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Inducible cAMP early repressor ICER down-regulation of CREB gene expression in Sertoli cells

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Abstract

The cAMP response element binding protein (CREB) and the cAMP-responsive element modulator (CREM) are cyclically expressed in the seminiferous tubules during spermatogenesis. In the somatic Sertoli cells, which are the major supporters of germ cell development in the seminiferous tubules, the expression of CREB is cyclical and appears to be regulated by the levels of cAMP produced in response to the pituitary derived follicle-stimulating hormone FSH. Cyclic AMP response elements (CREs) located in the promoter of the CREB gene were shown earlier to be implicated in an autopositive feedback loop that up-regulates the expression of CREB. Here we show that in Sertoli cells FSH-mediated induction of the CREM repressor isoform, ICER (inducible cAMP early repressor) is correlated with the inhibition and delay of CREB gene expression in the seminiferous tubules. ICER binds to the two CREs located in the promoter of the CREB gene and in transient transfection assays of Sertoli cells, ICER expression vectors down-regulate transcription of a reporter gene driven by the CREB gene promoter. In addition, analyses of ICER and CREB gene expression in isolated segments of rat seminiferous tubules reveals stage-specific and cycle-dependent expression of ICER. The periods of enhanced expression of ICER correspond to the stages of spermatogenesis with the lowest levels of CREB expression. We suggest that the expression of ICER in Sertoli cells may contribute to the periodic repression of CREB gene expression during the repeated 12-day cycles of spermatogenesis, and may be required to reset the levels of activator CREB prior to the initiation of each new cycle of spermatogenesis.

Keywords: CREB; ICER; Sertoli cell; Follicle stimulating hormone; Spermatogenesis; Testis; Gene expression

1. Introduction

CREB (cAMP response element binding protein) and CREM (cAMP response element modulator) are cAMP-responsive members of the bZIP family of transcription factors. They are so named because of their highly conserved DNA-binding domains consisting of an amino-proximal DNA-recognition basic region (b) and a carboxyl-terminal dimerization domain (ZIP, leucine zipper) (Hoeffler and Habener, 1990; McKnight, 1991; Meyer and Habener, 1993; Habener et al., 1995). CREB and CREM (and activating transcription factor-1, ATF-1) are distinguished amongst the bZIP proteins by the marked responsivity of their transcriptional transactivation functions to phosphorylation by cAMP-dependent protein kinase A (Meyer and Habener, 1993; Habener et al., 1995). These bZIP proteins bind to specific cAMP response elements (CREs) located in the promoters of cAMP-responsive genes and activate gene transcription in response to

Abbreviations: CREB, cAMP response element binding protein; CREM, cAMP response element modulator; ICER, inducible cAMP early repressor; EMSA, electrophoretic mobility shift assay; FSH, follicle-stimulating hormone; bZIP, basic region leucine zipper; DBDI, II, DNA-binding domains I or II of CREM..

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phosphorylation (Meyer and Habener, 1993; Habener et al., 1995). Both CREB and CREM are expressed at high levels in the testis and their expression is controlled by cAMP signalling mediated by the interactions of the pituitary gonadotropic hormone, FSH with stimulatory G-protein-coupled receptors found on the somatic Sertoli cells, (Steinberger et al., 1978; Waeber et al., 1991; Foulkes et al., 1993).

In Sertoli cells CREB mRNA levels are transiently induced in a repeated cyclical pattern corresponding to the specific 12-day temporal and anatomical cell association stages of spermatogenesis (Waeber et al., 1991). Levels of CREB mRNA increase in cell association stages II–V (Waeber et al., 1991), after some delay, following increases in FSH-induced cAMP levels in stages XII–V (Kangasniemi et al., 1990; Walker and Habener, 1996). CREB mRNA levels then fall rapidly to nearly undetectable levels in stages VII–XIV as cAMP levels decrease due to internalization of FSH receptors and the down-regulation of the FSH receptor (Themmen et al., 1991). The characterization of the promoter of the CREB gene identified cAMP response elements (CREs) that contribute to the cAMP induction of the transcription of the CREB gene (Meyer et al., 1993; Meyer and Habener, 1993). Subsequent studies showed that phosphorylation of CREB bound to a CRE by cAMP-dependent PKA stimulates CREB gene transcription and the consequent production of additional CREB, indicating the existence of an autopositive feedback loop (Walker et al., 1995). This autopositive regulation of CREB gene expression is proposed to account for the large increase in CREB mRNA levels that accumulate in the nuclei of Sertoli cells during stages II–VI of the spermatogenic cycle (Waeber et al., 1991). However, the delay in initiation of the positive feedback of the CREB gene until well after cAMP levels initially rise in stage XII has remained unexplained.

ICER, the cAMP-responsive repressor form of CREM is one candidate regulator of the CREB gene in Sertoli cells. Transcription of the ICER is also autoregulated by cAMP signalling as an internal promoter (P2) located in the 3′ region of the CREM gene is activated by cAMP (Molina et al., 1993). cAMP-responsive CREB and CREB-like activator proteins interact with four CAREs (cAMP autoregulatory response elements) in the P2 promoter to stimulate transcription (Molina et al., 1993). The mRNA that encodes the ICER repressor consists of a short ICER-specific region followed by the bZIP DNA-binding domain (Molina et al., 1993).

Earlier we reported that the autopositive upregulation of CREB gene expression in testicular germ cells is interrupted by the switch in expression from activator to inhibitor CREBs (I-CREBs) (Girardet et al., 1996; Walker et al., 1996; Walker and Habener, 1996). However, I-CREBs appear to be expressed at low levels in the somatic Sertoli cells leaving unanswered the mechanisms by which the cyclical upregulation of CREB is interrupted in Sertoli cells (Walker and Habener, 1996). Recently, it was reported that the ICER repressor is expressed in primary rat Sertoli cells in response to FSH and is proposed to down regulate the transcription of the FSH receptor gene in these cells (Monaco et al., 1995). Here we report findings that FSH-induced expression of ICER down regulates the expression of the CREB gene in primary rat Sertoli cells and thereby provides a potential explanation for the cyclical fluctuations in CREB gene expression during spermatogenesis specifically in Sertoli cells. Further, we show by studies of isolated rat seminiferous tubules that CREB and ICER are expressed reciprocally at different stages of the 12-day cycle of spermatogenesis.

2. Experimental procedures

2.1. Isolation of Sertoli cells, seminiferous tubules and preparation of protein extracts

Sertoli cells isolated from 16-day Sprague–Dawley rat testis after collagenase and trypsin digestion (Walker et al., 1995) were cultured on matrigel coated plates (Collaborative Research, Bedford, MA) in serum free medium containing 50% Dulbecco's modified Eagle's medium, 50% Ham's F-12. Media was supplemented with 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 1 μM retinoic acid, 10 ng ml^{-1} epidermal growth factor, 3 μg ml^{-1} cytosine β-D-arabinofuranoside, 2 mM glutamine, 1 mM sodium pyruvate, 100 u ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Walker et al., 1995). Sertoli cells were routinely greater than 95% pure as determined by phase microscopy and alkaline phosphatase staining (Chapin et al., 1987). Animal studies were conducted in accordance with the principles and procedures outlined in 'Guidelines for Care and Use of Experimental Animals'.

