NF-κB Induces cAMP-response Element-binding Protein Gene Transcription in Sertoli Cells*

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Spermatogenesis is dependent upon Sertoli cells, which relay hormonal signals and provide factors required for the differentiation and proliferation of germ cells. NF-κB transcription factors are constitutively expressed in the nuclei of Sertoli cells in rodent testis. Electrophoretic mobility shift assays demonstrated that Sertoli NF-κB proteins specifically bind to κB enhancer motifs within the promoter of the cAMP-response element-binding protein (CREB) gene, an important mediator of hormonal signals that control spermatogenesis. Overexpression of NF-κB proteins in primary Sertoli and NIH 3T3 fibroblast cells induced the CREB promoter in transient transfection assays. Stimulation of Sertoli cells with tumor necrosis factor-α, an NF-κB-activating cytokine produced by round spermatids located adjacent to Sertoli cells, stimulated the elimination of IκB, the translocation of additional NF-κB to the nucleus, and increased NF-κB binding to CREB promoter κB enhancer elements. Tumor necrosis factor-α also stimulated transcription from the CREB promoter. These data demonstrate that NF-κB contributes to the up-regulation of CREB expression in Sertoli cells and raises the possibility that NF-κB may induce other Sertoli genes required for spermatogenesis. Furthermore, the CREB promoter is also inducible by NF-κB in NIH 3T3 cells suggesting that NF-κB may be a general regulator of CREB in non-testis tissues.

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The abbreviations used are: FSH, follicle-stimulating hormone; CREB, cAMP-response element-binding protein; CRE, cAMP-response element; NF-κB, nuclear factor κB; IκB, inhibitor of κB; TNF-α, tumor necrosis factor α; bp, base pair(s); CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis.

The modulation of CREB expression levels represents a potential mechanism to alter Sertoli cell responsiveness to FSH. CREB expression in Sertoli cells has been demonstrated to vary in a stage-specific manner during the spermatogenesis cycle, (4–6). FSH-induced changes in cAMP levels have been implicated in the cyclical control of Sertoli cell CREB expression through CREs within the CREB promoter (7, 8). Additional signaling pathways may also control stage-specific expression of CREB. Specifically, NF-κB transcription factors that were recently identified as activating gene expression in Sertoli cells (9) are potential regulators of CREB expression as the CREB promoter contains consensus NF-κB binding motifs. The family of NF-κB or Rel transcription factors consists of five known mammalian subunits (RelA, RelB, c-Rel, p50, and p52). Multiple combinations of homo- and heterodimers are possible, thus providing the potential to generate both trans-activators and trans-repressors of transcription (10–13). In most cells NF-κB dimers remain sequestered in the cytoplasm by inhibitor proteins (IκB-α, IκB-β, IκB-γ, IκB-ε, and IκB-δ). Upon stimulation by diverse stimuli such as TNF-α, phorbol myristic acid, viral proteins, and interleukins, IκB is phosphorylated and ubiquinated leading to proteosome-mediated degradation. The NF-κB nuclear localization signal is then unmasked, and NF-κB is free to translocate to the nucleus and regulate gene expression via interactions with κB enhancer elements (reviewed in Ref. 14). In addition to being regulated via stimulus-induced release from IκB, the activity of free NF-κB can also be modulated through direct phosphorylation of the RelA subunit by protein kinase A (15, 16). The cytokine TNF-α is a regulator of NF-κB activity (reviewed in Ref. 11) and therefore is a candidate regulator of CREB gene expression. In the testis TNF-α is secreted primarily by round spermatids within the seminiferous tubules, and the 55-kDa TNF-α receptor has been detected in Sertoli cells (17, 18). Recently, we demonstrated that TNF-α increases the activity of NF-κB in rat Sertoli cells and that NF-κB levels in the nuclei of Sertoli cells are highest during the stages in which round spermatids are present (9).

