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Expression of the Basic Helix-Loop-Helix Protein REBα in Rat Testicular Sertoli Cells[1]

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ABSTRACT

Sertoli cell differentiation is initiated in the embryo to promote testicular development and male sex determination. In the adult, Sertoli cells are critical for maintenance of the spermatogenic process. Previously, Sertoli cell differentiation has been shown to be regulated in part by basic helix-loop-helix (bHLH) transcription factors. This was based on the observation that promoters of a number of Sertoli cell genes contained bHLH-responsive E-box response elements and that overexpression of Id, a negatively acting HLH protein, down-regulates Sertoli cell differentiated functions. Analysis of Sertoli cell bHLH proteins demonstrated the expression of REBα in Sertoli cells. REBα and REBβ are spliced variants of the *REB* gene that is implicated in cell-specific gene expression as part of dimeric bHLH complexes acting on E-box response elements. Although both the transcripts of the *REB* gene are widely expressed, differential expression of the *REB* gene transcripts REBα and REBβ has been shown. In the current study, a polymerase chain reaction (PCR)-based approach demonstrated that *REB* gene transcripts are expressed in the testis. Characterization of the *REB* transcripts suggested that REBα is the major splice variant in Sertoli cells. PCR primers specifically designed to amplify either REBα or REBβ demonstrated that Sertoli cells express only REBα, not REBβ. REBβ was present in the RNA samples obtained from whole testis, suggesting expression in other testicular cell types. A Northern blot analysis of RNA from Sertoli cells treated with or without FSH or cAMP demonstrated that REBα is not hormone responsive. REBα was also found to be expressed in germ cells and peritubular cells. An immunocytochemical analysis demonstrated that REBα is predominantly expressed by Sertoli cells within the seminiferous tubules. The activity of REBα in Sertoli cells was demonstrated with an E-box gel shift with Sertoli cell nuclear extracts. The E-box gel shift was found to contain REBα and E47/E12 bHLH proteins. In summary, the Sertoli cell is one of the first cells shown to specifically express the REBα isoform of the *REB* gene. The results are discussed in relation to the possibility that Sertoli cells may express a cell-specific bHLH protein that can preferentially dimerize with REBα.

INTRODUCTION

In the adult, Sertoli cells are a postmitotic somatic cell population that forms the seminiferous epithelium to provide both structural and functional support to the developing germ cells [1]. Sertoli cell development requires a tightly regulated differentiation process that includes 1) commitment of cell lineage in the bipotential gonad in the urogenital ridge, 2) embryonic and prepubertal growth and differentiation, 3) pubertal differentiation, and 4) maintenance of Sertoli cell function and differentiation in the adult testis. It is postulated that a number of diverse transcription factors regulate the Sertoli cell developmental pathway. The primary role of SRY [2, 3] and possibly SOX-9 [4] in earlier stages of Sertoli cell development has been shown. Later during development, transcription factors such as c-*fos* [5], c-*myc* [6], SF-1 [7], and cAMP response element binding protein [8] may regulate or maintain Sertoli cell differentiated functions.

Previous observations have suggested that the basic helix-loop-helix (bHLH) class of transcription factors may influence Sertoli cell functions. Down-regulation of transferrin [9] and c-*fos* promoter (unpublished results) activity by overexpression of Id, a dominant negative HLH protein, suggests that bHLH proteins influence Sertoli cell differentiation. The role of bHLH proteins in Sertoli cells is also supported by the observation that several Sertoli cell genes contain highly conserved E-box sequences within their proximal promoter regions as potential targets of bHLH proteins [9].

Basic HLH proteins have previously been shown to regulate cell-specific gene expression. These proteins form heterodimers consisting of ubiquitous class A and tissue-specific class B bHLH proteins [10, 11]. This family is characterized by a common structural motif, the HLH domain, which mediates protein interactions to form homo- and heterodimers [12]. The presence of a highly charged basic region in each of the dimerization partners immediately upstream of the HLH region forms a specific DNA-binding domain that can recognize the consensus DNA sequence, CANNTG, known as an E-box. The activity of these factors is often negatively regulated by another class of HLH proteins that lack the basic domain and are known as inhibitors of differentiation or Id [13]. The class A bHLH proteins HEB [14], E2–2 [15], and the *E2A* gene products E12 and E47 [16] are capable of forming heterodimers with class B HLH proteins like MyoD [10, 11].

