the CAT reporter gene driven by the rat FSH receptor promoter (Monaco et al., 1995) Co-transfection with an ICER expression construct driven by the SV-40 promoter was used to show cAMP-induced, ICER-dependent down-regulation of reporter constructs in Sertoli cells (Monaco et al., 1995). Such overexpression of ICER may down-regulate the transgene by a different mechanism.

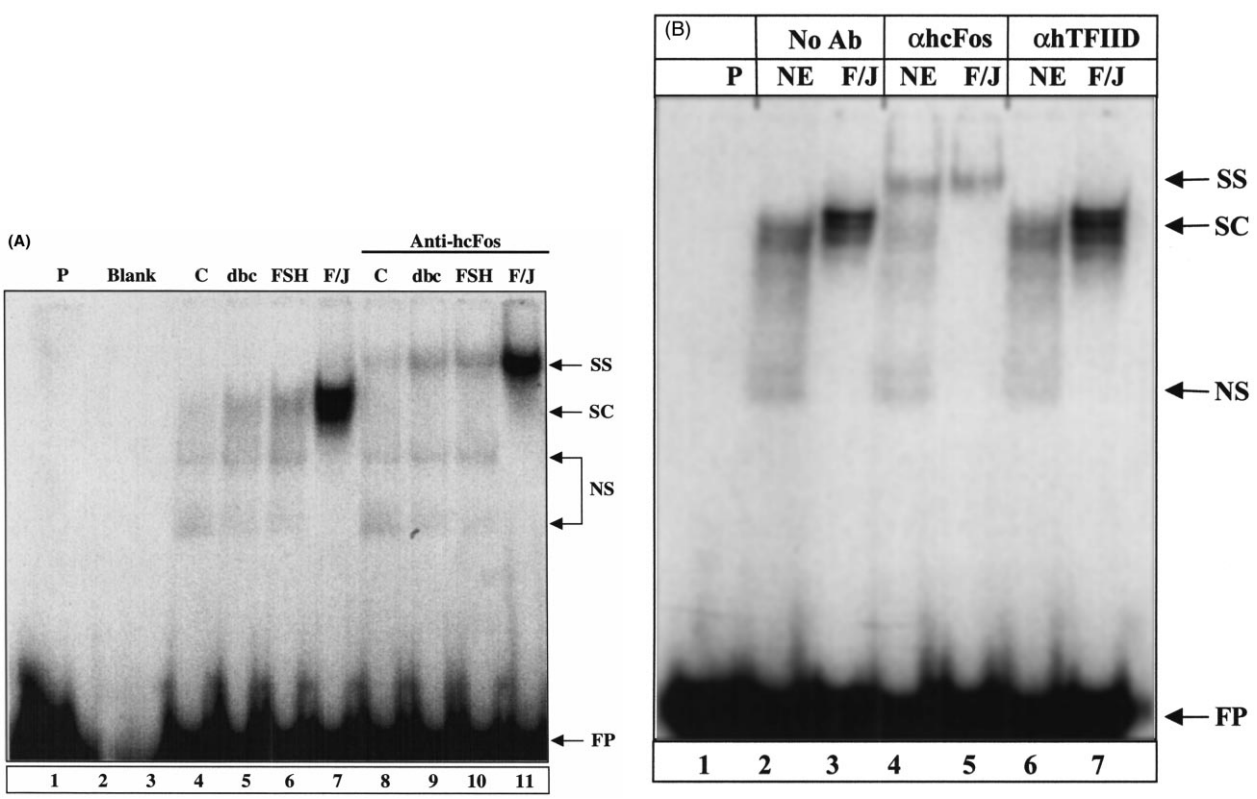

Fig. 6. The specific and inducible complex that forms on the AP1/CRE-like site in the wtAP1 oligonucleotide probe contains c-Fos. (A) EMSA Supershift analysis using anti-hcFos antibody (lanes 8–11). Sertoli cell nuclear extracts from untreated cells, cells treated with 1 mM $(Bu)_2$cAMP (dbc), cells treated with 2 U/ml oFSH or purified recombinant hcFos/Jun-B (F/J) were incubated with labeled wtAP1 oligonucleotide probe. Positions of the supershifted complexes (SS), specific complexes (SC), nonspecific complexes (NS), and free probe (FP) are indicated. Lane 1 contains a probe-only control. Lanes 2 and 3 are blank. (B) A supershifted complex does not form with a heterologous antibody. Sertoli cell nuclear extracts (NE) from untreated cells or recombinant human c-Fos/Jun-B heterodimers (F/J) were included in binding reactions with labeled wtAP1 oligonucleotide probe. Supershift EMSA with anti-hcFos antibody (lanes 4 and 5) or anti-hTFIID (transcription factor II-D) antibody (lanes 6 and 7). Control samples did not receive antibody (lanes 2 and 3).

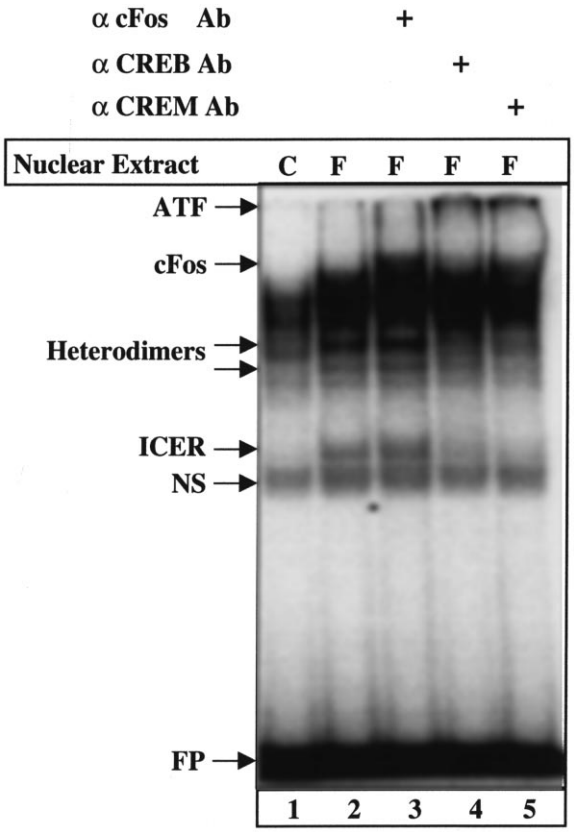