In this study we test the hypothesis that NF-κB and TNF-α are regulators of CREB expression. We demonstrate that Sertoli cell NF-κB proteins interact with NF-κB binding sites in the CREB promoter. We also show that overexpression of NF-κB subunits in Sertoli cells and NIH 3T3 cells increases CREB promoter activity. Stimulation of primary Sertoli cells with the cytokine TNF-α mediates a reduction in IκB-α and IκB-β levels, a concomitant increase in RelA nuclear translocation and the induction of NF-κB binding to a CREB promoter NF-κB enhancer motif. Transient transfection analyses dem-
onstrate that TNF-α also stimulates CREB gene promoter activity. These data suggest that NF-κB may be an important regulator of genes required for spermatogenesis and a general regulator of CREB gene expression in non-testis cells.

**EXPERIMENTAL PROCEDURES**

*Isolation of Primary Sertoli Cells and Cell Culture*—Sertoli cells were isolated from 16 day Harlan Sprague Dawley rats as described previously (7). Decapsulated testes were digested with collagenase (0.5 mg/ml, 37 °C, 20 min) in enriched Krebs-Ringer bicarbonate medium (19), followed by three washes in enriched Krebs-Ringer bicarbonate medium (3, 5, g, 3 min) to isolate seminiferous tubules. Tubules were digested with trypsin (0.5 mg/ml, 37 °C, 12 min), and cell aggregates were passed repeatedly through a Pasteur pipette. An equal volume of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was added to the Sertoli cells, which were then pelleted (500 × g, 5 min) and resuspended in serum-free media containing 50% Dulbecco's modified Eagle's medium, 50% Ham's F-12, 5 mg/ml insulin, 5 mg/ml transferrin, 0.5 mg/ml retinoic acid, 10 ng/ml epidermal growth factor, 3 mg/ml cytosine β-n-arabinofuranosidase, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 mg/ml streptomycin. Sertoli cells were cultured on matrigel (Collaborative Research, Bedford, MA) coated dishes (33 °C, 5% CO₂) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were also cultured in the presence of TNF-α (20). In some cases, cells were also cultured in the presence of TNF-α and dexamethasone (30). In some cases, cells were also cultured in the presence of TNF-α and dexamethasone (30).

*NF-κB* probe and nuclear extracts. Protein-binding comparisons to various NF-κB motifs, labeled probes with specific activities (≥20%) were used in simultaneous reactions. DNA-protein complex formation was quantified using NIH Image 1.6 software analysis of digitized images.

Western Immunoblotted and Immunocytochemistry—Sertoli cell nuclear and cytoplasmic extracts were generated by digitizing a Chameleon double-stranded, site-directed mutagenesis kit instructions (Stratagene). The following oligonucleotides were utilized to indicate the introduced underlined point mutations: 5'-ACACCCCTACAGGGCCACCTGGGACCTT-3' for the κB site and 5'-GTGCGGGCCAGAAAACACAA-3', for the κB2 site. The NF-κB expression vectors pCMV4p50 (23) and pCMV5p65 (24) contain cDNAs for NF-κB p50 and p65, respectively, positioned downstream of the CMV promoter/enhancer in the pCMV4 and pCMV5 expression vectors (25, 26).

Primary Sertoli cells were transfected by calcium phosphate coprecipitation as described (7) using 1 μg of luciferase reporter plasmid and 1 μg of empty pCMV expression vector or 1 μg of pCMV expression vectors encoding p50 and p65. DNA precipitates were added to Sertoli cells in the presence of 2% fetal bovine serum, and serum was removed after the addition of DNA to the cells for 4 h. For studies employing TNF-α, DNA was added to the cells in the presence of PuGene reagent (Roche Molecular Biochemicals) and 2% serum for 24 h. After washing off the PuGene reagent, the cells were maintained in 2% serum until harvesting 6 h later. NIH 3T3 cells and HEK 293 cells were transfected by the calcium phosphate coprecipitation method with the cells being maintained in 10% serum throughout the transfection experiment. Total cellular proteins were extracted in reporter lysis buffer (Promega), and luciferase assays were performed using a luminometer and the Promega luciferase assay reagent. Luciferase activity of the extracts was normalized for total protein as determined by Bradford assay.