REBα is a class A bHLH protein and is the rat homologue of the human E-box-binding protein (HEB, [14]). HEB/REBα is expressed in a wide variety of tissues [17]. Expression of REBα/HEB in the testis and more specifically in the Sertoli cells has not been reported. In contrast to its human homologue, *REB* is expressed as two alternatively spliced variants, REBα and REBβ. REBβ is characterized by a 24-amino acid insertion in the leucine heptad repeat near the amino terminal of the bHLH domain and may function as an inhibitory domain. The tissue-specific expression of *REB* RNA splicing may be involved in determining tissue-specific combinations of heterodimeric complexes between ubiquitous and tissue-restricted bHLH proteins.

The current study demonstrates that Sertoli cells specifically express REBα. This is the first cell type shown to specifically express only one transcript of the *REB* gene. The demonstration that REBα is expressed in Sertoli cells supports previous observations that bHLH proteins appear to act as regulators of Sertoli cell function and differenti-

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ation. The hypothesis is discussed that an as-yet-unidentified class B Sertoli cell-specific bHLH protein may be available for dimerization with the class A bHLH protein REBα.

MATERIALS AND METHODS

Isolation of Sertoli Cells

Sertoli cells were isolated from the testis of 20-day-old rats by a modified procedure described previously [9]. The purified Sertoli cells were more than 98% pure, with 2% contamination of peritubular cells as determined cytochemically [9]. The isolated Sertoli cells were then plated under serum-free conditions in 150 × 20-mm tissue culture dishes (Nunclon; Nunc, Naperville, IL) at a concentration 10^8 cells per plate. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco, Grand Island, NY) with 0.01% BSA at 32°C. Sertoli cells were treated with either FSH (100 ng/ml; ovine FSH-16, National Pituitary Agency, Harbor-UCLA Medical Center, Torrance, CA), dibutryl cAMP (dbcAMP; 100 μM/μl), or vehicle alone (Ham's F-12, control). These optimal concentrations of FSH and cAMP have previously been shown to dramatically stimulate cultured Sertoli cell differentiated functions [18, 19]. The cells were cultured under serum-free conditions for a maximum of 5 days with a change of medium and treatment after 48 h of culture. Cell number and viability did not change during the culture in the absence or presence of treatment.

RNA Preparation

Freshly isolated or cultured Sertoli cells were lysed directly using TRI Reagent (no. T9424; Sigma Chemical Co., St. Louis, MO). The cell lysate was then passed several times through a Pasteur pipette to form homogenous lysate. Tissues (skeletal muscle, brain, liver, kidney, and detunicated testis) were prepared in TRI Reagent (5% w:v) and were homogenized in a tissue homogenizer (Tissue Tearor; BioSpec, Bartlesville, OK). To avoid any possible contamination of extracellular material and high-molecular weight DNA in the final RNA samples, the homogenate was centrifuged at 12 000 × *g* for 10 min at 4°C. Total RNA was then isolated from the cell lysate and whole tissue homogenate according to the manufacturer's protocol for RNA isolation using TRI Reagent. The final RNA pellet was dissolved in distilled water at a concentration of 1 mg/ml.

Polymerase Chain Reaction (PCR)

Total RNA (2 μg) was reverse-transcribed in a final volume of 20 μl containing 20 U RNasin (Promega, Madison, WI), 200 μM each of dATP, dCTP, dTTP, and dGTP, 1 μg oligo dT (Pharmacia, Piscataway, NJ), 10 μM dithiothreitol, and 200 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (BRL, Gaithersburg, MD) in the MMLV first-strand synthesis buffer supplied by the manufacturer (BRL). The RNA and oligo dT primer in the buffer were first denatured for 5 min at 65°C and then cooled on ice before addition of nucleotides and enzyme. The reverse transcriptase reaction was carried out at 37°C for 1 h. PCR was performed using the GeneAmp 9600 (Perkin Elmer Cetus, Norwalk, CT) with 30 cycles as follows: 94°C, 1 min (denaturation); 58°C, 2 min (primer annealing); 72°C, 1 min (primer extension). Each PCR reaction contained 250 pg reverse-transcribed DNA; 1 μM of each 5′ and 3′ oligonucleotide primer; 2.5 U *Taq* polymerase (AmpliTaq; Perkin Elmer); and 200 μM of each dATP, dCTP, dGTP, and dTTP. After amplification, the product of each reaction was subjected to electrophoresis through a 1.5% agarose gel, and bands were visualized by ethidium bromide staining.