Fig. 7. Electrophoretic mobility shift and supershift assay with palindromic CRE oligonucleotide probe and Sertoli cell nuclear proteins showing the presence of ICER in the protein extracts. Sertoli cell nuclear extracts from untreated cells, or cells treated with 2 U/ml oFSH (F) were used in binding reactions with radiolabeled CREpal oligonucleotide probe. The presence of an antibody in a binding reaction is indicated (+). The identity of the complexes is shown on the left as follows: ATF (activating transcription factor family), cFos, heterodimers (complexes that contain ICER and some unknown protein(s), ICER, NS (non-specific complex). The band on the bottom corresponds to unbound, free probe (FP).

The role, if any, of c-Fos in the regulation of the FSH receptor gene is poorly understood. Evidence presented in this paper that c-Fos/Jun-B heterodimers can bind to the AP1/CRE-like in EMSA experiments is the first reported involvement of the AP-1 family of transcription factors in the regulation of the FSH receptor gene. These studies do not support the previously described role of ICER and a putative AP-1/CRE site in the down-regulation of the FSHR gene. The site is clearly shown to be a functional AP-1 site. When the rat FSHR promoter was reconstituted into nucleosomes and mapped by hydroxyl radical footprinting we found that the Ap-1 site was not accessible to transcription factors (unpublished data). The AP-1 site mapped close to the surface on a very stable nucleosome. Thus it is possible that the Ap-1 site plays no role in either transcription or down-regulation of the FSHR gene in vivo.

