Identification of a Novel Gene Product, Sertoli Cell Gene with a Zinc Finger Domain, That Is Important for FSH Activation of Testicular Sertoli Cells

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Sertoli cells provide the cytoarchitectural support and microenvironment necessary for the process of spermatogenesis. A novel, ubiquitously expressed cDNA clone was isolated from Sertoli cells and termed Sertoli cell gene with a zinc finger domain (SERZ). A significant homology of SERZ was found with a mouse genomic sequence that suggested the presence of at least 10 exons. An open reading frame at the 5′-end of the cDNA, termed SERZ-α, had a cryptic basic helix-loop-helix (bHLH) domain, but no start codon. When a start codon was engineered into the 5′-end of the cDNA, an in vitro translation product of SERZ-α was obtained. The longest second open reading frame with an ATG start site at 304 bp from the 5′-end coded for a 308-amino acid SERZ-β polypeptide. Motif analysis and BLAST search of SERZ-β showed significant homology to the DHHC domain of conserved zinc finger proteins. A number of potential phosphorylation sites were observed in the SERZ-β polypeptide sequence. The long 5′-untranslated region of SERZ-β prompted an investigation of both potential alternate polypeptide products, SERZ-α and SERZ-β. Both SERZ-α and SERZ-β proteins were detected with specific antibodies to SERZ-β and the 5′-end open reading frame SERZ-α in a Western blot analysis of total Sertoli cell proteins. The presence of the SERZ-β polypeptide was also confirmed by in vitro translation of the cDNA, but SERZ-α was not translated in vitro in the absence of an engineered start codon. The expression pattern of SERZ mRNA was observed in all tissues examined. The transcript size of SERZ as determined by Northern blot analysis is approximately 2.7 kb. An antisense oligonucleotide to SERZ was found not to influence basal levels of transferrin promoter activation, but significantly blocked FSH-induced transferrin promoter activation. SERZ mRNA expression was not regulated by FSH treatment of Sertoli cell cultures. In summary, a novel gene product, SERZ, was identified that appears to have a role in maintaining Sertoli cell differentiated functions and mediating FSH actions. Translation of SERZ may give rise to two gene products; however, the SERZ-β containing the zinc finger domain is probably the principal product of the SERZ gene. (Endocrinology 143: 426–435, 2002)

S ERTOLI CELLS ARE the testicular epithelial cells that form the seminiferous tubule and provide the cytoarchitectural support and microenvironment for developing germ cells (1, 2). This microenvironment is created through the expression of many different gene products. Sertoli cell gene products include growth factors, metabolic enzymes, transport proteins such as transferrin and androgen-binding protein, proteases, antiproteases, energy metabolites, and structural components (3). The gonadotropin FSH is an important endocrine hormone required for the regulation of Sertoli cell function (4, 5). FSH has been shown to regulate the expression of most Sertoli cell genes, including the FSH receptor, androgen-binding protein, transferrin, plasminogen activator, and aromatase (6–9). The signaling mechanism by which FSH acts on Sertoli cells involves the cAMP PKA pathway (8, 10). Promoter analysis of most Sertoli cell genes indicated the absence of a consensus cAMP response element site (11). Interestingly, the response element that was found conserved was an E box response element that is bound by the transcription factors of the bHLH family (11). bHLH transcription factors are considered to be master regulatory genes that can initiate and maintain cellular differentiation

(12, 13). Recent observations involving the overexpression of a negatively acting bHLH protein Id and mutations/deletions in the E box in specific promoters have suggested that bHLH proteins are required to maintain Sertoli cell differentiated functions (11, 14–18).

To identify bHLH transcription factors in Sertoli cells, RT-PCR was carried out using degenerate oligonucleotides designed toward the highly conserved basic and helix II domain of bHLH proteins (19). All of the PCR products obtained were of the ubiquitously expressed bHLH proteins E47 (19) and REB (20, 21). Screening of a rat Sertoli cell cDNA library with REB bHLH domain PCR product resulted in the identification of full-length REB gene and an additional clone with no homology to REB (22). Sequence analysis of this clone, termed Sertoli cell gene with a zinc finger domain (SERZ), demonstrated no sequence homology to any known cDNA sequence in GenBank.

The current study reports the nucleotide sequence of SERZ, its predicted amino acid sequences, and possible motifs. Two potential products of the SERZ transcript were investigated, SERZ-α with a potential bHLH domain and SERZ-β with a conserved zinc finger motif. The expression profile based on Northern blot analysis and RT-PCR suggests that SERZ is expressed by all tissues examined. A transiently transfected reporter construct driven by the transferrin promoter was inhibited by the presence of an antisense to SERZ,

suggesting SERZ may be important in regulating Sertoli cell function and hormone responsiveness.

Materials and Methods

Isolation of Sertoli cells

Sertoli cells were isolated from the testis of 20-d-old rats as previously described (23, 24). The isolated Sertoli cells were plated under serum-free conditions in 150 × 20-mm tissue culture dishes (Nunclon) at a concentration 10^8 cells/plate. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Life Technologies, Inc., Gaithersburg, MD) with 0.01% BSA at 32 C. Sertoli cells were treated with either FSH (100 ng/ml; o-FSH-16, National Pituitary Agency) or vehicle alone (Ham's F-12, control). The cells were cultured under serum-free conditions for a maximum of 5 d with a medium change and treatment after 48 h of culture. Cell number and viability did not change during the culture in the absence or presence of treatment (25, 26).