The primer pair sequences used were obtained from published sequences of REBα (GenBank acc. no. S53920) and REBβ (GenBank acc. no. S53921) and were synthesized from commercial sources. Primer pairs were designed to amplify a region 5′ toward the bHLH domain of the *REB* gene and to selectively amplify either REBα or REBβ (Fig. 1). To distinguish possible contamination of RNA with DNA, the reverse transcription (RT) reaction was performed without MMLV reverse transcriptase. The absence of any product in the amplification reaction using such a reverse-transcribed preparation indicated the absence of any contaminating DNA in our RNA samples. The sequence and position of the primers were as follows: REBα 5′ primer (939)—5′ CTC ACT TCA CTC GCA GTC TCG AAT GGA; REBβ 5′ primer (939)—5′ TGC AAT GTC CCT CTT AAA GGA TGT TGA GC; REBα and REBβ 3′ primer (REBα: 1578; REBβ: 1650)—5′ GTA AGT TCC TCG AGC CGG CTT ACA GA. As shown in Figure 1, the REBα-specific 5′ primer was designed to span the junction of 72-base pair (bp) insertion specific to REBβ. The REBβ-specific 5′ primer was designed within the 72-bp insertion absent in REBα. Each RT reaction was performed using three different samples. The PCR-based amplification reactions were carried out in duplicate on each reverse-transcribed RNA sample. Simultaneous PCR reactions were also carried out using primers designed to rat cyclophilin to monitor the efficiency of the PCR and to verify that cDNA samples contained equivalent amounts of material [20]. Cyclophilin was faithfully and equally amplified in all the PCR reactions, indicating consistency in the quality of RT and PCR reactions. The data presented are representative of three different RT-PCR reactions carried out in duplicate.

Subcloning and Sequencing

The PCR fragments were run on 1.5% agarose gel, stained with ethidium bromide, and visualized. The bands were then dissected out, and the DNA was isolated from the gel using Glass MAX DNA isolation system (BRL). The purified DNA fragments were subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA) plasmid. The cloned DNA fragments were sequenced using standard M13 forward and reverse primers in an automated fluorescence-based sequencer (Applied Biosystems, Foster City, CA). Internal sequences were obtained using nested primers that were designed based on previous sequences. All the sequences reported are consensus of two different experiments. The sequence alignments (Genetics Computer Group DNA analysis software, Madison, WI) were carried out using the available sequence of REBα and REBβ.

Northern Analysis

Total RNA from Sertoli cells, germ cells, and peritubular cells were isolated as described above using TRI Reagent (Sigma). Approximately 10 μg of total RNA was fractionated on a 1% formaldehyde-agarose gel. After fractionation, the RNA in the gel was transferred onto nylon membrane (Hybond N; Amersham, Piscataway, NJ) in 10-strength SSC (single-strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) buffer and UV cross-linked as de-