In previous studies we presented evidence that the down-regulation of the FSH receptor gene did not result from changes in the stability of the FSHR mRNA (Maguire et al., 1997). We were able to show that the kinetics of mRNA decay was the same in the presence or absence of actinomycin D. Also, the addition of FSH or actinomycin D to cultured Sertoli cells appeared to produce similar kinetics suggesting that FSH inhibited transcription. While it is still possible that mRNA stability plays a role we think it is unlikely to be the primary mechanism. The FSH receptor gene has a relatively low transcription rate and direct measurement of transcription with nuclear run-on assays was not possible. Therefore we utilized a quantitative PCR-based assay designed to measure the levels of FSH receptor hnRNA. It has been demonstrated that this hnRNA RT-PCR assay is an acceptable substitute for the nuclear run-on assay (Elferink and Reiners, 1996). Both the hnRNA RT-PCR assay and the nuclear run-on assay detected an equivalent increase in transcription of Cyp1a-1 in cultured murine Hepa 1c1c7 cells following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The RT-PCR assay also revealed TCDD-dependent transcriptional activation of the Cyp1a-1 gene in murine skin, a tissue unsuited to the nuclear run-on assay because of inherent difficulties associated with the isolation of nuclei (Elferink and Reiners, 1996). The results presented here showed that the steady state level of hnRNA transcribed from the FSHR gene was decreased 5 h after the addition of FSH. Since the levels of FSHR mRNA also decrease and mRNA turnover does not change, it follows that the decreased amount of hnRNA must result from decreased transcription and not from decreased hnRNA processing.

Two observations originally suggested that endogenous chromatin structure might play a role in the FSH-induced repression of the gene. First, none of the transiently transfected constructs used in this study, or by Monaco et al. (1995), directed homologous down-

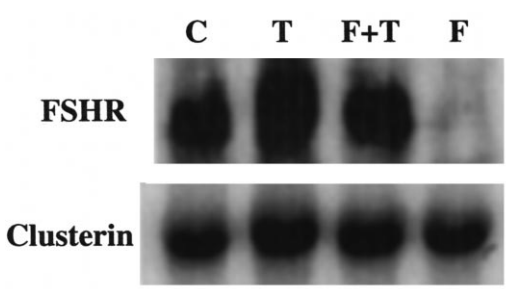

Fig. 8. Upper panel: Northern blot analysis of FSH receptor mRNA in total RNA from Sertoli cells that were untreated (Lane 1), treated with TSA alone (Lane 2), TSA and oFSH (Lane 3), or oFSH alone (Lane 4) as described in Section 2. Lower panel: the same blot as in the upper panel that has been stripped and reprobed with rat clusterin cDNA.

regulation of the FSH receptor gene. The down-regulation of the mRNA level was not seen when 2.7 kb or 383 base pairs of the promoter driving a reporter gene were transfected into Sertoli cells. In fact, in each of these constructs the luciferase reporter gene activity was increased in the presence of FSH. Second, in previous studies from this laboratory we showed that the tissue-specific pattern of expression of the FSH receptor gene was also not recreated in transient transfection assays (Linder et al., 1994). Indeed, reporter genes driven by FSH receptor gene promoters of various lengths were promiscuously expressed in a number of cell types in transient transfection assays whereas the expression of such genes in transgenic mice was restricted to the gonads in one study (Linder et al., 1994).

The hypothesis that chromatin structure is involved in the FSH-induced repression of the FSH receptor gene in Sertoli cells was tested in this study by adding TSA to Sertoli cells 1 h prior to the addition of FSH. TSA inhibits histone deacetylase activity and blocks the formation of repressive chromatin structure that is caused by the recruitment of histone deacetylase activity. Treatment of Sertoli cells with TSA prior to the addition of FSH completely prevented the down-regulation of the gene (Fig. 8). Taken together, these observations are consistent with, but certainly do not prove, the hypothesis that changes in chromatin structure are involved in the homologous down-regulation of the FSH receptor gene in Sertoli cells. The E-box and the E-box binding proteins USF-1 and USF-2 play critical roles in the regulation of the FSH receptor gene in Sertoli cells (Goetz et al., 1996; Heckert et al., 1998). In a recent publication, the phosphorylation of a cAMP-responsive activator was shown to be modulated by means of a chromatin-dependent mechanism (Michael et al., 2000). Similarly, to our studies it was shown that histone deacetylase inhibitors influenced CREB activity but only on chromatin templates. The FSHR gene could also utilize a mode of regulation that links the control of gene expression by signal transduction and chromatin structure. Additional studies will be needed to establish the exact mechanism of transcriptional down-regulation.

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