Sertoli cells (1×10^9 cells) were collected after 0.5–24 h of stimulation with 100 ng ml^{-1} FSH (ovine pituitary FSH, Sigma, St. Louis) and 0.5 mM 3-isobutyl-1 methylxanthine (IBMX) or empty vehicle. Nuclear extracts of Sertoli cells were prepared as described (Schreiber et al., 1989). Seminiferous tubules were isolated from 60-day Sprague Dawley rats and maintained in enriched Krebs–Ringer bicarbonate buffer (Bellvé et al., 1977). The stage dependent transillumination pattern was identified using a stereomicroscope and 2 mm sections were sequentially cut and flash frozen beginning with the stage VIII | IX border identified by the distinct darklight interface (Toppari and Parvinen, 1985, Kangasniemi et al., 1990). Whole cell protein extracts were prepared from the tubule sections by shaking the tissue for 15 min in ELB buffer (250 mM

NaCl, 0.1% NP40, 50 mM Hepes pH 7.0, 5 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail) at 4°C (Walker, et al., 1992), followed by centrifugation (12000 × g) for 5 min to remove cellular debris. Protein concentrations were determined using the BioRad Protein Assay.

2.2. DNA-binding assays

Electrophoretic mobility shifts (EMSA) binding reactions were performed in the presence of 1 μg poly (dI–dC) using 5 μg of protein extract as described (Deutsch et al., 1988) and ^{32}P-labeled oligonucleotide probes containing either a consensus CRE (COLCRE) (5′-GATCCGGCTGACGTCATGAAGCTAGATC-3′, or the wild type CRE1 and CRE2 region of the CREB promoter (CREBCRE) (5′-GATCCGTTGGTGAG TGACGCGGCGGAGGTGTAGTTTGACGCGGTG TGAG-3′). For immune supershift assays, extracts from Cos-1 cells expressing ICER were incubated with the ^{32}P-labeled oligonucleotide probes in the presence of rabbit pre-immune sera, CREMS4 rabbit antisera that recognizes the carboxyl-terminal region of CREM and ICER, guinea pig pre-immune sera or ICER-specific peptide guinea pig antisera. DNA-protein complexes were resolved on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. For competition EMSA experiments to estimate relative DNA-protein affinities, ICER and CREB proteins (2 μl) derived from coupled in vitro transcription-translation reactions (TnT, Promega) were incubated with CREBCRE probe (0.5 pmol) and increasing amounts of unlabeled CREBCRE probe (0.5–50 pmol). The relative levels of DNA-protein complexes were determined by densitometric scanning using the BIO-RAD Molecular Analyst system.

2.3. Construction of ICER expression vectors

The ICERIγ isoform was isolated from rat testis RNA using reverse transcriptase and polymerase chain reaction (PCR). A dT_{17} 3′ oligonucleotide was used to reverse transcribe cDNAs from 5 μg of Sertoli cell RNA. The ICERIγ isoform was amplified by PCR using an oligonucleotide corresponding to the ICER 5′ untranslated region (5′-ACTCTATATGCAAAAAGC CC-3′) and the CREMR5 oligonucleotide (5′-GAGCTCGAATTCCCAATTCACACTCTACAGCA G-3′) corresponding to the DNA-binding domain I (DBDI) region of CREM (exon Ia) (8) located 120 bp downstream of the translation termination codon. ICER cDNAs were subcloned into the PCRII vector (Invitrogen, San Diego) for expression of ICER in vitro. For expression in eukaryotic cells, ICERIγ was excised from the PCRII vector with EcoR1 and inserted into pCMV5 (Chen et al., 1991) linearized with EcoR1.

2.4. Northern blotting

RNA from primary Sertoli cells treated with FSH and IBMX or empty vehicle was prepared with Trizol reagent (Gibco-BRL) according to the manufacturers instructions and 10 μg of each RNA sample was subjected to Northern analysis as described (Walker et al., 1995). Northern blots were probed with ^{32}P labeled ICERIγ or CREB cDNA probes prepared by random priming. Equal loading of RNA samples was confirmed by staining the gel with SyBR Green II or Ethidium Bromide dye. The RNA products on the autoradiograms were scanned with a computing densitometer to provide a semiquantitative evaluation of the relative levels of the RNAs (ImageQuaNT, Molecular Dynamics).

2.5. Immunocytochemistry

Frozen sections from adult rat testis were immunostained with preimmune sera or ICER-specific antisera (rabbit) directed against amino acids 2–11 of ICER (Bodor et al., 1996 PNAS) and the antigen–antibody immune complex was visualized using a Cy3 fluorescent secondary anti-rabbit serum (Jackson Immunoresearch Laboratories) or an anti-rabbit biotinylated antibody and colorimetric staining as described in the Vectastain Elite kit (Vector Laboratories).

2.6. Expression of proteins in bacteria and Cos-1 cells

Synthesis of recombinant proteins was induced in *E. coli* BL21 (DE3) pLysS. CREB and ICER were produced from plasmids containing the appropriate cDNA under the control of the T7 polymerase promoter in the pET-3b prokaryotic expression vector (Vallejo et al., 1992). ICER and CREB were produced in Cos-1 cells transiently transfected with pCMV5 ICER or pCMV5 CREB expression vectors (5 μg) (Seldon 1989). Cos-1 whole cell extracts were prepared 48 h post-transfection after lysis of cells in ElB buffer (Walker et al., 1992).

2.7. Sertoli cell transfections and CAT assays

Primary Sertoli cells were transfected 3 days after isolation as described (Walker et al., 1995), except that 1 μg of CAT reporter plasmids in a total of 5 μg DNA were used per 60 mM dish. The cells were cotransfected with either the wild-type ICERIγ or the mutated ICERmut expression plasmids. The ICERmut has a deletion in the leucine zipper dimerization domain, so it cannot dimerize or bind DNA (Bodor et al., 1995). Cells were harvested 48 h post-transfection and CAT activity determined as described (Walker et al., 1995). To activate CREB, certain transfections included the plasmid pRSVCat-β that expresses the C_β isoform of the cata-