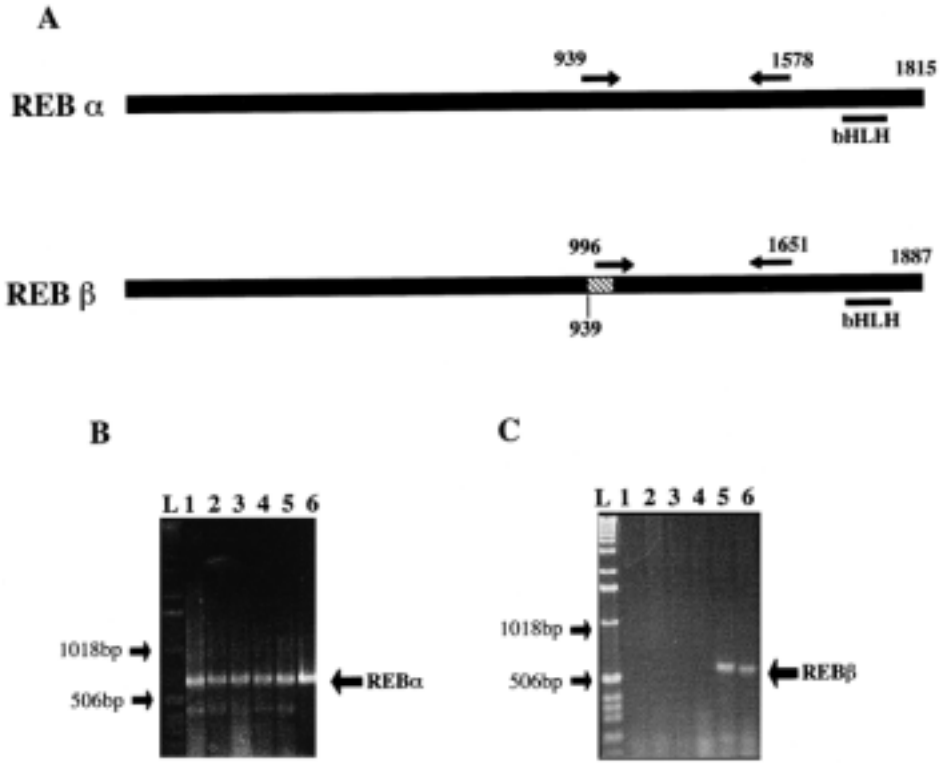

FIG. 1. **A**) Schematic showing the location of PCR primers within REBα and REBβ sequences. REBβ is almost identical to REBα except for a 72-bp insertion (hatched region within REBβ) that is absent in REBα. The 5′ primer specific for REBα was therefore designed across the junction of 72-bp insertion. The 5′ primer specific for REBβ was designed within this REBβ-specific 72-bp insertion. The 3′ primer is common to both REBα and REBβ. **B**) PCR using REBα-specific 5′ primer on RNA from whole testis (lane 1), freshly isolated Sertoli cells (lane 2), and cultured Sertoli cells untreated (lane 3) or treated with either FSH (lane 4) or dbcAMP (lane 5). RNA from kidney (lane 6) was used as a positive control. **C**) PCR using REBβ-specific 5′ primer on RNA from freshly isolated Sertoli cells (lane 1), untreated cultured Sertoli cells (lane 2), cultured Sertoli cells treated with either FSH (lane 3) or dbcAMP (lane 4), whole testis (lane 5), and lungs (lane 6). The data are representative of three separate PCRs carried out on at least three different RT-mRNA samples.

scribed previously [21]. The membranes were then prehybridized in quick hybridization buffer (Stratagene, La Jolla, CA) for 30 min at 60°C. The hybridization was carried out at 60°C for 1 h with ^{32}P-labeled REBα probe obtained by RT-PCR of Sertoli cell RNA using primer set 2. The membrane was subsequently stripped and rehybridized with rat cyclophilin. All the probes were labeled using Prime-it II kit from Stratagene. Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) was exposed to the membrane overnight at −80°C, and densitometry values were obtained by scanning with Molecular Dynamics ImageQuant Digital Image analysis system (Sunnyvale, CA). The statistical significance of the relative expression between various tissues and treatments was determined by ANOVA.

Immunocytochemistry

Sections (6 mm thick) of paraffin-embedded testes from 20-day-old rats were fixed in buffered formaldehyde (4% in 0.1 M PBS) and incubated either with rabbit anti-human HEB (Santa Cruz Laboratories, Santa Cruz, CA; 1:200) or with normal rabbit serum (negative control) for 2 h at room temperature. Before application of the primary antibody, a microwave antigen retrieval technique was used as described previously [22]. The sections were then rinsed in Tris-buffered saline and incubated with goat anti-rabbit biotinylated antibody (Vectastain Elite kit; Vector Laboratories, Burlingame, CA; dilution 1:100). After rinsing, the sections were incubated in ABC complex (Vector Laboratories; dilution 1:100), and the reaction was visualized with diaminobenzidine (Sigma).

Gel Mobility Shift and Blot Assay

Gel-shift assays were performed with nuclear extracts of cultured Sertoli cells. The Sertoli cells were isolated as described above and cultured in 150 × 20-mm tissue culture dishes (Nunclon). The cells either were treated after 48 h in culture with FSH or were not treated (for controls). After 72 h, the cells were scraped off the tissue culture dishes and washed once with PBS. The nuclear extracts of these cells were then prepared as described by Guillou et al. [23]. Typically 70–100 μg of protein was obtained from 10^8 plated cells. The double-stranded DNA probe used in the gel retardation assay was a 20-bp mouse transferrin promoter proximal E-box (-327CAGCTG) containing flanking sequence. The single-stranded oligonucleotides were 5′ ^{32}P end-labeled with [γ-^{32}P]ATP (150 μCi/μl; NEN, Boston, MA) and T4 polynucleotide kinase (Boehringer-Mannheim, Indianapolis, IN). The complementary oligonucleotides were annealed, electrophoretically purified, and then used as probes in gel-shift assays [9].

For immunoblot analysis, a gel-shift assay was performed as described above in duplicate on the same gel, using radioactive and nonradioactive E-box oligonucleotides. A gel-shift assay using nonradioactive E-box probe on the polyacrylamide gel was electrophoretically transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell, Keene, NH) by electrophoresis in Tris-glycine buffer containing 12% methanol. The blot was then blocked in 5% nonfat milk in TBSN (TBSN: 50 mM Tris [pH 7.4], 150 mM NaCl, and 0.05% Nonidet P-40) and incubated with 1:3000 dilution of antibodies to either E12/E47 or HEB (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h. After three washes of 15 min each, the blot was hybridized with a secondary antibody (1:3000 dilution; directed against rabbit IgG) conjugated to alkaline phosphatase for 1 h at room temperature. After five washes in TBSN, the immune complex was detected using an Immun-Star chemiluminescent protein detection kit (Bio-Rad, Hercules, CA). The nonimmune serum was included to determine the specificity of the blot. As a positive control, a gel shift was carried out using AP-1 oligo and blotted with c-*fos*, E12, USF, and SRF antibody. When AP-1 was used in the gel

shift, a band in the blot was observed only with the c-*fos* antibody (data not shown).