RNA preparation

Freshly isolated or cultured Sertoli cells were lysed directly using Tri-Reagent (Sigma, St. Louis, MO). The cell lysate was then passed several times through a Pasteur pipette to form a homogenous lysate. The freshly dissected tissue (skeletal muscle, brain, and detunicated testis) was homogenized in Tri-Reagent (5%, wt/vol) with a tissue homogenizer (Tissue Tearor, BioSpec Products, Inc., Bartlesville, OK). To avoid any possible contamination of extracellular material and high mol wt 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.

PCR

Total RNA (2 μg) was reverse transcribed in a final volume of 20 μl containing 20 U RNasin (Promega Corp., Madison, WI); 200 μM each of dATP, dCTP, dTTP, and dGTP; 1 μg oligo(deoxythymidine) (Pharmacia Biotech, Piscataway, NJ), 10 μM dithiothreitol, and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies, Inc.) in the MMLV reverse transcriptase first strand synthesis buffer supplied by the manufacturer (Life Technologies, Inc.). The RNA and oligo(deoxythymidine) primer in the buffer were first denatured for 5 min at 65 C, 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 kit (Perkin-Elmer Corp./ Cetus, Emeryville, CA) with 30 cycles as follows: 94 C for 1 min (denaturation), 50 C for 2 min (primer annealing), and 72 C for 1 min (primer extension). Each PCR contained 250 pg reverse transcribed DNA, 1 μM of each 5'- and 3'-oligonucleotide primer, 2.5 U Taq polymerase (AmpliTaq, Perkin-Elmer Corp.), and 200 μM of each dATP, dCTP, dGTP, and dTTP. The 5'-primer was derived from the amino acid sequence RR(K/M/I)(AN)(AN)(TN)(M/L/A)RER(RL) of the basic domain, whereas the 3'-primer was derived from the degenerate amino acid sequence (L/V)(L/E)(IT)L(HQ)(QN)(AT)(VL)(QN)(YC)(VA) of the Helix II domain. The sequences used for the primers were based on the comparison of at least 20 human, rat, and mouse bHLH proteins.

Cloning and sequencing

The PCR fragments were run on 1.5% agarose gels, stained with ethidium bromide, and visualized. The bands were then dissected out, and the DNA was isolated from the gel using the Glass MAX DNA isolation system (Life Technologies, Inc.). The purified DNA fragments were subcloned into pBluescript (Stratagene, La Jolla, CA) plasmid. The cloned DNA fragments were sequenced using standard M13 forward and reverse primers in an automated fluorescence-based sequencer (PE Applied Biosystems, Foster City, CA). The sequences were then analyzed against the known sequences in GenBank using a FASTA and BLATSn search.

Sertoli cell cDNA library

The rat Sertoli cell cDNA library was constructed in HybriZapII by Stratagene from poly(A)$^+$ RNA isolated from purified Sertoli cells of 20-d-old rats. The HybriZap library is primarily designed for use in the yeast two-hybrid screening, but also allows for nucleic acid screening. The standard procedures for nucleic acid screening were followed as mentioned previously (27). The REB probe was digested from the pBluescript plasmid using EcoRI, dephosphorylated, and end labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase (Life Technologies, Inc.). Individual plaques (1.5 × 10^6) were screened with the 170-bp REBα PCR fragment. The positive clones were excised from the HybriZap library to obtain pAD plasmid-containing clones. The pAD plasmids were analyzed by restriction mapping and sequencing. The sequences were subjected to a FASTA and BLASTn search against known sequences in GenBank.

The primer pair sequences used to investigate the presence of SERZ in various tissues by RT-PCR were determined empirically and synthesized from commercial sources. Primer pairs were designed to amplify an approximately 523-bp region of SERZ (1490–2013 bp). The possible contamination of RNA with DNA was determined by performing an RT reaction 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: SERZ 5'-primer, 5' (1490–1510)-AGC ACA GCA GCT GCT GGG GTC; and SERZ 3'-primer, 5' (2013–1993)-AGT GCT GGG TCT CAG CAC AGG.

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 PCRs were also carried out using primers designed to rat cyclophilin to monitor the efficiency of the PCR (28). Cyclophilin was faithfully amplified in all PCRs, indicating consistency in the quality of RT and PCRs. The data presented are representative of three different RT-PCRs carried out in duplicate.

Northern analysis

Total RNA from Sertoli cells or tissues were isolated as described above using Tri-Reagent (Sigma). Approximately 10 μg total RNA were fractionated on a 1% formaldehyde-agarose gel. After fractionation, the RNA was transferred onto Nylon membrane (Hybond N$^+$, Amersham Pharmacia Biotech, Arlington Heights, IL) in 10× SSC buffer and UV cross-linked as described previously (27). The membranes were then prehybridized in Quick Hybridization buffer (Stratagene) for 30 min at 60 C. The hybridization was carried out at 60 C for 1 h with ^{32}P-labeled SERZ probe. The membrane was subsequently stripped and rehybridized with rat cyclophilin. All probes were labeled using the Prime-It II kit from Stratagene.

Antibodies to SERZ-α and SERZ-β

Peptides (15 amino acids) were synthesized (Center for Reproductive Biology, Molecular Biology Core Laboratory, Washington State University, Pullman, WA) toward the N-terminal (SERZ-α) and C-terminal (SERZ-β) of the predicted SERZ protein sequences (Fig. 1). Antibodies to SERZ-α and -β were generated in the rabbits by Strategic Bio-Solutions (Ramona, CA). To generate an immune response SERZ-α was coupled to BSA, and SERZ-β was coupled to keyhole limpet hemocyanin. Antibody titers were determined with ELISAs using the peptide antigens (Strategic Bio-Solutions). Antibodies were aliquoted and stored at −20 C until use.