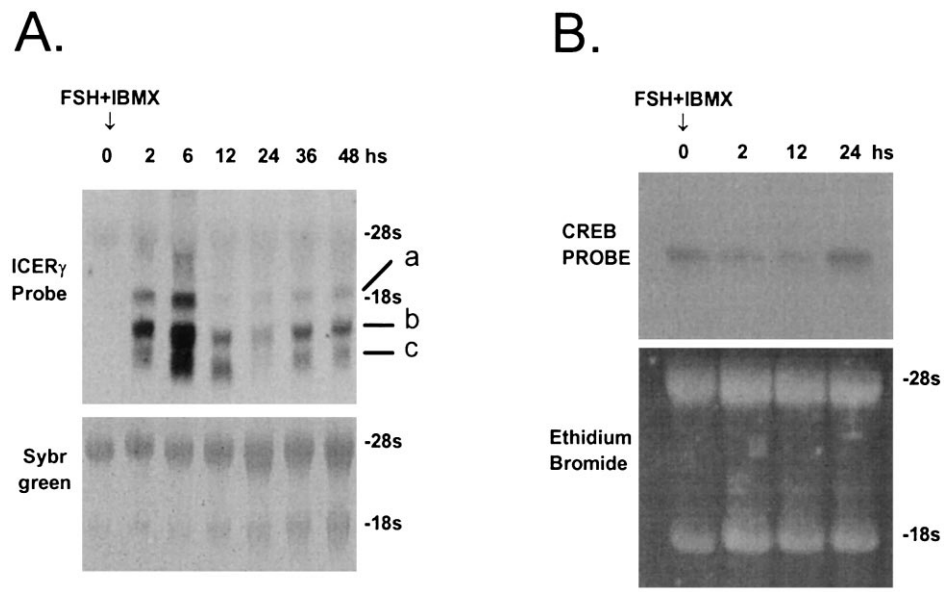

Fig. 1. FSH and IBMX induce ICER and repress CREB mRNAs in primary rat Sertoli cells. (A) Northern blot of RNA isolated from primary Sertoli cells, either untreated (0 h) or stimulated with FSH and IBMX for 2–48 h. Top, ICER mRNA was hybridized with a ^{32}P-labeled RNA derived from ICERIγ cDNA; a, b, and c refer to the three alternatively spliced forms of ICER detected by the ICER specific probe. Bottom, SyBR Green staining of the gel used to characterize ICER mRNA levels showing equal loading of RNA (28S and 18S ribosomal RNA) for all time points. (B) Northern blot of RNA prepared from rat Sertoli cells after their treatment with FSH and IBMX. Membrane-bound RNA was probed with a CREB cDNA probe. Bottom, Ethidium Bromide staining of the gel used to show equal loading of RNA (28S and 18S ribosomal RNA) for all time points.

lytic subunit of protein kinase A (Maurer, 1989). Cells were also transfected with the cAMP responsive pENKAT-12 plasmid as a positive control (Comb et al., 1986). The pENKAT-12 consists of the CAT gene under control of the proenkephalin promoter. Relative CAT activities of the various experimental conditions were normalized to the activities of-278CREBCAT containing 278 bp upstream of the CREB translation start site (Walker et al., 1995) or pENKAT-12 co-transfected with pRSVCat-β which were taken as 100% activity.

3. Results

3.1. ICER and CREB mRNA levels are regulated by FSH and IBMX in primary rat Sertoli cells

To determine the mechanism accounting for the downregulation of the transcriptional expression of the CREB gene in Sertoli cells, the role of ICER in the regulation of the expression of the CREB gene was investigated in primary Sertoli cells treated with FSH and 3-isobutyl-methylxanthine (IBMX) over a time course of 48 h. Measurements of ICER and CREB mRNA levels in extracts of Sertoli cells at increasing times after the addition of FSH and IBMX was taken as an initial index of the effects of cAMP signalling on the expression of the ICER and CREB genes. Time-dependent, cyclical fluctuations in the levels of ICER and CREB mRNAs were observed by Northern RNA blot analyses (Fig. 1). Before the addition of FSH and IBMX, no ICER mRNA was detectable (Fig. 1(A)). By 2 h after the addition of FSH and IBMX, ICER mRNA became readily detectable, increased markedly by 6 h, and then decreased from 12 to 24 h, only to increase again at 36–48 h. In addition, at least three ICER mRNAs were detected that likely correspond to alternatively spliced isoforms of the mRNA (Rauchaud et al., 1997). These observations are typical for the expression of ICER which is itself under cyclical autoregulatory control in response to cAMP signalling. When levels of ICER rise they feed back on the ICER gene promoter to repress it (Molina et al., 1993). In contrast, treatment of Sertoli cells with FSH initially causes a small apparent decrease in CREB mRNA levels. However, in agreement with earlier studies employing 8-Br cAMP as a stimulant (Walker et al., 1995), addition of FSH and IBMX first results in the elevation of CREB mRNA levels between 12 and 24 h (Fig. 1(B)). Because ICER levels are high immediately after FSH-stimulation and the induction of CREB occurs much later, the possibility that ICER was responsible for the repression and delay in CREB gene transcription was investigated.