**RESULTS**

Sertoli Cell Proteins Bind CREB NF-κB Enhancer Elements—Four potential κB enhancer elements within the CREB gene in the pGL2-Basic vector (Promega, Madison, WI) by blunt end ligation. This CREB promoter fragment included the entire region from −1264 to −51 bp upstream of the CREB translation start site. To generate the −537 CREBLUC plasmid an Asp718-Stul fragment containing upstream sequences upstream of −537 bp was removed from the −1264 CREBLUC construct. The −278 CREBLUC was constructed by eliminating a Xhol fragment of −1264 CREBLUC containing CREB promoter sequences upstream of −278 bp. The following CREB promoter deletion constructs were also generated from the −1264 CREBLUC vector: ΔκB III-IV (a 480-bp FstI-Ralf deletion from −1160 to −680 bp), ΔκB I-II (a 265-bp RalRivar1 deletion from −680 to −385 bp), and ΔκB I-II deletion from −680 to −577 bp. The ΔκB I-II deletion construct was created by mutating the CREB promoter κB1 and κB2 binding motifs according to the Chameleon double-stranded, site-directed mutagenesis kit instructions (Stratagene). The dominant-negative CREB plasmid (S-targeted mutants) was created by mutating CREB promoter κB1 and κB2 binding motifs according to the Chameleon double-stranded, site-directed mutagenesis kit instructions (Stratagene). The dominant-negative CREB plasmid (S-targeted mutants) was created by mutating CREB promoter κB1 and κB2 binding motifs according to the Chameleon double-stranded, site-directed mutagenesis kit instructions (Stratagene). The dominant-negative CREB plasmid (S-targeted mutants) was created by mutating CREB promoter κB1 and κB2 binding motifs according to the Chameleon double-stranded, site-directed mutagenesis kit instructions (Stratagene). The dominant-negative CREB plasmid (S-targeted mutants) was created by mutating CREB promoter κB1 and κB2 binding motifs according to the Chameleon double-stranded, site-directed mutagenesis kit instructions (Stratagene). The dominant-negative CREB plasmid (S-targeted mutants) was created by mutating CREB promoter κB1 and κB2 binding motifs according to the Chameleon double-stranded, site-directed mutagenesis kit instructions (Stratagene).
promoter (CREB-B1–4) (Fig. 1A) were tested for interactions with Sertoli cell proteins using EMSA. A series of radiolabeled oligonucleotide probes having similar specific activities (±20%), each containing one of the CREB promoter κB enhancer elements plus 22 bp of promoter flanking sequences, were used in EMSA studies. Following incubation of the probes with nuclear protein extracts prepared from rat primary Sertoli cell cultures, DNA-protein complexes exhibiting varying degrees of binding affinity were generated (Fig. 1B). In the CREB promoter, CREB-B1 and CREB-B2 containing probes were most effective at forming complexes with Sertoli cell nuclear extracts; however, lower levels of probe-protein interactions were also observed with the CREB-B4 probe. Specifically, if CREB-B1 binding activity was arbitrarily set to 100%, then the binding activity of CREB-B2 and CREB-B4 was 95 ± 10.0% and 15.9 ± 2.9%, respectively. No detectable binding was observed following incubation with the CREB-B3 probe. All complexes observed for each probe represented specific protein interactions with the κB enhancer elements as complex formation was effectively inhibited by a 20-fold excess of an unlabeled probe containing a consensus κB binding site (κB) but not by an excess of competitor oligonucleotides containing an Sp1 binding site or a CRE (Fig. 1C). These data suggest that the complexes formed are because of the specific binding of NF-κB proteins to the probes.

**Fig. 1.** Sertoli cell proteins bind to the CREB promoter κB enhancer motifs. A, the positions of putative CREB promoter NF-κB binding elements κB1-κB4 are indicated relative to the translation start site of the CREB gene. B, the binding of Sertoli cell protein nuclear extracts to the CREB κB enhancer motifs was examined using EMSA. ϕ32P-labeled oligonucleotides containing the indicated NF-κB binding sites (κB1–κB4) plus flanking sequences were incubated with Sertoli cell nuclear protein extracts (1.5 μg). Data are representative of at least two independent experiments. C, Sertoli cell proteins specifically interact with CREB promoter κB enhancer elements. Nuclear proteins (1.5 μg) from Sertoli cell cultures were incubated with the indicated ϕ32P-labeled CREB κB enhancer probes in the absence (−) or presence of a 20-fold excess of either an unlabeled consensus κB enhancer probe (κB), an oligonucleotide containing an Sp1 binding site (Sp1), or a CRE-containing oligonucleotide (CRE). Data are representative of three independent experiments. For B and C, DNA-protein complexes were resolved via 5% nondenaturing PAGE and visualized by autoradiography. The unbound probe was run off the gel.