Western blotting

Sertoli cells were cultured in 150-mm plates and treated with FSH as described above. After 72 h of treatment the cells were washed twice with HBSS and lysed with 1 ml M-PER lysis buffer (Pierce Chemical Co., Rockford, IL) supplemented with mini-protean protease inhibitor cocktail (Roche, Indianapolis, IN) at 4C for 30 min. Lysates were centrifuged at 10,000 × g for 30 min at 4 C, and supernatants were collected. The protein concentration in the supernatants was estimated using a Bradford assay (Bio-Rad Laboratories, Inc., Richmond, CA). Approximately 50–150 μg protein in SDS sample loading buffer were boiled for 5 min

SERZα
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   1 CAGCTCCGC GTCGGGCTC CCGCTTCTG CTGGTCCGC TGCCTCCCG CACACCTGC CGGGCCGGA CCGAGCCGA ACGGAACTG AGCGGATCC
     Q   L   R   V   G   L   P   L   L   L   V   R   C   L   P   H   T   C   R   A   G   P   S   R   T   E   L   S   G   S

  91 GGGTCCACC CGGCCGGGC CATGAGCGC TGCCGCATG ATGCGTCGC CGCCCGCGC CGCTGCAGC CGCCGGCCG CGAGGAGCC TCAGCACTG
     G   S   T   R   P   G   H   E   R   C   R   M   M   R   R   R   P   R   R   C   S   R   R   P   R   G   A   S   A   L

 181 GGCGACCCC GCCCGGGCC TCGGGCCAC ATCACACAG GACAGCATG CGGCTTACG GTCCTGAAA AAAAGGCAC AAGTGCTCC CATGATGCT
     G   D   P   A   R   A   S   G   H   I   T   Q   D   S   M   R   L   T   V   L   K   K   R   H   K   C   S   H   D   A
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SERZβ
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 271 TAGAAGCAA CTTGTAAGG CTGTGCCAG GGAATCATG CAGCCGTCA GGACACAGG CTCCGGGAC ATTGAGCAC CATCCTCTC CTGACTGAC
     *                                       M   Q   P   S   G   H   R   L   R   D   I   E   H   H   P   L   L   T   D

 361 AATGACAAT TATGACTCT GCGTCCTCC TCGTCCTCC GAGGCTGAC ATGGCAGAC AGGGTGTGG TTCATCCGA GATGGCTGT GGCATGGTC
     N   D   N   Y   D   S   A   S   S   S   S   S   E   A   D   M   A   D   R   V   W   F   I   R   D   G   C   G   M   V

 451 TGTGCTGTC ATGACATGG CTTCTGGTC GTCTATGCA GACTTTGTG GTGACCTTC GTCATGCTG CTGCCTTCC AAAGACTTC TGGTACTCC
     C   A   V   M   T   W   L   L   V   V   Y   A   D   F   V   V   T   F   V   M   L   L   P   S   K   D   F   W   Y   S

 541 GTGGTGAAC GGGGTCCTC TTCAACTGC TTGGCGGTG CTCGCGCTG TCCTCCCAC CTAAGAACC ATGCTCACT GACCCGGGG CTGTCCCC
     V   V   N   G   V   L   F   N   C   L   A   V   L   A   L   S   S   H   L   R   T   M   L   T   D   P   G   A   V   P

 631 AAAGGCAAC GCCACGAAG GAGTACATG GAGAGCTTG CAGCTGAAG CCGGGCGAG GTGATCTAC AAGTGCCCC AAGTGTTGC TGCATCAAG
     K   G   N   A   T   K   E   Y   M   E   S   L   Q   L   K   P   G   E   V   I   Y   K   C   P   K   C   C   C   I   K

 721 CCGGAGCGT GCCCACCAC TGCAGTATC TGCAAGAGG TGCATTCGA AAGATGGAC CACCACTGC CCGTGGGTG AACAACTGT GTGGGAGAG
     P   E   R   A   H   H   C   S   I   C   K   R   C   I   R   K   M   D   H   H   C   P   W   V   N   N   C   V   G   E

 811 AAGAACCAG CGCTTCTTC GTGCTCTTC ACCATGTAC ATAGCTCTG TCTTCAATC CATGCTCTG ATTCTCTGC GGGCTTCAG TTCATCTCT
     K   N   Q   R   F   F   V   L   F   T   M   Y   I   A   L   S   S   I   H   A   L   I   L   C   G   L   Q   F   I   S

 901 TGCGTCCGA GGGCAGTGG ACAGAGTGC AGTGACTTC TCTCCTCCC ATAACTGTA ATCCTGTTG GTCTTCCTG TGCCTTGAG GGCCTCCTG
     C   V   R   G   Q   W   T   E   C   S   D   F   S   P   P   I   T   V   I   L   L   V   F   L   C   L   E   G   L   L

 991 TTCTTCACT TTCACCGCA GTCATGTTC GGCACCCAG ATCCACTCA ATATGCAAT GACGAAACT GAGATCGAG AGGCTGAAG AGTGAGAAG
     F   F   T   F   T   A   V   M   F   G   T   Q   I   H   S   I   C   N   D   E   T   E   I   E   R   L   K   S   E   K