RESULTS

An RT-PCR of RNA from cultured Sertoli cells and whole testis was performed to determine whether the transcripts of the *REB* gene are present in the Sertoli cells. The primers for RT-PCR were designed toward the 5′ end of the bHLH domain of the *REB* gene encoding approximately a 650-bp fragment. The 3′ primer was common to both REBα and REBβ, whereas the two 5′ primers were designed to selectively amplify either REBα or REBβ transcripts (Fig. 1A). As shown in Figure 1B, when REBα-specific 5′ primer was used in the PCR reaction, a single predominant band of approximately 640 bp was observed in the RNA from whole testis (lane 1), freshly isolated Sertoli cells (lane 2), cultured Sertoli cells (lane 3), cultured Sertoli cells treated with either FSH (lane 4) or dbcAMP (lane 5), and kidney (lane 6, positive control). The 640-bp PCR fragment was isolated from the gel, purified, and subcloned for sequencing. Alignment of the sequence obtained to the available nucleotide sequence of REBα confirmed that the 640-bp band was REBα. The expression of REBα appears constitutive because of its presence in untreated and FSH- or dbcAMP-treated Sertoli cells. The identity of the 450-bp testis-specific band seen in Figure 1B (lanes 1, 2, 3, 4, and 5) is under investigation. A PCR analysis for a constitutively expressed cyclophilin gene demonstrated an equivalent amount of cyclophilin PCR product in all RNA samples (data not shown).

In most of the tissues examined, REBα and REBβ were coexpressed [17]. The relative abundance of the expression of REBα and REBβ was, however, differentially regulated. In the rat head, for example, a distinct difference in the relative levels of REBα and REBβ was observed [17]. The REBβ transcript predominated over REBα, and the levels of both the species exhibited a general decrease with continuing embryonic development. With the exception of the lung, where REBα is predominantly overexpressed, most other tissues express similar levels of REBα and REBβ transcripts [17]. To investigate the presence of REBβ in Sertoli cells we examined the expression of REBβ transcripts in Sertoli cells and whole testis by a PCR-based protocol that distinguishes between the two *REB* gene transcripts. The total RNA from cultured Sertoli cells treated with FSH and from whole testis were reverse-transcribed using oligo dT primer. To specifically amplify the REBβ transcript, a 5′ primer in the 72-bp insertion of REBβ was used in the PCR reaction. With this 5′-REBβ-specific primer, a 655-bp band corresponding to the predicted length between the two primers was observed in the whole testis (lane 5) and lung (lane 6), but not in Sertoli cells (Fig. 1C, lanes 1–4). The REBβ transcript in Sertoli cells was also not observed in a PCR reaction carried out for 45 cycles (data not shown). Subsequent subcloning and sequencing of the 655-bp band confirmed its identity as REBβ. The differential expression of REBα and not REBβ in Sertoli cells is a unique observation. To our knowledge, no other system has been reported in which one splice variant of the *REB* gene is expressed exclusively.

A Northern blot analysis was performed to investigate the potential hormonal regulation of *REB*α gene expression. A [^{32}P]dCTP-labeled random-primed probe was used from a 640-bp REBα-specific PCR product from Sertoli cells as described above. As shown in Figure 2A, the predicted size of the RNA species migrating at approximately 4.2 kilobases (kb) corresponding to an *REB* transcript was detected. The abundance of this message was similar in untreated and FSH-treated Sertoli cells (Fig. 2B). Cyclophilin expression was used as a constitutively expressed gene to normalize for the integrity and amount of RNA (Fig. 2B). Although hormones did not appear to influence REBα expression, higher levels of expression were observed in the cultured Sertoli cells in comparison to freshly isolated cells (Fig. 2).