**NF-κB Regulates CREB Promoter Activity in Sertoli Cells**—The hypothesis that CREB transcription is regulated by NF-κB proteins in Sertoli cells was tested in transient transfection assays. Primary cultures of rat Sertoli cells were initially transfected with luciferase reporter plasmids containing either the full-length CREB promoter (−1264 CREBLUC), a CREB promoter fragment lacking the entire NF-κB3- and NF-κB4-containing region upstream of −537 bp (−537 CREBLUC), or a CREB promoter sequence missing the entire NF-κB-containing region upstream of −278 bp (−278 CREBLUC). B, NIH 3T3 cells were transfected with reporter plasmids containing CREB promoter sequences as in A. In A and B, cells were cotransfected with either an empty expression vector or with expression vectors for RelA alone or p50 and RelA in combination as indicated. Luciferase activity was measured using a luminometer, and relative light units were adjusted for total protein levels and expressed as a percentage of −1264 CREBLUC activity. Data represent the mean ± S.E. of three independent experiments in duplicate.

**Fig. 2.** NF-κB stimulates CREB promoter activity in Sertoli and NIH 3T3 cells. A, primary cultures of 16 day rat Sertoli cells were transfected with reporter plasmids containing either the full-length CREB promoter (−1264 CREBLUC), a CREB promoter fragment lacking the entire NF-κB3- and NF-κB4-containing region upstream of −537 bp (−537 CREBLUC), or a CREB promoter sequence missing the entire NF-κB-containing region upstream of −278 bp (−278 CREBLUC). B, NIH 3T3 cells were transfected with reporter plasmids containing CREB promoter sequences as in A. In A and B, cells were cotransfected with either an empty expression vector or with expression vectors for RelA alone or p50 and RelA in combination as indicated. Luciferase activity was measured using a luminometer, and relative light units were adjusted for total protein levels and expressed as a percentage of −1264 CREBLUC activity. Data represent the mean ± S.E. of three independent experiments in duplicate.
NF-κB Regulates CREB

NF-κB induces the CREB promoter predominately through the CREB κB1 and κB2 motifs. Primary cultures of 16 day rat Sertoli cells or NIH 3T3 cells were transfected with luciferase reporter plasmids containing either the full-length CREB promoter (−1264 CREBLUC), a CREB promoter construct lacking the NF-κB3 and NF-κB4 sites (ΔκBIII-IV), a CREB promoter sequence in which NF-κB1 and NF-κB2 sites have been deleted (ΔκBII-II), a full-length CREB promoter containing 5-bp point mutations in the NF-κB1 and NF-κB2 sites (κB1-IImt), or a CREB promoter sequence lacking the entire NF-κB containing sequence (ΔκBII-IV). Schematic representations of the deletion constructs are shown to the left. Mutations within an NF-κB binding motif are designated by an X. Cells were cotransfected with either an empty expression vector or with expression vectors for RelA alone or p50 and RelA in combination as indicated. Luciferase activity was measured using a luminometer, and relative light units were adjusted for total protein levels and expressed as a percentage of −1264 CREBLUC activity. Data represent the mean ± S.E. of three independent experiments in duplicate.