1081 CCTACGTGG GAGCGAAGG CTGCGGTGG GAAGGGATG AAGTCTGTC TTCGGGGGC CCTCCCTCC CTCCTCTGG ATGAACCCC TTTGTGGGC
     P   T   W   E   R   R   L   R   W   E   G   M   K   S   V   F   G   G   P   P   S   L   L   W   M   N   P   F   V   G

1171 TTCCGATTC AGGCGGCTG CAGATGAGG ACCAGGAAG GGAGGCCCC GAGTTCTCT GTATGAGCC TTCAGGGCA GGATGTCTG CAGACATCA
     F   R   F   R   R   L   Q   M   R   T   R   K   G   G   P   E   F   S   V   *
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1261 TACTCAGTG GCCGCAAGC ACAGCAGCC TTGTGGGCA GATGGAGCT GGCCAGCAG ACGGGGCCA TGAGCACTT CATGGACCT AGGGCCACA
1351 GGCACTTGT CATTTCTGT GTGTACTGC CAGGAGAAG CAGTCCCGG GAGGCCATC CTGACCATC CTGACCCAG GATGTCTCG TGGGCTGCG
1441 AGCACCCAC AGCACAGGT GCCGTTGCT TCTTGGTCA TGAAGGAAG CCAGCACAG CAGCTGCTG GGGTCCTGC TGGCCCATC CCCAGCCCA
1531 CTTCTTACT GGAAGGGGT TTCCACAGA GACTCTGGC TTGGAGGGC TCTGACACC TAGCACTGA ACAGTCAAA GCGGCTTCC AGAACACTC
1621 CATGCTGCC CCAGAACGG GCATCCTTT GACCCTTCC AGCAAGTCT GTGTGTGCT CATCTGTGT GTCTGTGTG GTGTGTGTG CGTGAGCAT
1711 GTGTCAGAA TCAGCAGTG TTGTTCAAG CACGCGTGT GGTAGACAA GCTGTCTTT CAGACTCCA GTGTTTCAG GGAAGTGGG ACGCACTGC
1801 TGGCCCTTG CTGGCCTCC TATGAATGT ATCCTCCAG TGTCCAGAT CCAAAGAAC AGGCATGCC TGCGCATGG GCCCGGCCT CCTGCTTTT
1891 ATTTCACTG AAAAACTTAG TGTTTGAGG GCATGGAGC TGAGGTTTT GTTGTTCCA GCCAAACTA GCCACTGCA ACATTCTTC TCAACAAGG
1981 AAAGTGGTA GCCCTGTGC TGAGACCCA GCACTGTGG GCGACTGTGG TGTGGGGAG CTCAGCATG AGAAGCCGC CTTGGGCCC CTTTGGAGA
2071 GGTGCTGCA GGCTGAGAC AGCCCGGTG CCTGAGCTA TCAAGAGTG GGGACATGT GACTATTTC TTTACCCAT GGCACTTCA GTCAGTGGA
2161 CCCTTGGTG TGGGCTTAG TATTATGTC AGCACACAC AGCTCCCCT CAGGGGACT TGCCTTAAA CTCGTAGGT TGTCCTTTG CTGCTGGGT
2251 GAAAGCAGT CTGACAGAA GCTGGGGAC TTCCGGAGAG TGTTCTCCC ATGCAGACA CCAGTGTTT CCCTTCTGG GACCAGCTG CCTTTCTGT
2341 CCCCGTGAC ACCATGACT GTTCTTTCG GTGTCAGTC GCTTGTGCC CTGCCCTTG TCTGGGCTC CTATGCTAC TGAGGTGAA CCCGGGGGA
2431 CCTGAGCCC TCTCATGCA GTGAGCAAA CTCTGGGGC CTGAGTCCC TGCAGAGGG ACATCGTGG CTTCAGATT GTCTCTGCG TTCCTTTGC
2521 ACATGTGTC TGGGCATGT AGGCTCTGG CAGTCTCAC CCTCCAGTG TGGAGTCCG TGGCGGTGA ATCCAAGCT GAGTATACT TGACTTTTC
2611 AATTCCTCA GTGTGATTT TTTATGAAA TAAAGTAAC CCGTCCACA GTTCAAAAA AAAAAAAAA AAACTCGAG CTCGTGCC
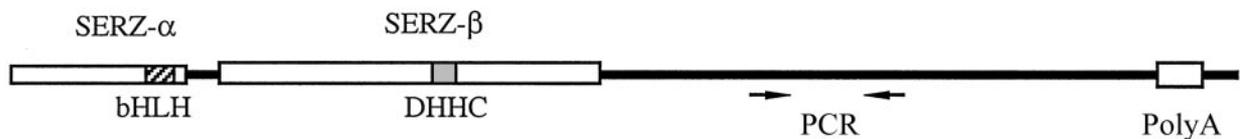```

SERZ-α      SERZ-β

bHLH      DHHC      PCR      PolyA

FIG. 1. Nucleic acid and predicted amino acid sequence of SERZ isolated from a Sertoli cell cDNA library (HybriZap). Products of both SERZ-α and SERZ-β open reading frame are indicated. The *asterisk* denotes the termination codon for the corresponding reading frame. The *gray box* at the 5′-end represents the sequence used to generate antisense oligonucleotides. The *underlined bold* sequence box near the 5′- and 3′-ends shows the potential ATG start site and a consensus poly(A) site, respectively. The sequence is a consensus of a minimum of six separate sequence analysis (accession no. AY040615). The *underlined* and *bold* amino acid sequences represent the corresponding peptides used to generate SERZ-α and -β antibodies. At the *bottom* is a schematic of the SERZ mRNA and translation products.

and electrophoresed on a 5–20% SDS gradient gels (Bio-Rad Laboratories, Inc.). The protein was subsequently transferred onto nitrocellulose membrane and probed with specific rabbit polyclonal antibodies to SERZ-$\alpha$ or SERZ-$\beta$. The specific antigen-antibody complex was visualized using an alkaline phosphatase chemiluminescent detection kit (Bio-Rad Laboratories, Inc.).