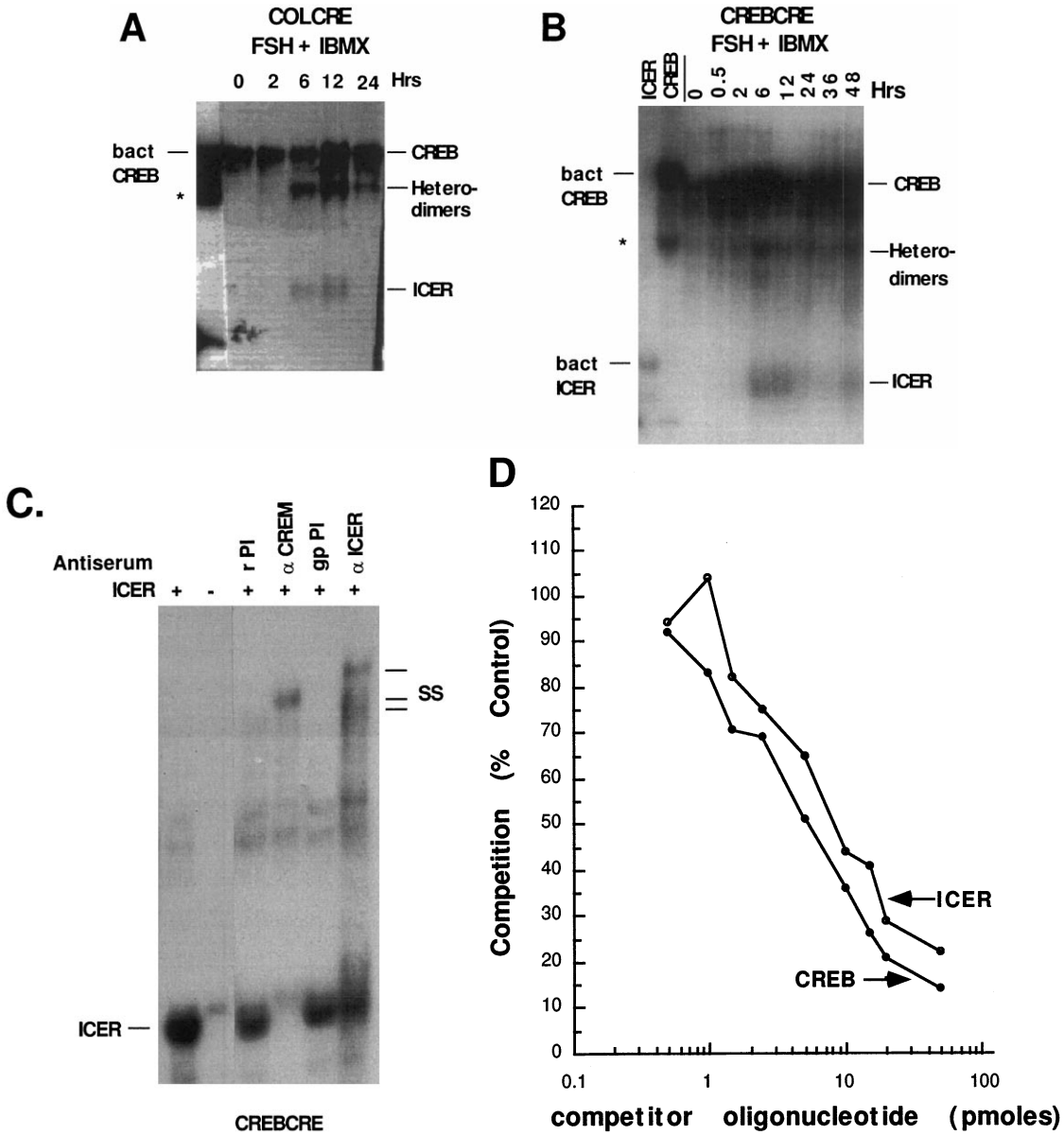

Fig. 2. Electrophoretic mobility gel shift assays showing that FSH and IBMX transiently induces ICER in primary Sertoli cells. (A) A consensus CRE probe (COLCRE) (Deutsch et al., 1988) or (B) a CREB gene promoter CRE (CREBCRE) (Walker et al., 1995) were incubated with bacterially expressed CREB, bacterially expressed ICERIγ or nuclear extracts from primary Sertoli cells, either untreated (0 h) or stimulated with FSH and IBMX for 0.5, 2, 6, 12, 24, 36, or 48 h. CREB, and ICER homodimer-probe and CREB-ICER heterodimer-probe complexes are indicated. Complexes formed with bacterially expressed CREB and ICER are slightly larger due to added vector-encoded amino acids. In A and B an asterisk denotes complexes formed with smaller CREB-like proteins produced by processing of overexpressed CREB in bacteria. (C) extracts from Cos-1 cells transfected with an ICERIγ expression vector were incubated with an oligonucleotide containing the CREB CRE element of the CREB promoter in EMSA analysis. In immunosupershift analyses, rabbit preimmune serum (αrPI), rabbit CREMS4 (αCREM) antiserum directed against the carboxyl-terminus of CREM and ICER, guinea pig preimmune serum (αgp PI), or guinea pig ICER-specific antisera directed against the seven unique amino terminal amino acids of ICER (αICER) were added to the DNA-protein binding reactions. The ICER-probe complex and antiserum-dependent ICER up-shifted complexes (SS) are indicated. (D) competition analysis to determine the relative affinities of CREB and ICER for the CREB promoter CREs. CREB and ICER proteins produced by coupled in vitro transcription-translation reactions were incubated with radiolabeled CREBCRE probe and increasing amounts of unlabeled CREBCRE competitor as shown. The DNA-protein complexes were resolved by nondenaturing PAGE and the intensities of the DNA-protein complexes were quantitated using the BIO-RAD image analysis system. The results shown are an average of four experiments. The standard error was less than 20% for each observation.

3.2. ICER and CREB protein levels are regulated by FSH and IBMX in primary rat Sertoli cells

In DNA-binding EMSA studies, nuclear extracts from Sertoli cells treated with FSH and IBMX were incubated with a ^{32}P-labeled oligonucleotide probe containing an optimized symmetrical palindromic CRE (COLCRE) (Fig. 2(A)). By 6 h after stimulation of the Sertoli cells with FSH and IBMX, a CRE-binding protein corresponding to the mobility of ICER was induced. To determine whether the ICER-like protein could bind to the CRE motifs of the CREB promoter, an oligonucleotide consisting of the two tandemly-arranged asymmetrical CREs, as they appear in the context of the promoter of the CREB gene (CREBCRE) (Meyer et al., 1993), was used in binding studies of FSH-treated primary Sertoli extracts (Fig. 2(B)). A DNA-protein complex that migrated close to that observed due to the binding of bacterially expressed ICER protein was induced 6 and 12 h after FSH treatment with levels falling significantly by 24 h at a time when CREB levels begin to rise. Concomitant with the appearance of the faster migrating ICER complex, a new complex appeared intermediate in mobility between those of ICER and CREB. This intermediate complex likely represents heterodimers of ICER and CREB or CREB-like proteins because it migrates in the same position as complexes derived from mixtures of partially purified bacterial extracts of CREB and ICER (data not shown). In fact, the relative intensities of the complexes formed suggests that in Sertoli cells, ICER is more likely to form heterodimers with CREB than it is to form homodimers. The co-regulation of these two ICER-containing complexes are maintained after longer periods of FSH treatment since both CREB/ICER heterodimers and ICER homodimers appear to be deinduced after 24–36 h, reappearing again at 48 h (Fig. 2(B)).

That the inducible ICER complex contained the ICER repressor protein is supported by the findings that the induced DNA-protein complex comigrated with a complex formed by ICER produced in bacteria (Fig. 2(B)), the pattern of induction matched that of ICER RNA produced in Sertoli cells after FSH treatment (Fig. 1(A, B)) and that of FSH-induced ICER protein previously described in Sertoli cells (Monaco et al., 1995). To further confirm that ICER binds to the CREBCRE probe, supershift EMSA analysis was performed using antisera raised either against CREM (αCREM) which recognizes the carboxyl-terminal region of CREM that is shared with ICER or an ICER-specific antiserum (Fig. 2(C)). For this study, extracts from Cos-1 cells transfected with CREB or ICER expression vectors were used in the binding reactions. Both of the ICER antisera, but not pre-immune sera, caused disruption and upshifting of the FSH and IBMX-inducible ICER-containing complex (Fig. 2(C)).

For ICER to be an effective repressor of CREB transcription it must be able to bind to the CREB promoter CRE motifs with affinity similar to that of CREB. This is particularly important because members of the CREB family of transcription factors have been found to bind to asymmetrical CREs, such as those within the CREB promoter, with lower affinity than to symmetrical CREs (Nichols et al., 1992). The relative affinities of ICER and CREB for the CREB promoter CREs were measured using a competitive EMSA approach. These assays were performed with constant inputs of CREB or ICER protein and ^{32}P labeled CREB CRE probe in the presence of increasing amounts of non-labeled CREB CRE probe. The intensities of the DNA-protein complexes were measured using image analysis software (Molecular Analyst, Bio-Rad Laboratories) and competition curves were generated. The relative affinities of each protein for the CREs was estimated from the competitor values at which 50% of the binding was inhibited. The relative affinity of ICER for the CREB promoter CREs was found to be close to that of CREB: 9.1 pmol of CRE CREB competitor probe was required to compete 50% of ICER binding compared to the 5.2 pmol of competitor required to reduce CREB-binding by 50% (Fig. 2(D)). These data support the idea that the FSH-induced increases in ICER expression are capable of competing effectively with CREB for occupancy of the CREs.

3.3. Cyclical expression of ICER and CREB in rat seminiferous tubules in vivo

Although a transient induction of the expression of ICER by FSH has been shown in cultured Sertoli cells in vitro (Monaco et al., 1995), the dynamics of such expression in vivo have not been investigated. Therefore, we examined the temporal pattern of the induction of ICER in Sertoli cells within the context of the intact seminiferous tubule. In the rat, 14 stages of spermatogenesis define specific cell associations of developing germ cells in the seminiferous epithelium (Leblond and Clermont, 1952; Perey et al., 1961). These 14 stages of development appear in succession along the length of seminiferous tubules in 12-day repeating cycles designated as the waves of the seminiferous epithelium (Leblond and Clermont, 1952; Perey et al., 1961). Whole cell extracts prepared from a contiguous series of tubule segments isolated by microdissection and representing the stages of spermatogenesis were analyzed in EMSA studies to determine the stages of spermatogenesis in which ICER is expressed. Incubation of the tubule extracts with a probe containing the CREB promoter CRE motifs (CREBCRE) resulted in the formation of complexes comigrating with those formed in the presence of ICER produced by trans-