To identify NF-κB inducible regions of the CREB promoter, a series of CREB promoter deletion constructs were generated. Primary Sertoli cells were transfected with luciferase reporter vectors driven by various CREB promoter mutants in the presence or absence of RelA and p50 expression vectors (Fig. 3). Deletion of a region containing the two distal κB enhancer elements (ΔκBII-II) resulted in a 2-fold increase in basal promoter activity suggesting that the distal κB enhancer motifs were not required for basal CREB promoter activity and/or negative regulatory elements may be present in the deleted region. Overexpression of RelA and p50 induced ΔκBIII-IV CREB promoter activity 3-fold over the elevated basal levels and slightly higher than the NF-κB-induced wild type promoter thus providing further evidence that the distal κB enhancer sequences were not essential for CREB promoter induction. In contrast, deletion of a fragment including the two proximal κB enhancer elements (ΔκBII-II) reduced basal activity by 50% and abolished NF-κB-mediated induction of the CREB promoter. Introduction of 5 bp changes into each of the two proximal κB enhancer motifs (κB1-IImt) also reduced basal activity by 50% and reduced NF-κB induction of the CREB promoter to 3-fold. Basal activity was dramatically reduced and induction by NF-κB transcription factors was completely eliminated following removal of a fragment containing all four κB enhancers (ΔκBII-IV). The response of the CREB promoter mutants to overexpression of RelA or p50 and RelA in NIH 3T3 cells was very similar to that observed in primary Sertoli cells. However, one apparent difference was the further diminished response of κB1-IImt to the overexpression of NF-κB in NIH 3T3 cells. Together, the data in Fig. 3 suggest that basal and NF-κB inducible promoter activity can be regulated through κB motifs I-IV but that the region containing κB1 and κBII is more responsive to NF-κB.

TNF-α Induces Degradation of IκB-α and IκB-β, Nuclear Translocation of RelA, and NF-κB Binding to CREB Promoter κB Motifs in Sertoli Cells—TNF-α, a known activator of NF-κB in Sertoli cells (9), is secreted by the adjacent round spermatids (17). Stimulation of cells with TNF-α has been demonstrated to result in phosphorylation-dependent degradation of IκB-α and subsequent translocation of NF-κB from the cytoplasm to the nucleus. Therefore, the TNF-α-mediated effects on IκB family members in Sertoli cells was investigated. Because IκB expression has not been previously characterized in Sertoli cells, studies focused on IκB-α and IκB-β, as IκB-α degradation is a prerequisite for NF-κB translocation in most cells and high levels of IκB-β mRNA have been detected in whole testis (27). Cytoplasmic extracts from untreated primary rat Sertoli cells or from cells treated with TNF-α for 30 min were subjected to Western blot analyses using antiserum directed against IκB-α (Fig. 4A) or IκB-β (Fig. 4B). TNF-α induced a reduction in the concentration of IκB-α and IκB-β compared with untreated cells.

Fig. 3. NF-κB induces the CREB promoter predominately through the CREB κB1 and κB2 motifs. Primary cultures of 16 day rat Sertoli cells or NIH 3T3 cells were transfected with luciferase reporter plasmids containing either the full-length CREB promoter (−1264 CREBLUC), a CREB promoter construct lacking the NF-κB3 and NF-κB4 sites (ΔκBIII-IV), a CREB promoter sequence in which NF-κB1 and NF-κB2 sites have been deleted (ΔκBII-II), a full-length CREB promoter containing 5-bp point mutations in the NF-κB1 and NF-κB2 sites (κB1-IImt), or a CREB promoter sequence lacking the entire NF-κB containing sequence (ΔκBII-IV). Schematic representations of the deletion constructs are shown to the left. Mutations within an NF-κB binding motif are designated by an X. Cells were cotransfected with either an empty expression vector or with expression vectors for RelA alone or p50 and RelA in combination as indicated. Luciferase activity was measured using a luminometer, and relative light units were adjusted for total protein levels and expressed as a percentage of −1264 CREBLUC activity. Data represent the mean ± S.E. of three independent experiments in duplicate.

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TNF-α Induces Degradation of IκB-α and IκB-β, Nuclear Translocation of RelA, and NF-κB Binding to CREB Promoter κB Motifs in Sertoli Cells—TNF-α, a known activator of NF-κB in Sertoli cells (9), is secreted by the adjacent round spermatids (17). Stimulation of cells with TNF-α has been demonstrated to result in phosphorylation-dependent degradation of IκB-α and subsequent translocation of NF-κB from the cytoplasm to the nucleus. Therefore, the TNF-α-mediated effects on IκB family members in Sertoli cells was investigated. Because IκB expression has not been previously characterized in Sertoli cells, studies focused on IκB-α and IκB-β, as IκB-α degradation is a prerequisite for NF-κB translocation in most cells and high levels of IκB-β mRNA have been detected in whole testis (27). Cytoplasmic extracts from untreated primary rat Sertoli cells or from cells treated with TNF-α for 30 min were subjected to Western blot analyses using antiserum directed against IκB-α (Fig. 4A) or IκB-β (Fig. 4B). TNF-α induced a reduction in the
cytoplasmic levels of both IκB-α (4.33 ± 1.1-fold, n = 3) and IκB-β (3.4 ± 0.1-fold, n = 3).