### In vitro translations

To perform *in vitro* translation, the SERZ gene was subcloned into pBluescript plasmid downstream of the T7 promoter. The plasmid was linearized downstream of the SERZ gene, and *in vitro* translations were performed using Promega Corp.'s TNT coupled system in the presence of [$^{35}$S]methionine (NEN Life Science Products, Boston, MA). The reactions were run on SDS gels and fluorographed. An aliquot of the reaction was also blotted with SERZ$\alpha$ and -$\beta$ antibodies after gel fractionation.

### Transfections

The chloramphenicol acetyltransferase (CAT) reporter plasmid (pUC8-CAT) containing −581 bp (−581 bp mTf-CAT) of the proximal mouse transferrin promoter was provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA) (29). The mouse transferrin promoter used in the present study included the transcriptional initiation site of the transferrin gene, which is 54 bp upstream of the translation start site. Sertoli cells cultured in 24-well cell culture plates for 48 h were transfected with the reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as previously described (30, 31). In each experiment the transfection efficiency was monitored by transfecting the Sertoli cells by the plasmid containing $\beta$-galactosidase gene driven by a cytomegalovirus promoter. Subsequent staining and counting the cells expressing $\beta$-galactosidase (blue color) resulted in 25% transfection efficiency. The antisense to SERZ was synthesized using phosphorthiote modification. The antisense oligonucleotide to SERZ (4 $\mu$M) was added to the Sertoli cells 4 h after transfection. The cells were subsequently treated with FSH. The antisense oligonucleotide was added to the culture wells every 12 h for a total period of 48 h, after which the cells were harvested for the CAT assay as described previously (31). The average conversion of CAT substrate for treated cells ranged between 20–30%. This assay was linear with the protein concentration used.

### Sequence motif analysis

GCG DNA analysis software was used to generate the restriction map and translation to the corresponding protein sequence. FASTA and BLASTn (NCBI BLAST) against GenBank, Entrez, and SRS links were used to generate a homology table of SERZ. The MOTIF and PATTERNS analysis package available on the worldwide web was used to identify motifs in the SERZ sequence. Phosphorylation patterns in SERZ were determined by PROSITE analysis.

### Results

To identify transcription factors of the bHLH family in Sertoli cells, an RT-PCR of the RNA obtained from cultured Sertoli cells was performed using degenerate oligonucleotide primers designed toward the highly conserved basic and helix II domain of bHLH proteins. The approximately 170-bp PCR fragment spanning the bHLH domain was cloned into a pBluescript plasmid. Ten different clones were isolated, sequenced, and analyzed. All of the clones matched 100% the ubiquitously expressed bHLH transcription factors REB or E47. Based on the observation that the bHLH domain is highly conserved, a rat Sertoli cell cDNA library was screened with the 170-bp bHLH domain PCR product of REB (20). The screening of the library resulted in the identification of a full-length clone of the REB-$\alpha$ gene, a spliced variant of the REB gene (20), and an additional clone, termed SERZ. SERZ showed no homology to the REB nucleotide sequence at the nucleotide level. The nucleotide sequence of SERZ revealed an insert of 2690 bp with a consensus poly(A)* site (AAUAAA) present toward the 3′-end of the sequence, followed by a poly(A) region (Fig. 1). To predict the corresponding protein structure, SERZ nucleotide sequence was translated into all three forward reading frames. A potential ATG start site corresponding to the longest open reading frame of 308 amino acids (924 bp) was observed at 304 bp (Fig. 1). The stop codon at 1228 bp corresponding to this reading frame resulted in an unusually long 1462 bp of the 3′-untranslated region (Fig. 1). The product of this open reading frame was referred to as SERZ-$\beta$. A computer search using BLASTn and tBLASTX for the matching sequences in the GenBank including updates and EST databases against SERZ as a query sequence revealed more than 95% similarity with the mouse genomic fragment (GenBank accession no. AF059580). Alignment of the SERZ sequence with AF059580 using ClustalW suggested the presence of at least 10 exons (Fig. 2). This alignment also revealed the presence of unusually large introns of 8090 and 4129 bp separating potential exons 1 and 2 (corresponding to SERZ, 202–288 bp) and 3 (SERZ, 9–203 bp), respectively. The BLASTn search also identified SERZ sequence similarity with cDNA sequences from human (NM_017740 and NM_016598), mouse (BF100004), and rat (AW914001) (Table 1). A strong sequence homology was also observed with the largest open reading frames of the corresponding human, mouse, and rat sequences aligned with the predicted SERZ-$\beta$ sequence (Fig. 3). The alignment shown in Fig. 3 also suggests the presence of spliced variants of SERZ. To determine the possible function/identity of SERZ-$\beta$, a detailed motif analysis was performed. The motif that was found to be highly con-
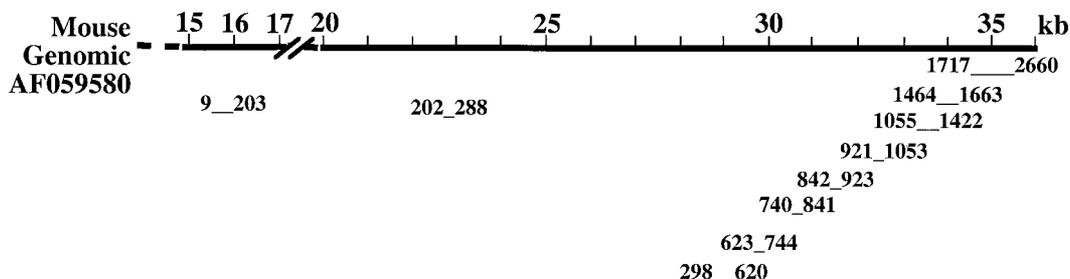