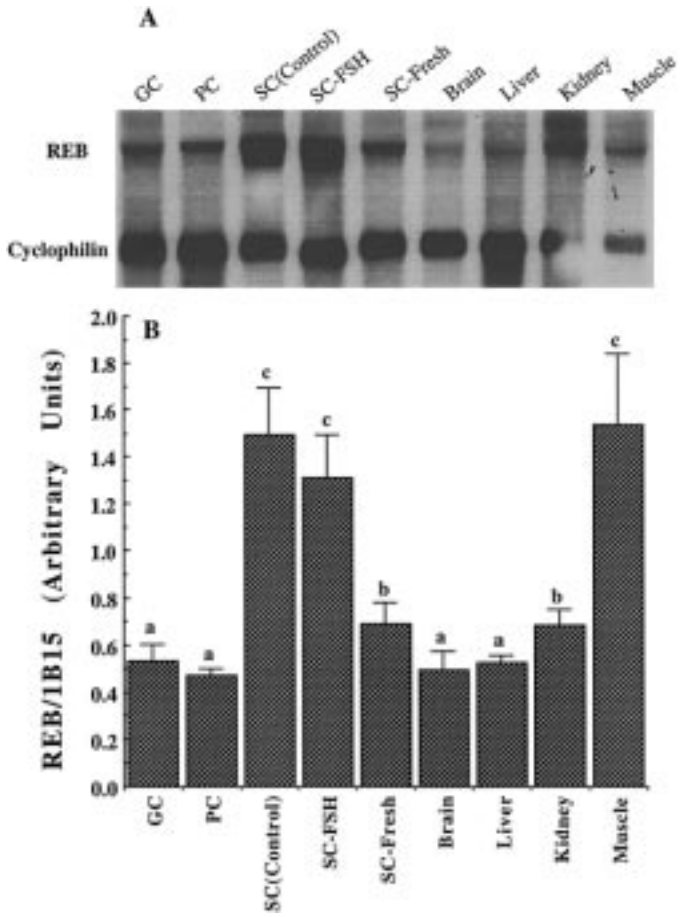

FIG. 2. **A**) Northern blot analysis of REB. The predicted transcript size of approximately 4.2 kb was detected when random-primed 640-bp REBα probe was used. Also at the bottom is the blot for the constitutively expressed cyclophilin gene. (GC, Germ cells; PC, peritubular cells; SC, cultured untreated Sertoli cells or Sertoli cells treated with FSH or testicular paracrine factor PModS [S300]). The data are representative of three different Northern blots performed on separate RNA samples collected at different times. **B**) Scanning densitometry of the blots was used to quantitate the bands, with mean ± SEM presented for three different experiments. Data were normalized for cyclophilin expression, and different letters indicate a statistically significant difference ($p < 0.001$).

The RT-PCR data presented in Figure 1 were obtained from RNA collected from Sertoli cells and whole testis from 20-day-old rats. This represents a stage when Sertoli cells are functionally differentiated and have formed a blood-testis barrier. The transcripts of the *REB* gene were also detected in the whole testis by RT-PCR of RNA obtained from embryonic Day 18 and postnatal Day 0 and 10 testis (Fig. 3). Similar results were obtained for REBβ (data not shown). An RT-PCR for cyclophilin demonstrated a PCR product in all the RNA samples (data not shown).

Immunohistochemistry was performed to complement the PCR and Northern blot data obtained and to show that the REB protein is also expressed in Sertoli cells. As shown

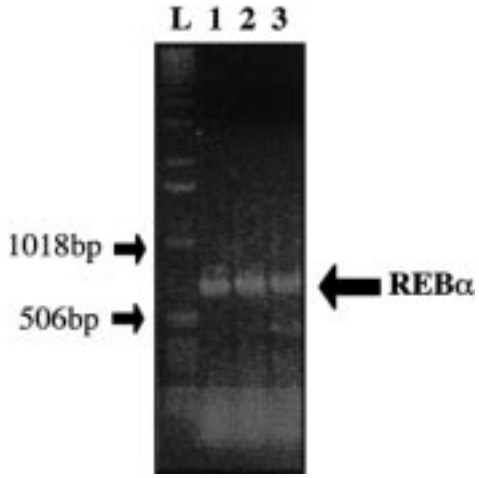

FIG. 3. Expression of REBα during various stages of testicular development (whole testis from embryonic Day 18 [lane 1]; postnatal Day 0 [lane 2], and postnatal Day 10 [lane 3]) as detected by PCR using 5′ primer specific to REBα. The data are representative of three separate PCRs carried out on at least three different RT-mRNA samples.

in Figure 4A, REB was localized in the nucleus of the Sertoli cells, suggesting that the REB protein is expressed by Sertoli cells. The HEB antibody used in the present study cross-reacts with its rat homologue REB. This antibody does not distinguishes between REBα and REBβ proteins. Since the REBβ transcript was not expressed in Sertoli cells (Fig. 3), it is concluded that the antibody detected REBα protein in Sertoli cells. REB staining was also observed in the interstitial and peritubular cells surrounding the seminiferous tubules (Fig. 4A).