The ability of TNF-α to induce nuclear translocation of NF-κB in Sertoli cells was studied using indirect immunofluorescence and Western blot analyses. The RelA subunit was chosen for investigation because it is required to maximize NF-κB transactivation activity (see Figs. 2 and 3). In western immunoblotting assays, a 30-min TNF-α (20 ng/ml) treatment of rat Sertoli cell cultures caused a significant increase in nuclear RelA levels that was accompanied by a reduction in cytoplasmic RelA (Fig. 5A). The TNF-α-induced translocation of RelA was confirmed in immunofluorescence localization studies. These studies demonstrated that RelA was present in the nucleus of untreated Sertoli cells; however, a rapid and dramatic increase in nuclear RelA levels occurred following TNF-α treatment (Fig. 5B). To confirm that TNF-α treatment of Sertoli cells could increase the levels of NF-κB available to bind to the CREB promoter, EMSA analyses were performed with a probe containing the CREBxB1 binding site, which exhibited the most efficient binding of Sertoli cell NF-κB protein. Stimulation of primary rat Sertoli cells with TNF-α (20 ng/ml) for 30 min caused a dramatic induction of NF-κB binding activity in nuclear extracts, whereas a loss of NF-κB binding activity was observed in cytoplasmic extracts (Fig. 5C).

Together, the data in Figs. 4 and 5 suggest that TNF-α stimulates rapid IκB-α and IκB-β degradation in Sertoli cells leading to the translocation of NF-κB to the nucleus and subsequent binding to κB enhancer elements.

**TNF-α Stimulates the CREB Promoter**—Because TNF-α efficiently stimulated NF-κB activation in Sertoli cells, TNF-α induction of the NF-κB-responsive CREB promoter was investigated. Transient transfections of primary Sertoli cells (Fig. 6A) and HEK 293 cells (Fig. 6B) were performed with the −1264 CREBLUC and ΔxB1–4 CREBLUC constructs. The −1264 CREBLUC construct was induced 2.2-fold in both cell lines following stimulation with TNF-α for 6 h. In contrast, the ΔxB1–4 CREBLUC construct was not inducible by TNF-α. Interestingly, TNF-α induction of −1264 CREBLUC in Sertoli cells was only possible after employing a new, more efficient transfection protocol. Transfections in which Sertoli cells were incubated with FuGENE reagent (Roche Molecular Biochemicals) and plasmid DNA for 24 h in the presence of 2% fetal bovine serum followed by continued incubation with TNF-α in 2% serum resulted in significant induction of −1264 CREBLUC. Serum alone did not activate the CREB promoter and in the absence of serum, the CREB promoter was not stimulated by TNF-α (data not shown).

![Figure 4](image-url) **Fig. 4.** Sertoli cell IκBa and IκBβ are rapidly degraded after TNF-α treatment. For Western immunoblot analyses, cytoplasmic extracts from untreated Sertoli cells or Sertoli cells treated with TNF-α (20 ng/ml) for 30 min were fractionated by SDS-PAGE, transferred to membranes, and probed with antiserum directed against IκB-α (A) or IκB-β (B). Immunodetected IκB proteins and molecular weight standards are indicated. Nonspecific proteins recognized by IκB antisera are shown to confirm equal loading of protein. Data in A and B are representative of two independent experiments.