FIG. 2. Schematic showing the alignment of the potential SERZ exons with the mouse genomic clone AF059850. The numbers on *top* indicate the location in kilobases. The starting and ending base pair numbers for all exons are presented.

**TABLE 1.** Homology of SERZ with available sequences in the GenBank

| Species | Accession no. (sequence length, bp) | Homology [sequence length, bp (%)] | Comments |
|---|---|---|---|
| Mouse (genomic) | AF059580 (36326) | 93 | Multiple introns (see Fig. 2) |
| Human cDNA | NM_017740 (3172) | 87 (1062) | Hypothetical protein FLJ20279, other similar GenBank entries: AK001654, AK000286, AU132797, AW752704, AW388078 |
| Human cDNA | NM_016598 (2720) | 82 (252) | Human DHCC1 protein; other human DHCC1 GenBank entry: AF247703 |
| Rat cDNA | AW914001 (560) | 99 (560) | Normalized EST (EST294779); other similar GenBank entries: AW142744, AW535430, AW142599BF281748, AW525333, AW520536, BF281756, AA944811, AI180401, BF290300, AI453965 |
| Mouse cDNA | BF100004 (878) | 91 (623) | EST; other similar GenBank entries: AA260796, AW610648, AL024087, AA266371, AI506174, AA543107, AI561852 |

The table lists the percent homology within a specified number of base pairs (in *parentheses*) of the GenBank sequences when compared with 2.7-kb od SERZ. Additional sequences with known homology and predicted motifs are listed in the *Comments* column.

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SERZ-β(r)       MQPSGHRLRDIEHHPLLTDNDNYDSASSSSSEADMADRVWFIRDGCGMVCAVMTWLLVVY 60
NM_01774(h)     MQPSGHRLRDVEHHPLLAENDDYDSSSSSSSEADVADRVWFIRDGCGMICAVMTWLLVAY 60
BF_100004(m)    -----------------------------------------------ASRVLEFEPVCF 12
                                                             . *:  :   * :


SERZ-β(r)       ADFVVTFVMLLPSKDFWYSVVNGVLFNCLAVLALSSHLRTMLTDPGAVPKGNATKEYMES 120
NM_01774(h)     ADFVVTFVMLLPSKDFWYSVVNGVIFNCLAVLALSSHLRTMLTDPGAVPKGNATKEYMES 120
BF_100004(m)    S--------------------------ASFSPNLNSICVHRGAVPKGNATKEYMES 42
                :                          ::*.:*.::  .. **************


SERZ-β(r)       LQLKPGEVIYKCPKCCCIKPERAHHCSICKRCIRKMDHHCPWVNNCVGEKNQRFFVLFTM 180
NM_01774(h)     LQLKPGEVIYKCPKCCCIKPERAHHCSICKRCIRKMDHHCPWVNNCVGEKNQRFFVLFTM 180
BF_100004(m)    LQLKPGEVIYKCPKCCCIKPERAHHCSICKRCIRKMDHHCPWVNNCVGEKNQRFFVLFTM 102
                ************************************************************


SERZ-β(r)       YIALSSIHALILCGLQFISCVRGQWTECSDFSPPITVILLVFLCLEGLLFFTFTAVMFGT 240
NM_01774(h)     YIALSSVHALILCGFQFISCVRGQWTECSDFSPPITVILLIFLCLEGLLFFTFTAVMFGT 240
BF_100004(m)    YIALSSVHALILCGLQFISCVRGQWTECSDFSPPITVILLVFLCLEGLLFFTFTAVMFGT 162
                ******:*******:****************************:****************


SERZ-β(r)       QIHSICNDETEIERLKSEKPTWERRLRWEGMKSVFGGP-PSLLWMNPFVGFRFRRLQMRT 299
NM_01774(h)     QIHSICNDETEIERLKSEKPTWERRLRWEGMKSVFGGP-PSLLWMNPFVGFRFRRLPTRP 299
BF_100004(m)    QIHSICNDETEIERLKSEKPTWERRAAVGRNEVCLRGASPSLLWMNPFVGSRLRRLQMRD 222
                *********************     :  :  *. **********  *:***   *