The presence of REBα in Sertoli cells suggests that it can potentially bind to E-box sequences present in the promoters of a number of Sertoli cell genes. To test this hypothesis, both gel-shift and gel-shift immunoblot experiments were performed. Nuclear extracts from cultured Sertoli cells treated with FSH were incubated with the proximal transferrin promoter E-box sequence and resolved in a native polyacrylamide gel. The presence of a retarded band (Fig. 5, gel shift) suggested that E-box binding proteins are present in the Sertoli cells. To determine the identity of these E-box binding proteins, the binding reaction with unlabeled E-box was electrophoresed on a native polyacrylamide gel and blotted onto a nitrocellulose membrane. The membrane was subsequently probed with HEB antibody. A similar gel-shift blot was also probed with E12/E47 antibody. The presence of HEB immunoreactive band similar in migration to the gel shift retarded band suggested that REBα is part of the complex binding to the E-box oligonucleotide (Fig. 5, blot). Interestingly, a band similar in migration to REBα was also observed when E12/E47 antibody was used to probe the blotted E-box gel shift. A control Oct1 oligonucleotide gel shift had no immunoreactive band (Fig. 5). A control with nonimmune serum also had no immunoreactive band.

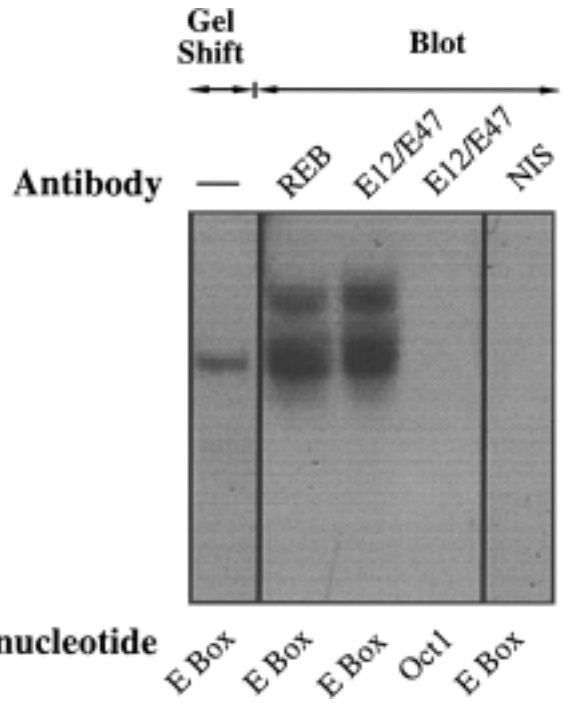

FIG. 5. Gel-shift immunoblot using antibodies to REB and E12/E47. The gel shift used an E-box oligonucleotide (CAGCTG) with flanking sequences and nuclear extracts from cultured Sertoli cells treated with FSH (gel shift, far left). This gel shift was transferred to nitrocellulose membrane and blotted with antibody to either REB or E12/E47. A band similar in migration to the gel-shift band was detected using both the antibodies. Controls with an Oct1 oligonucleotide gel shift and nonimmune antiserum were performed. The gel-shift and blot data are representative of three different experiments.

DISCUSSION

The bHLH class of transcriptional regulators play a role in the control of cell differentiation and development [24]. Members of the bHLH family have been demonstrated to be important in controlling both cell proliferation and differentiation. This class of transcription factors influences processes such as hematopoiesis, sex determination, myogenesis, and neurogenesis [24]. Most of these differentiated functions are regulated by cell-specific bHLH proteins. Recent studies have suggested that bHLH proteins regulate Sertoli cell differentiated functions such as transferrin [9]

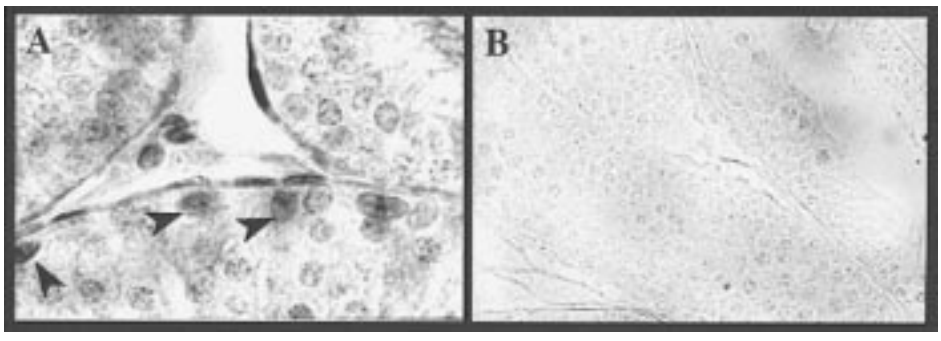

FIG. 4. **A**) Immunocytochemical localization of *REB*. REB protein is localized in the nucleus of Sertoli cells (arrow). REB expression is also observed in the interstitium in the Leydig cells and in the peritubular cells surrounding the seminiferous tubules. **B**) Control using nonimmune serum. The data are representative of immunocytochemistry performed in triplicate on three different testis samples from 20-day-old rats.

and c-*fos* gene expression (unpublished results). Identification of functional E-box sequences in the promoters of a number of Sertoli cell genes suggests the potential importance of bHLH proteins in these cells [9]. The current study shows that a class A bHLH transcriptional activator REBα is also present in Sertoli cells.