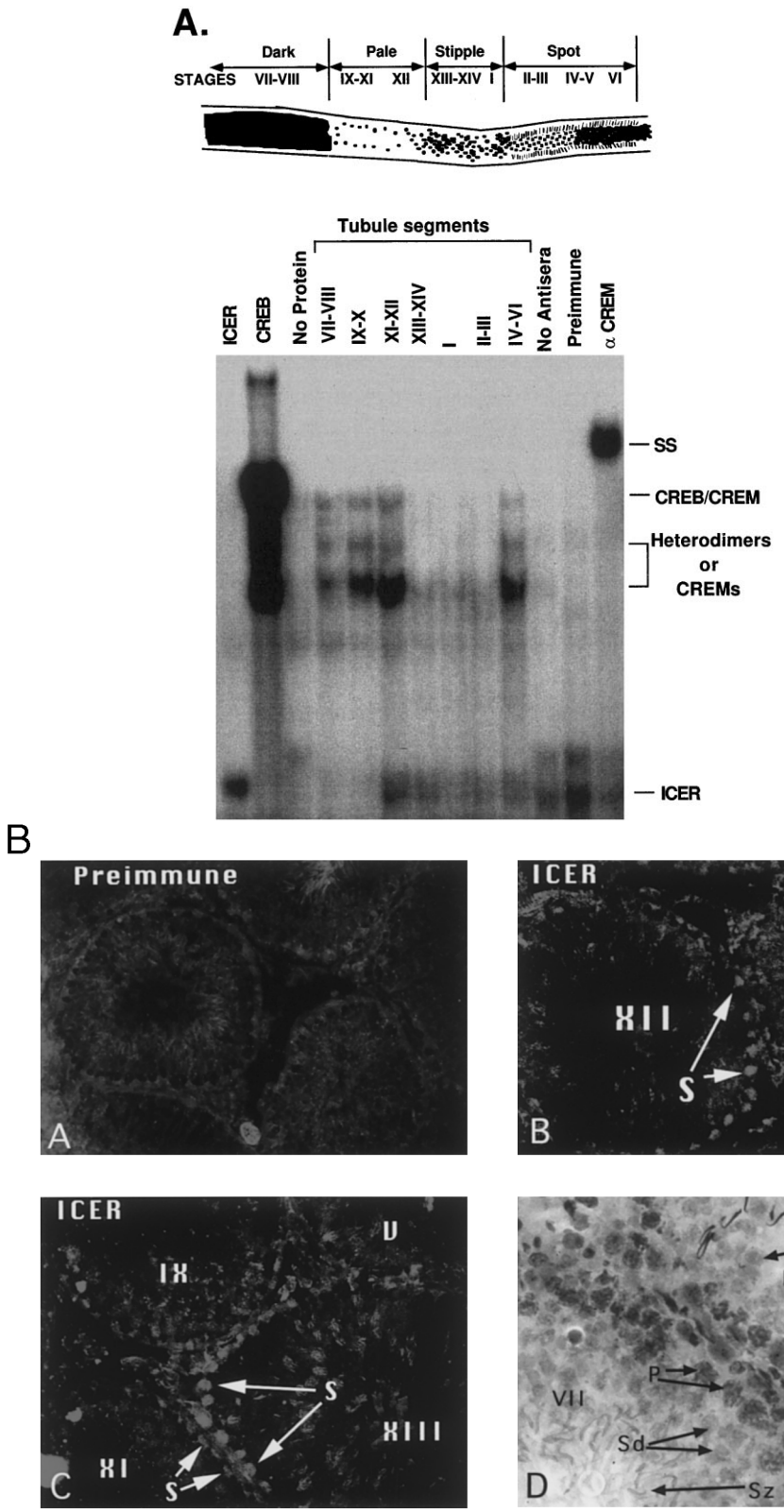
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Fig. 3.

formed bacteria (Fig. 3(A)). ICER-like binding activity is induced at approximately stage XI–XII with levels falling to basal levels by stages I–II. Interestingly, the induction of ICER-like binding activity corresponds to the initial increase in cAMP levels in Sertoli cells during stages XII–XIV (Kangasniemi et al., 1990).

In the seminiferous tubules of the adult rat, Sertoli cells make up only 3% of the cell population with germ cells accounting for greater than 95% of the cells (Bellvé et al., 1977). In contrast to ICER, which has only been detected in Sertoli cells (Delmas et al., 1993; Molina et al., 1993; Monaco et al., 1995), the production of various CREB and CREM isoforms in germ cells complicates the interpretation of the relative levels of Sertoli-derived CREB present in the stage-specific seminiferous tubule extracts. In addition, due to the low ratio of Sertoli to germ cells in microdissected seminiferous tubules it is technically difficult to purify sufficient numbers of Sertoli cells to directly compare the relative CREB and ICER levels at each stage of spermatogenesis using immunoblot or immunoprecipitation assays. Therefore, to confirm that ICER is induced stage-specifically in Sertoli cells, immunocytochemistry of adult rat testis was performed. Using an ICER-specific antiserum and a flourescent secondary antibody, immunostaining was restricted to nuclei along the basement membrane of the seminiferous tubule (Fig. 3(B)). Periodic acid Scheiff-hematoxalin staining of adjacent serial sections showed that ICER immunostaining was induced in the nuclei of Sertoli cells at stages XI-I (data not shown). Basal levels of ICER were seen in all other stages examined. Further examination of ICER expression using a colorimetric immunocytochemistry assay showed stage-specific staining of Sertoli nuclei with the ICER antisera. The more mature spermatocyte and spermatid germ cells were not stained with the ICER antisera; however, we are unable to rule out the possibility that spermatogonia may express ICER. The results of the immunocytochemistry studies support the idea that ICER is induced transiently in a stage-specific manner.

3.4. Inhibition of CREB-induced gene transcription by ICER

The functional properties of ICER in Sertoli cells were further examined in transient transcription assays. Primary Sertoli cells were transfected with a chloramphenicol acetyl transferase (CAT) reporter plasmid containing a region of the CREB promoter that includes the two CREs (CREBCAT). As was shown previously (Walker et al., 1995), transcription of the CAT reporter gene was induced approximately 5–6 fold by cotransfection of an expression vector encoding the catalytic subunit of protein kinase A (Fig. 4(A and C)). In contrast, cotransfection of the ICER expression vector reduced PKA-stimulated CAT activity to basal levels, whereas a vector expressing a control carboxyl-terminal deleted ICER mutant deficient in dimerization (ICER-mut) (Bodor et al., 1995) had little effect upon transcriptional activity. In transfection experiments employing the proenkephalin promoter, which also contains non-consensus CREs, ICER was also an effective repressor of PKA-mediated transcription (Fig. 4(B)). Together, these data show that in Sertoli cells ICER competes with CREB for occupancy of asymmetrical CREs in vivo and block transcription induction by the cAMP-dependent PKA pathway.