![Figure 5](image-url) **Fig. 5.** TNF-α induces RelA nuclear translocation and NF-κB DNA binding activity in Sertoli cells. A, Western blot analyses were performed on 50 µg of nuclear (NE) or cytoplasmic (CE) extracts from untreated or TNF-α (20 ng/ml) stimulated primary cultures of 16 day rat Sertoli cells. Immunoblots were probed with RelA-specific antiserum. Immunodetected RelA protein is indicated. B, primary Sertoli cell cultures from 16 day rats receiving no stimulus (untreated) or TNF-α (20 ng/ml) for 30 min were fixed and probed with preimmune sera or antiserum against RelA. C, in EMSA analyses, nuclear or cytoplasmic extracts (5 µg) from untreated primary cultures of 16 day rat Sertoli cells or from those treated with TNF-α (20 ng/ml) for 30 min were incubated with the 32P-labeled CREBxB1 oligonucleotide probe. Protein-DNA complexes were resolved via non-denaturing PAGE and visualized by autoradiography. Cytoplasmic extracts were treated with detergent prior to incubation with the probe to liberate NF-κB from IκB proteins. Data in A and C are representative of three experiments. Data in B are representative of two independent experiments.
cells were transfected with the k present in Sertoli cells specifically bind k motifs as described earlier (7) as well as the proximal a BLUC reporter plasmids in the presence or absence of TNF.

Fig. 6. TNF-α stimulates the CREB promoter. Primary Sertoli cells were transfected with the −1264 CREB-LUC or ΔB1–4 CREB-LUC reporter plasmids in the presence of absence of TNF-α (20 ng/ml). Luciferase activity was measured using a luminometer, and relative light units were adjusted for total protein levels and expressed as fold induction over the untreated activity for each plasmid. Data represent the mean ± S.E. of three independent experiments in duplicate.

DISCUSSION

In this study, we have demonstrated that NF-κB proteins present in Sertoli cells specifically bind κB enhancer motifs within the CREB promoter. Four potential κB enhancer elements were identified in the CREB promoter by computer-assisted sequence analysis. The two gene proximal κB motifs bound NF-κB proteins more effectively than the distal consensus sequences. The binding of NF-κB was functionally significant as overexpression of NF-κB proteins in Sertoli cells stimulated transcription from the CREB promoter. Although NF-κB is constitutively expressed in the nucleus of Sertoli cells, TNF-α was shown to induce the degradation of IκB and further increase the levels of nuclear NF-κB in Sertoli cells. Furthermore, TNF-α was found to stimulate CREB gene expression in primary Sertoli cells and the HEK293 cell line.

In transient transfection studies of primary Sertoli cells, the basal activity of the −1264CREB promoter was 2- and 4-fold higher than the activities of the −537CREB and −278CREB promoter fragments, respectively. The decrease in −537CREB promoter activity in Sertoli cells but not NIH 3T3 cells may reflect the loss of two distal κB elements and the higher relative nuclear levels of NF-κB in Sertoli cells compared with NIH 3T3 cells. The more dramatic decrease in activity for the −278CREB promoter may be because of the elimination of Sp1 motifs as described earlier (7) as well as the proximal κB enhancer motifs. Although the relative basal activities of the −1264CREB, −537CREB and −278CREB promoter constructs in Sertoli cells were similar to that of an earlier report (7), it is difficult to make precise comparisons of the basal activity between various constructs as transfection efficiencies were not standardized using unregulated, control reporter constructs.

Nevertheless, NF-κB proved to be a potent inducer of the CREB promoter as overexpression of NF-κB p50 and RelA or RelA alone stimulated transcription from the full-length −1264CREB promoter six-fold. Although the distal κB3 and κB4 motifs contribute to the induction of the CREB promoter, deletion analysis of the CREB promoter showed that the region containing the proximal κB1 and κB2 motifs is required to maintain high basal expression and full NF-κB induction of the CREB promoter. The induction through κB1 and κB2 is not unexpected as these sequences most effectively bind NF-κB present in Sertoli extracts. Deletion analysis of the −680 to −1160 upstream region of the CREB promoter also suggests that this region may contain negative elements as removal of this region results in a 2-fold increase in basal activity.