The presence of REBα and not REBβ in Sertoli cells is a novel observation and suggests a cell-specific splicing event. The alternatively spliced 24-amino acid insert specific to the REBβ transcript is predicted to interact with the bHLH domain. The effect of such an interaction may mask the HLH domain from both homo- and heterodimeric interactions with other bHLH domains [17]. The leucine zipper domain common to both REBα and REBβ is also present in other bHLH proteins like AP-4 and USF. This domain is required for efficient homodimerization. Unlike these bHLH-ZIP molecules, REBα does not require its leucine repeat sequence for efficient dimerization and DNA binding [17]. REBα mutants lacking the leucine repeat can efficiently bind E-box sequences both as homodimers and heterodimers with native REBα and E47 [17]. This observation helps explain the gel-shift data of Figure 5. The gel-shift data presented in this figure suggest the potential of REBα forming heterodimers with E47. Alternatively, REBα may form homodimers similar to E47 homodimers and colocalize on the gel shift. Currently being investigated is the potential that yet-unknown Sertoli cell-specific bHLH protein may dimerize with either REBα and/or E47.

The tissue-specific alternative splicing of the *REB* gene observed in different tissues [17] suggests a potential distinct functional importance of the two gene products. The absence of REBβ suggests that the REBβ actions are not required or are detrimental to Sertoli cells. Given the apparent similarity of their heterodimerization capabilities and the presence of at least two class A bHLH proteins in Sertoli cells, REBα and E47, these two bHLH proteins may have a functional redundancy in the cells. This concept is supported by recent *E2A* and *REB* gene knockout studies. The *E2A* deficiency is lethal postnatally, and the surviving F2 progeny are severely growth retarded [25]. In the absence of *E2A* activity, the earliest committed B-cell precursors are undetectable in the bone marrow. This phenotype is consistent with role of the *E2A* gene in B-cell development. The females were significantly underrepresented in comparison to males, and the growth defect was more pronounced in the females than in the males. The phenotype of *REB* knockout [26] is less pronounced than that of the *E2A* knockout. Although the deficiency is lethal postnatally, the F2 progeny of *REB* (−/−) were born with close-to-expected frequency, but they died within the first 1 or 2 wk after birth. Although a detailed study of the *E2A* and *REB* knockout with respect to reproductive organs and fertility was not reported, it is clear that male sex determination was normal. During rat embryonic development, male sex determination is associated with the differentiation of Sertoli cells and their ability to secrete Müllerian inhibiting substance [2, 3]. It is possible that the expression of Sertoli cell E47 and REBα shows a high degree of functional compensation. This idea is supported by the recent report that human HEB cDNA under the control of the endogenous *E2A* promoter, when substituted for the mouse *E2A* gene, can functionally replace *E2A* in supporting B-cell commitment and differentiation and can rescue postnatal lethality associated with *E2A* disruption [27].

Expression of the bHLH E2A transcription factors has been shown to cause growth arrest in NIH 3T3 cells [28]. This function of the *E2A* gene products is consistent with its role in cell differentiation. Based on structural homology and functional compensation, it is speculated that REBα may also cause growth arrest followed by cellular differentiation. The postpubertal Sertoli cells represent a terminally differentiated nondividing cell population [1]. To ensure that these cells do not undergo dedifferentiation and enter the cell cycle, they may express high levels of REBα and E47. Previous observations have shown that overexpression of the dominant negative HLH protein Id in Sertoli cells is able to down-regulate the activation of transferrin [9] and c-*fos* promoters (unpublished results). Both these proteins are associated with differentiation of Sertoli cells. The hypothesis is being examined that bHLH proteins such as REBα have a role in regulating Sertoli cell differentiation.

Given the widespread expression pattern and sequence homology to other class A bHLH proteins, such as the *E2A* gene products E12/E47, REBα is likely to serve a similar function [17]. Analysis of in vivo dimerization between cell-specific class B bHLH proteins and ubiquitously expressed class A bHLH proteins has indicated the necessity for heterodimeric interactions [11]. The presence of REBα suggests that Sertoli cells may also express a cell-specific bHLH protein. The Sertoli cell-specific bHLH protein remains to be elucidated.

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