SERZ-β(r)       RK------------------------GGPEFSV 308
NM_01774(h)     RK------------------------GGPEFSV 308
BF_100004(m)    QERKARVLCLKPSGWECLRPSLARGWVGRSLLCDGGPLDGP 263
                ::                             ***    .
```

Fɪɢ. 3. Alignment of the largest open reading frame of SERZ-β with the largest open reading frames of mouse (m), human (h), and rat (r) sequences showing sequence homology. The representative sequences shown in Table 1 were translated and aligned using ClustalW. The *asterisk* denotes complete homology; a *colon* or *period* denotes homology within the same class of amino acids.

served in SERZ-β was the DHHC1-type domain (C..C..C. . . . . . .C), (32) (Fig. 4). The homology to the DHHC domain was also observed in the comparative BLASTn search (human cDNA accession no. NM_016598 and NM_017740, human hypothetical protein FLJ20279) with 87% homology in 1062 bp. A number of potential phosphorylation sites, such as PKC, casin kinase 2 (CK_2), and PKA, were also observed (Fig. 4). Two potential myristoylation sites, one each at N and C terminals, were also recognized. The presence of myristoylation sites suggests that SERZ-β may be membrane bound, as reported in the case of p60^{src} (33). In addition to phosphorylation and myristoylation sites, a site for ASN glycosylation was observed (34) (Fig. 4). Therefore, SERZ-β may be a glycosylated and membrane-associated zinc finger protein that is a potential target for phosphorylation by kinases.

```
MQPSGHRLRDIEHHPLLTDNDNYDSASSSSSEADMADRVWFIRDGCGMVCAVMTWLLVVY 60
                 C            C                GMVCAVM
                                                   E
ADFVVTFVMLLPSKDFWYSVVNGVLFNCLAVLALSSHLRTMLTDPGAVPKGNATKEYMES 120
                     VLFNC                         NATKE
                       E                              B
LQLKPGEVIYKCPKCCCIKPERAHHCSICKRCIRKMDHHCPWVNNCVGEKNQRFFVLFTM 180
  PGEVIYK             C..C..C.......C
    D
YIALSSIHALILCGLQFISCVRGQWTECSDFSPPITVILLVFLCLEGLLFFTFTAVMFGT 240