The demonstration of CREB promoter stimulation by NF-κB in Sertoli cells suggested that NF-κB may regulate this gene in other cell types. In this regard, we found that the CREB gene is up-regulated by NF-κB overexpression in NIH 3T3 cells (Figs. 2B and 3B), and TNF-α induces the CREB promoter in HEK 293 cells (Fig. 6B). The regulation of CREB by NF-κB may be a new method of cross-talk between NF-κB and CREB signaling pathways to compliment the competition for the CREB-binding protein/p300 coactivator displayed by these factors (28, 29). Alternatively, it is possible that CREB and NF-κB may cooperate for recruiting CREB-binding protein/p300 to the promoter as CREB and RelA interact with different regions of the coactivator. The opportunity for CREB-NF-κB interactions on the CREB promoter exists as the CREB promoter contains binding sites for CREB in close proximity to κB motifs. RelA has been shown to directly interact with other bZIP family members related to CREB (ATF-2, c-Jun, and c-Fos) through a mini leucine zipper located in the Rel homology domain of RelA (30, 31). Furthermore, in glutathione S-transferase-pulldown and co-immunoprecipitation experiments RelA has been shown to also directly interact with CREB.2 Studies are underway to investigate potential cooperativity of NF-κB and CREB in stimulating transcription from the CREB promoter.

In this initial characterization of IκB proteins from pure cultures of rat Sertoli cells, both IκBa and IκBβ were determined to be present in the cytoplasm, and the levels of both were dramatically reduced after the addition of TNF-α. A previous study failed to detect IκBa mRNA in extracts from whole mouse testis (27). The inability to detect Sertoli cell-derived IκBa in whole testis is likely because of the small proportion of Sertoli cells in the mammalian testis. Fewer than 5% of adult mouse testis cells are Sertoli cells (19). In addition to the immunodetection of IκBβ in Sertoli cells in the present report, IκBβ mRNA was previously shown to be enriched in mouse testis (27). Together, these data suggest that whereas IκBa and IκBβ are both present in Sertoli cells, the developing germ cells, which account for greater than 90% of testis cells, likely contain IκBβ but not IκBa. Differences in the levels of the two IκB isoforms may be important in cell-specific gene regulation as stimulation of IκBa degradation is rapid and transient, but IκBβ degradation can be delayed and persistent (27).

One factor that may maintain high nuclear levels of NF-κB in Sertoli cells and account for additional NF-κB translocation to the nucleus is the cytokine TNF-α. In Sertoli cells, TNF-α activates NF-κB via the elimination of IκB from the cytoplasm and the subsequent translocation of additional NF-κB to the nucleus. TNF-α-mediated stimulation of NF-κB in Sertoli cells may be physiologically important because germ cells adjacent to Sertoli cells secrete TNF-α in a stage-specific manner. Although it is possible that TNF-α may also act through mitogen-

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2 W. Walker, unpublished results.
activated protein kinase or other pathways, it is significant that NF-κB is activated by TNF-α in Sertoli cells. In the absence of nuclear NF-κB, apoptosis pathways may be initiated by TNF-α (32). In contrast to germ cell development in which some apoptosis is required to constrain the expansion of germ cells and maintain spermatogenesis (33, 34), nuclear NF-κB may protect Sertoli cells from apoptosis inducers. NF-κB-mediated protection from apoptosis agents would explain why few or no apoptotic Sertoli cells are detected in the testis (32, 35, 36).

In a previous study TNF-α alone was able to stimulate transcription from a minimal promoter containing two consensus κB enhancers (9). In contrast, TNF-α induction of the CREB promoter in primary Sertoli cells required the addition of serum. These latest findings suggest that serum factors in addition to TNF-α are required to allow the activation of the more complex CREB promoter or that serum-dependent signaling pathways must be activated to allow some Sertoli cell genes to be regulated by TNF-α.

Although other germ cell types secrete some TNF-α, most of the TNF-α is secreted by round spermatids (17). Because round spermatids are present during only the first eight stages (stages I-VIII) of the 14 stages of rat spermatogenesis, it is possible that TNF-α secretion may represent stage-specific communication between spermatids and Sertoli cells. In this regard, our previous studies (9) have shown that there are significant increases in the nuclear expression of NF-κB during stages I-VII of spermatogenesis, which would correspond to the time when TNF-α producing spermatids are present. Therefore, spermatid-Sertoli cell communication via TNF-α may signal Sertoli cells to activate NF-κB causing CREB to induce the production of specific factors that are required by spermatids or other germ cells. The stage-specific expression of TNF-α may be relevant to the previously reported dramatic cyclical changes in the testis (33, 34), nuclear NF-κB may be a general regulator of CREB expression in non-testis tissues.

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