4. Discussion

The finding that the cAMP-responsive early repressor, ICER, is induced by FSH in Sertoli cells, binds to the CREs of the CREB promoter, and appears to down-regulate the expression of CREB may be an important point in understanding the stage-specific regulation of CREB expression in Sertoli cells (Waeber et al., 1991). With the exception of ICER, other isoforms of CREM, including the transcriptional activator CREMτ, are not expressed at high levels in Sertoli cells (Foulkes et al., 1992; Monaco et al., 1995); therefore, CREB is a promising candidate as a positive regulator

Fig. 3. ICER is induced in stage XII–XIV seminiferous tubules. A, Electrophoretic mobility shift assay of extracts from segments of microdissected rat seminiferous tubules. Partially purified bacterially expressed ICER (lane 1), CREB proteins (lane 2), no proteins (lane 3), and whole cell protein extracts from segments of seminiferous tubules representing stages VII–VIII, IX–X, XI–XII, XIII–XIV, I, II–III, and IV–VI were incubated with a ^{32}P-radiolabeled oligonucleotide probe containing the CREB CREs. The DNA-protein complexes were fractionated through a native polyacrylamide gel and identified by autoradiography. Complexes containing homodimers of CREB/CREM or ICER and CREB/ICER heterodimers are noted. The last three lanes (right) show that the addition of CREM/CREB-specific antiserum (αCREM) to the binding reaction containing extract of stage XIII–XIV tubule segments results in a retardation of the DNA (CREBCRE)-protein complex (Supershift, SS) not seen in control reactions without serum or with preimmune serum. A diagram of a rat seminiferous tubule is shown above the autoradiogram to provide a guide to the identification of the tubule segments identified by transillumination. B, immunostaining of adult rat testis with ICER-specific antiserum. Frozen sections of adult rat testis tissue were immunostained with ICER-specific antisera and a Cy3 fluorescent secondary anti-rabbit serum (panels A–C) or a biotin conjugated secondary antiserum (panel D): panel A, control preimmune serum staining at low magnification (50X). panel B, ICER specific antisera staining at low magnification. panel C, ICER-specific antisera staining (70X), panel D, ICER-specific antisera staining (70X); the brown staining is indicative of the immune avidin-biotin complex, nuclei have been stained blue with hemotoxilin. Staining of Sertoli cell nuclei (S) is identified by arrows. Zygotene spermatocytes (Z) pachytene spermatocytes (P), Diplotene spermatocytes (D), round spermatids (Sd) elongated spermatids (ES) and spermatozoa (Sz) are also indicated. Seminiferous tubules at various stages are shown in Roman numerals.

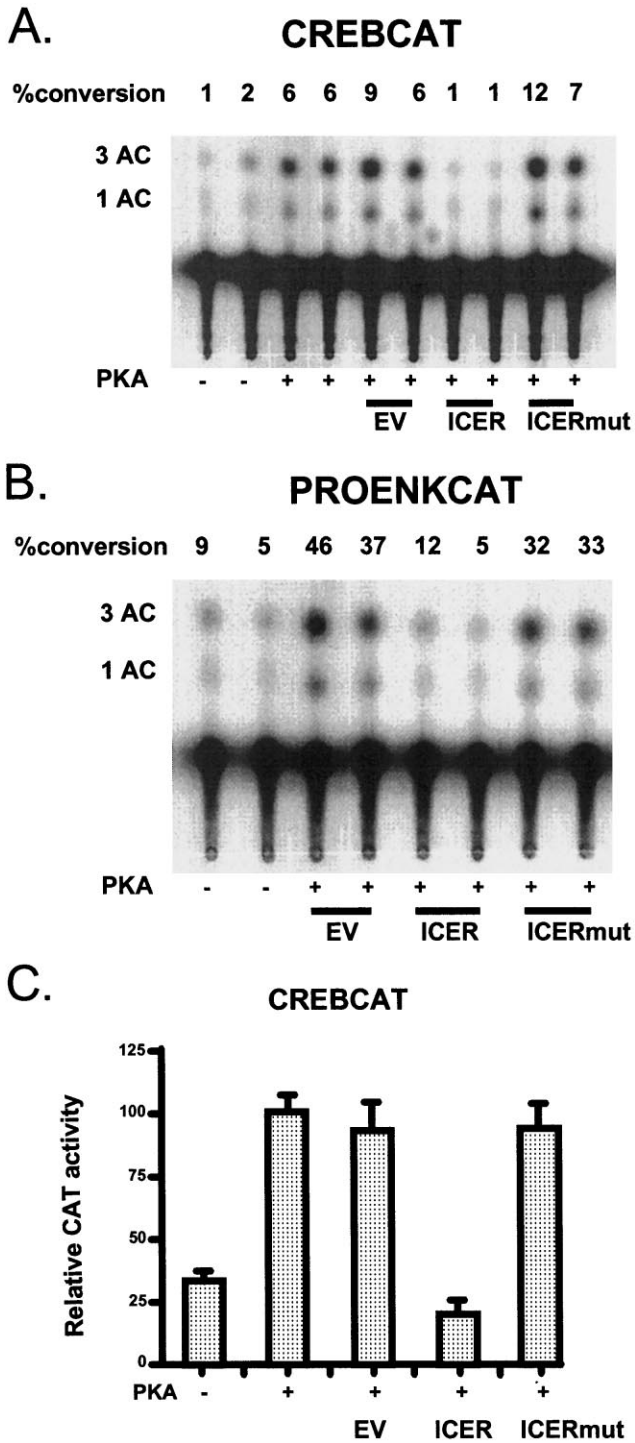

Fig. 4. ICER down-regulates the promoter of the CREB gene in Sertoli cells. Primary Sertoli cells were transfected with: A, the CREBCAT plasmid containing the CREB promoter region extending 278 bp 5′ to the translation start site linked to the CAT reporter gene (-278CREBCAT) (Walker et al., 1995), or B, PROENKCAT, the CREs in the context of the rat proenkephalin promoter (pENKAT-12) (Comb et al., 1986). Transfection assays were done with or without the PKA catalytic subunit (C_β) expression vector, pRSVCat-β (PKA) (Maurer, 1989) and empty pCMV5 expression vector (EV) pCMV5 containing the sequences encoding ICERIγ (ICER) or a mutant ICER in which the carboxyl-terminal leucine zipper is mutated (ICERmut) (Bodor et al., 1995). CAT activity is given as percent conversion of unacetylated to acetylated CAT products (3AC, 1AC). C, Summary of CREBCAT experiments. CAT activity is expressed relative to cells cotransfected with pRSVCat-β which was designated 100% activity. Results shown are from three separate transfections performed in duplicate. Standard errors of the mean are provided for each condition.

of cAMP-induced genes in Sertoli cells. CREB may be an important regulatory signal for a number of cAMP-regulated genes that have been studied in Sertoli cells, including the proto-oncogenes c-fos (Hall et al., 1988), junB (Smith et al., 1989), and α-inhibin (Najmabadi et al., 1993), as well as proenkephalin (Yoshikawa and Azawa, 1988) and androgen-binding protein (Joseph et al., 1988). The cAMP-regulated control of CREB gene expression, therefore, may be critical for the regulation of several genes required for the maturation of germ cells.

Our studies suggest that the expression of CREB and ICER in Sertoli cells in vivo may be cyclically regulated via the production of cAMP induced by FSH that occurs during cell association stages XII-V (Kangasniemi et al., 1990). The ICER gene appears to rapidly respond to increased cAMP levels inasmuch as the highest levels of ICER are detected by immunocytochemistry during stages XII–XIV. The levels of ICER then return to basal levels after stage I. In contrast to the temporal pattern of the induction of ICER, induction of the CREB gene by cAMP is delayed until stages II–V. Because ICER is a candidate repressor of the CREB gene, it may be an important factor responsible for the delayed induction of the CREB gene by FSH and cAMP in Sertoli cells. Together, the two regulators, CREB and ICER, may be responsible for limiting the expression of cAMP-inducible genes in Sertoli cells to specific stages of germ cell development.

As depicted in Fig. 5, ICER interrupts a hypothetical positive auto-feedback loop responsible for the high levels of CREB detected in Sertoli cells at specific stages of spermatogenesis. In response to stimulation by FSH, cAMP levels increase, resulting in the activation of the PKA catalytic subunit and the phosphorylation of CREB. The activation of CREB results in the stimulation of the CREB promoter and thereby increases levels of CREB mRNA and protein. At the same time, the ICER promoter is stimulated by CREB, causing ICER levels to increase, which eventually feed back to suppress the ICER gene promoter. The delay in the induction of CREB by FSH-mediated cAMP formation may be due to the induction of the ICER repressor which transiently occupies the cAMP-response elements present in the promoter of the CREB gene until ICER levels eventually fall (Molina et al., 1993).

The gene encoding the FSH receptor is also down-regulated by ICER in Sertoli cells in response to stimulation by FSH (Monaco et al., 1995). Therefore, ICER

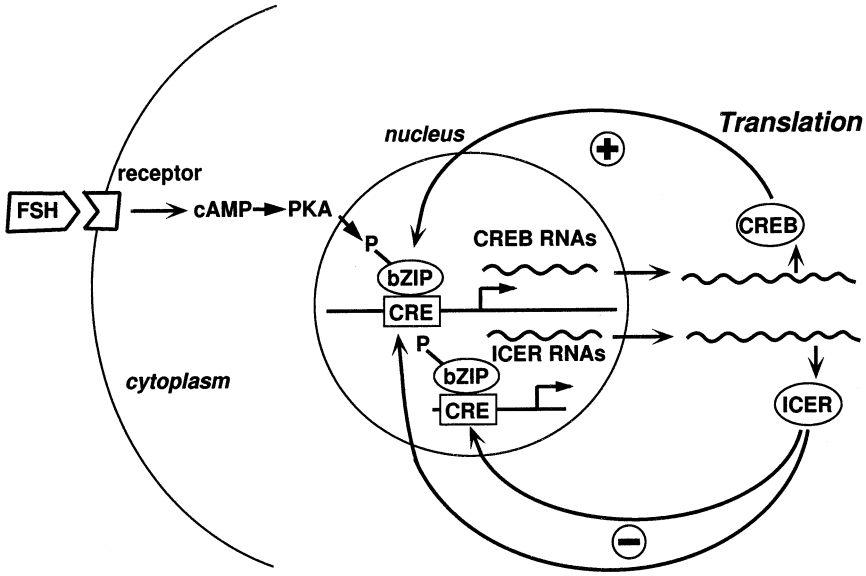

Fig. 5. CREB gene expression is cyclically stimulated and repressed during the spermatogenic cycle. A model of CREB gene expression during spermatogenesis shows that at the initiation of a new round of germ cell development FSH induces stimulation of the production of cAMP and activates PKA to phosphorylate CREB. When phosphorylated, CREB bound to the CREB promoter stimulates CREB transcription, producing more CREB. Meanwhile, CREB activates ICER transcription, causing the production of ICER repressor. Above some threshold level, ICER causes the down regulation of cAMP-induced genes including the CREB gene. Within 6–12 h ICER acts to repress transcription from the ICER promoter within the CREM gene. ICER then slowly degrades allowing cAMP promoters to be reset to basal activities until cAMP levels rise again.

may down-regulate the expression of the CREB gene by two independent but cooperative mechanisms: by eliminating the ability of FSH to raise cellular cAMP levels or by a direct blockade of the activation of the CREB promoter. This level of coordinate control of CREB gene expression suggests that CREB may be an important regulatory factor in Sertoli cells during the spermatogenic cycle.

It is notable that in vitro, ICER-binding activity in Sertoli cells is induced by 6–12 h after FSH stimulation and that binding activity declines by 24 h and reappears at 36–48 h. FSH-induced ICER levels in Sertoli cells as detected by Western immunoblot, however, remain elevated for up to 36 h (Monaco et al., 1995). In the seminiferous tubule, the time from the beginning of the induction of ICER (Stage XII) to the time of deinduction (Stage XIV) is approximately 48 h, based on the accuracy by which we can estimate the expression of ICER in the segments of seminiferous tubules. Therefore, it would appear that the interval for the induction of ICER in the seminiferous tubule in vivo, approximates that of Sertoli cells in culture.

The long half-life of ICER does not reconcile with the reported findings that FSH receptor mRNA levels are repressed for 2–4 h after FSH-stimulated production of ICER but rebound to pretreatment levels after 8–24 h of FSH treatment (Monaco et al., 1995). A similar pattern of regulation was reported earlier in Sertoli cells for the induction of the expression of the CREB gene in response to FSH (Walker et al., 1995). It would appear, therefore, that although ICER levels may remain elevated 12–24 h after stimulation, the major repressor activity of ICER on some genes is short-lived. Perhaps the action of ICER is to promptly inhibit CRE-mediated transcription of cAMP responsive target genes including the CREB and FSH receptor genes, and then to allow basal levels of transcription to be reinitiated. The reaccumulation of FSH receptors and CREB then allows the system to be reset and to be triggered by the cAMP-mediated phosphorylation of CREB again.

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References

Bellvé, A.R., Millette, C.F., Bhatnagar, Y.M., O'Brien, D.A., 1977. Dissociation of the mouse testis and characterization of isolated spermatogenic cells. J. Histochem. Cytochem. 25, 480–494.

Bodor, J., Walker, W.H., Flemington, E., Spetz, A.-L., Habener, J.F., 1995. Modulation of tax and PKA-mediated expression of HTLV-I promoter via cAMP response element binding and modulator proteins CREB and CREM. FEBS Lett. 377, 413–418.

Bodor, J., Spetz, A.-L., Strominger, J.L., Habener, J.F., 1996. cAMP inducibility of transcriptional repressor ICER in developing and mature human T lymphocytes. Proc. Natl. Acad. Sci. USA 93, 3536–3541.

Chapin, R.E., Phelps, J.L., Miller, B.E., Gary, T.J.B., 1987. Alkaline phosphatase histochemistry discriminates peritubular cells in primary rat testicular cell culture. J. Androl. 8, 155–161.

Chen, W.J., Andres, D.A., Goldstein, J.L., Russell, D.W., Brown, M.S., 1991. cDNA cloning and expression of the peptide-binding beta subunit of rat p21ras farnesyltransferase, the counterpart of yeast DPR1/RAM1. Cell 66, 327–334.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., Goodman, H.M., 1986. A cyclic AMP- and phorbol ester-inducible DNA element. Nature 323, 353–356.

Delmas, V., van der Hoorn, F., Mellstrom, B., Jegou, B., Sassone-Corsi, P., 1993. Induction of CREM activator proteins in spermatids: down-stream targets and implications for haploid germ cell differentiation. Mol. Endocrinol. 7, 1502–1514.

Deutsch, P.J., Hoeffler, J.P., Jameson, J.L., Lin, J.C., Habener, J.F., 1988. Structural determinants for transcriptional activation by cAMP-responsive DNA elements. J. Biol. Chem. 263, 18466–18472.

Foulkes, N.S., Mellstrom, B., Benusiglio, E., Sassone-Corsi, P., 1992. Developmental switch of CREM function during spermatogenesis: from antagonist to activator. Nature 355, 80–84.

Foulkes, N.S., Schlotter, F., Pevet, P., Sassone-Corsi, P., 1993. Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. Nature 362, 264–267.

Girardet, C., Walker, W.H., Habener, J.F., 1996. An alternatively spliced polycistronic mRNA encoding cyclic adenosine 3′,5′-monophosphate (cAMP) responsive element binding protein (CREB) in human testis extinguishes expression of an internally translated inhibitor CREB isoform. Mol. Endocrinol. 10, 879–891.

Habener, J.F., Miller, C.P., Vallejo, M., 1995. cAMP-dependent regulation of gene transcription by cAMP response element-binding protein and cAMP response element modulator. In: Litwack, G. (Ed.) Vitamins and Hormones. Academic Press, San Diego, pp. 1–57

Hall, S.H., Joeseph, D.R., French, F.S., Conti, M., 1988. Follicle-stimulating hormone induces transient expression of the protooncogene c-fos in primary Sertoli cell cultures. Mol. Endocrinol. 2, 55–61.

Hoeffler, J.P., Habener, J.F., 1990. Characterization of a cAMP regulatory element binding protein. Trends Endocrinol. Metab. 13, 184–188.

Joseph, D.R., Hall, S.H., Conti, M., French, F.S., 1988. The gene structure of rat androgen binding protein: identification of potential regulatory deoxyribonucleic acid elements of a follicle-stimulating hormone-regulated protein. Mol. Endocrinol. 2, 3–13.

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. Record 227, 62–76.

Leblond, C.P., Clermont, Y., 1952. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann. NY Acad. Sci. 55, 548–573.

Maurer, R.J., 1989. Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. J. Biol. Chem. 264, 6870–6873.

McKnight, S.L., 1991. Molecular zippers in gene regulation. Sci. Am. April, 54–64.

Meyer, T.E., Waeber, G., Lin, J., Beckmann, W., Habener, J.F., 1993. The promoter of the gene encoding 3′, 5′-cyclic adenosine monophosphate (cAMP) response element binding protein contains cAMP response elements: evidence for positive autoregulation of gene transcription. Endocrinology 132, 770–780.

Meyer, T.E., Habener, J.F., 1993. Cyclic AMP response element binding protein CREB and related transcription-activating DNA binding proteins. Endocrine Rev. 14, 269–290.

Molina, C.A., Foulkes, N.S., Lalli, E., Sassone-Corsi, P., 1993. Inducibility and negative autoregulation of CREM: An alternative promoter directs the expression of ICER, an early response repressor. Cell 75, 875–886.

Monaco, L., Foulkes, N., Sassone-Corsi, P., 1995. Pituitary follicle-stimulating hormone (FSH) induces CREM gene expression in Sertoli cells: Involvement in long term desensitization of the FSH receptor. Proc. Natl. Acad. Sci. USA 92, 10673–10677.

Najmabadi, H., Rosenberg, L.A., Yuan, Q.X., Bhatia, N., Albiston, A.L., Berger, H., Bhasin, S., 1993. Transcriptional and posttranscriptional regulation of inhibin α–subunit gene expression in rat Sertoli cells by 8–Bromo-3′,5′-cyclic-adenosine monophosphate. Mol. Endocrinol. 7, 469–476.

Nichols, M., Weih, F., Schmid, W., DeVack, C., Kowenz-Leutz, E., Luckow, B., Boshart, M., Schutz, G., 1992. Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. EMBO J. 11, 3337–3346.

Perey, B., Cleremont, Y., Leblond, C.P., 1961. The wave of the seminiferous pithelium in the rat. Am. J. Anat. 108, 47–77.

Rauchaud, S., Seite, P., Foulkes, N.S., Sassone-Corsi, P., Lanotte, M., 1997. The transcriptional repressor ICER and cAMP-induced programmed cell death. Oncogene 15, 827–836.

Schreiber, E., Matthias, P., Muller, M.M., Schaffner, W., 1989. Rapid detection of octamer binding proteins with miniextracts prepared from a small number of cells. Nucleic Acids Res. 17, 6419.

Seldon, R., 1989. Transfection using DEAE-dextran. In: Ausubel, F., Brent, R., Kingston, R., Seidman, J., Smith, J., Struhl, K. (Eds.), Current protocols in molecular biology. Wiley/Greene, New York, pp. 921–926.

Smith, E.P., Hall, S.H., Monaco, L., French, F.S., Wilson, E.M., Conti, M., 1989. A rat factor similar to basic fibroblast growth factor increases c-fos messenger ribonucleic acid in cultured Sertoli cells. Mol. Endocrinol. 3, 954–961.

Steinberger, A., Hintz, M., Heindel, J., 1978. Changes in cAMP responses to FSH in isolated rat Sertoli cells during sexual maturation. Biol. Reprod. 19, 566–572.

Themmen, A.P.N., Blok, L.J., Post, M., Baarends, W.M., Hoogerbrugge, J.W., Vassart, G., Anton, J., Grootengoed, J.A., 1991. Follitropin receptor down-regulation involves a cAMP-dependent post-transcriptional decrease of receptor mRNA expression. Mol. Cell Endocinol. 78, R7–R13.

Toppari, J., Parvinen, M., 1985. In vitro differentiation of rat seminiferous tubular segments from defined stages of the epithelial cycle: Morphological and immunolocalization analysis. J. Androl. 6, 334–343.

Vallejo, M., Penchuck, L., Habener, J.F., 1992. Somatostatin gene upstream enhancer element activated by a protein complex consisting of CREB, Isl-1-like, and α-CBF-like transcription factors. J. Biol. Chem. 267, 12876–12884.

Waeber, G., Meyer, T.E., LeSieur, M., Hermann, H., Gérard, N., Habener, J.F., 1991. Developmental stage-specific expression of the cyclic AMP response element binding protein CREB during spermatogenesis involves alternative exon splicing. Mol. Endocrinol. 5, 1418–1430.

Walker, W.H., Stein, B., Ganchi, P.A., Hoffman, J.A., Kaufman, P.A., Ballard, D.W., Hannink, M., Greene, W.C., 1992. The v-rel oncogene: insights into the mechanism of transcriptional activation, repression, and transformation. J. Virol. 66, 5018–5029.

Walker, W.H., Fucci, L., Habener, J.F., 1995. Expression of the gene encoding transcription factor adenosine 3′, 5′-monophosphate (cAMP) response element-binding protein: regulation by follicle-stimulating hormone-induced cAMP signalling in primary rat Sertoli cells. Endocrinology 136, 3534–3545.

Walker, W.H., Habener, J.F., 1996. Role of transcription factors CREB and CREM in cAMP-regulated transcription during spermatogenesis. Trends Endocrinol. Metab. 7, 133–138.

Walker, W.H., Girardet, C., Habener, J.F., 1996. Alternative exon splicing controls a translational switch from activator to repressor isoforms of transcription factor CREB during spermatogenesis. J. Biol. Chem. 271, 20145–20150.

Yoshikawa, K., Azawa, T., 1988. Expression of the enkephalin precursor in rat Sertoli cells. FEBS Lett. 237, 183–186.