QIHSICNDETEIERLKSEKPTWERRLRWEGMKSVFGGPPSLLWMNPFVGFRFRRLQMRTR 300
         TEIE               GGPPSL                    R
            C    F A                E                      A
KGGPEFSV 310
G
```

FIG. 4. Sequence analysis of SERZ-β with the zinc finger motif (DHHC1 domain) shown in *bold*. Potential phosphorylation and myristoylation sites in the predicted SERZ-β polypeptide are marked. The sites indicated are as follows: A, PKC; B, ASN-glycosylation; C, CK-2; D, Tyr phosphorylation; E, myristoylation; and F, PKA.

A bHLH domain signature was not observed in the SERZ-β open reading frame motif search.

Analysis of the SERZ sequence by physically aligning the potential SERZ amino acid sequences with the conserved bHLH domain sequence indicating the presence of a potential bHLH domain. This potential bHLH domain was upstream of the ATG start site at 304 bp of SERZ-β. The start site for the SERZ-β polypeptide was observed at about 307 bp downstream of the 5′-end of the nucleotide sequence. A single stop codon at 271 bp was observed between the 5′-end and the start of the SERZ-β polypeptide. The polypeptide sequence starting at the 5′-end and coded by 271 bp of the 5′-end of the SERZ nucleotide sequence was termed SERZ-α. As shown in Fig. 1, SERZ-α had a sequence with some homology to a bHLH domain.

The absence of a start codon sequence for the SERZ-α sequence shown in Fig. 1 suggests that the SERZ sequence may be incomplete at the 5′-end. The presence of a consensus ATG translational initiation codon 305 bp downstream of the 5′-end of the sequence suggests that either SERZ-β has a long 5′-untranslated region or a possible sequencing error or there is an additional sequence at the 5′-end. The sequence error possibility prompted a repeat of the sequencing using a different set of internal primers every 200 bp in both the forward and reverse directions to confirm the absence of any sequencing error. Using 20 different primers in forward and reverse directions, no sequence error was observed that would either change the reading frame or extend the length of the longest open reading frame. Rapid amplification of 5-′-cDNA ends (5′-RACE) on Sertoli cell RNA was performed with primers designed 300 bp downstream of the 5′-end. No additional sequence using 5′-RACE was obtained (data not shown). Observations suggest that either the reported SERZ sequence is complete, or there is a GC-rich region that was not being transcribed in the 5′-RACE reaction. Similar results were obtained when the 5′-RACE was performed using two different kits from different vendors [*i.e.* Ambion, Inc. (Austin, TX) and Invitrogen]. No additional sequences were obtained at the 5′-end. To further explore the possibility that additional sequences may be present at the 5′-end, the mouse genomic sequence AF059580 was explored for the presence of additional upstream exons. PCR 5′-primers were designed in the two predicted exons, and PCR was performed with the 3′-primers used in the RACE reaction on reverse transcribed Sertoli cell mRNA. No specific PCR prod-
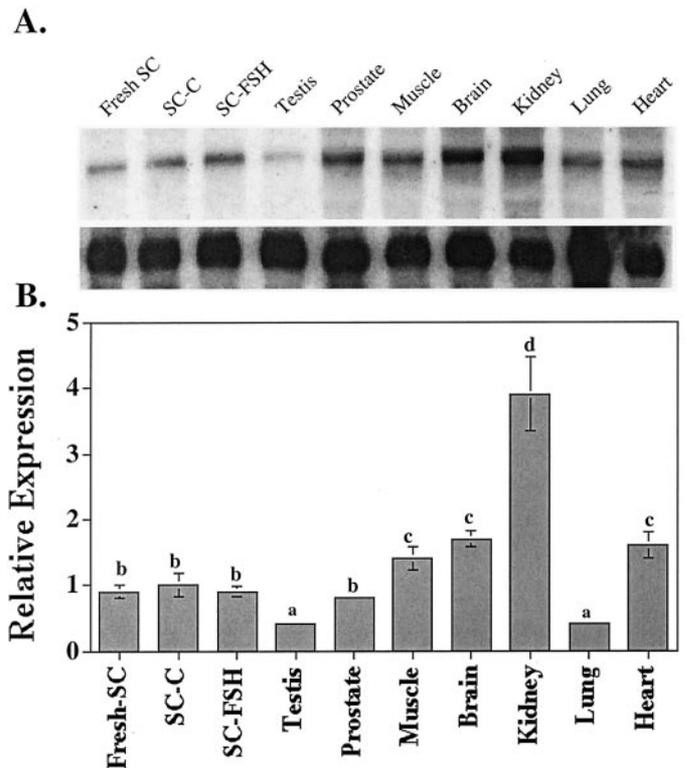

FIG. 5. A, Expression of SERZ (*upper panel*) and cyclophilin (*lower panel*) mRNA by Northern blot analysis in freshly isolated Sertoli cells (SC), cultured untreated Sertoli cells (SC-C), cultured FSH treated Sertoli cells (SC-FSH), whole testis, prostate, muscle, brain, kidney, lung, and heart. B, Quantitation of the SERZ expression by densitometric scan of the Northern blot. The expression of SERZ was normalized to the constitutively expressed gene cyclophilin. A representative of three different experiments is shown for A, and combined data for B with the mean ± SEM are presented. *Different letter superscripts* on the *bars* are statistically different by ANOVA ($P < 0.01$).

ucts were observed. Taken together, these results suggest that either the SERZ sequence reported in this study is complete or the presence of strong secondary structure/high CG content is detrimental to the PCRs used, including RACE.

Northern blot analysis of the whole testis RNA with SERZ as a probe suggested that the SERZ transcript size is approximately 2.7 kb (Fig. 5). This 2.7-kb SERZ transcript was expressed in the testis as well as all other tissues examined,

such as prostate, muscle, brain, kidney, lung, and heart. The Northern blots for expression of SERZ in Sertoli cells cultured with or without FSH were subjected to a densitometric scan to determine whether SERZ expression is regulated by FSH. Quantitation of the Northern blot densitometric scans suggested the expression of SERZ by Sertoli cells is not regulated by FSH (Fig. 5). To further analyze the expression of SERZ, an RT-PCR with SERZ-specific primers using RNA obtained from the testis and various other tissues was performed. As shown in Fig. 6, SERZ expression (520-bp band) was observed in Sertoli cells and all other tissues examined (Fig. 6). This observation compliments the Northern blot data.

Western blotting was performed to verify whether the predicted SERZ-β protein is expressed by Sertoli cells. A 15-amino acid peptide (SERZ-β peptide) within the predicted SERZ-β protein sequence (amino acid CNDETEIERCKSEKP; Fig. 1) was synthesized and used as an antigen to raise antibodies in rabbits. Purified rabbit anti-SERZ-β IgG (protein A affinity column, Pharmacia Biotech) was used to perform Western blots on electrophoretically fractionated total Sertoli cell proteins. As shown in Fig. 7A, a 36-kDa band was observed when total proteins from Sertoli cells were run on an SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and blotted with SERZ-β antibody. To confirm the specificity of the antigen-antibody complex on the Western blot, the SERZ-β antibody was preabsorbed with the SERZ-β peptide for 16–18 h at 4 C and used to probe the Sertoli cell proteins. As shown in Fig. 7A, the absence of a band in the Western blot confirmed that the 36-kDa band observed in Fig. 7A was specific. This also suggests that the predicted SERZ-β protein is expressed in Sertoli cells. To further confirm that the predicted SERZ-β protein is translated an *in vitro* translation assay was performed. A SERZ-β full-length sequence plasmid in pBluescript was translated *in*
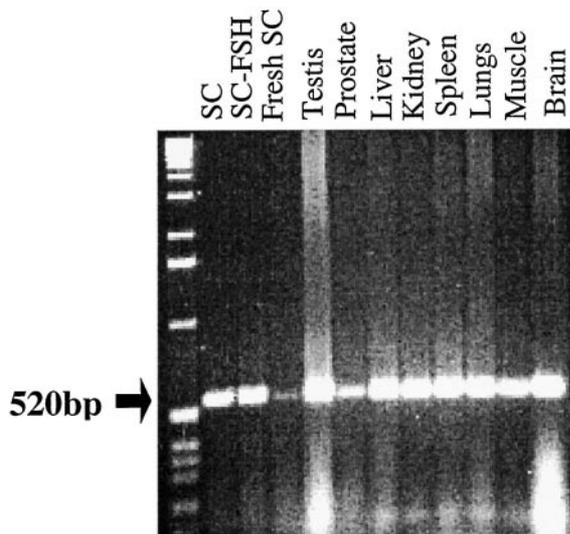
FIG. 6. Expression of SERZ as determined by RT-PCR analysis in cultured untreated Sertoli cells (SC), freshly isolated Sertoli cells (Fresh SC), cultured FSH-treated Sertoli cells (SC-FSH), testis of postnatal 20-d-old testis, and various tissues as indicated. The data shown are representative of three different experiments. The SERZ 520-bp PCR product is indicated.
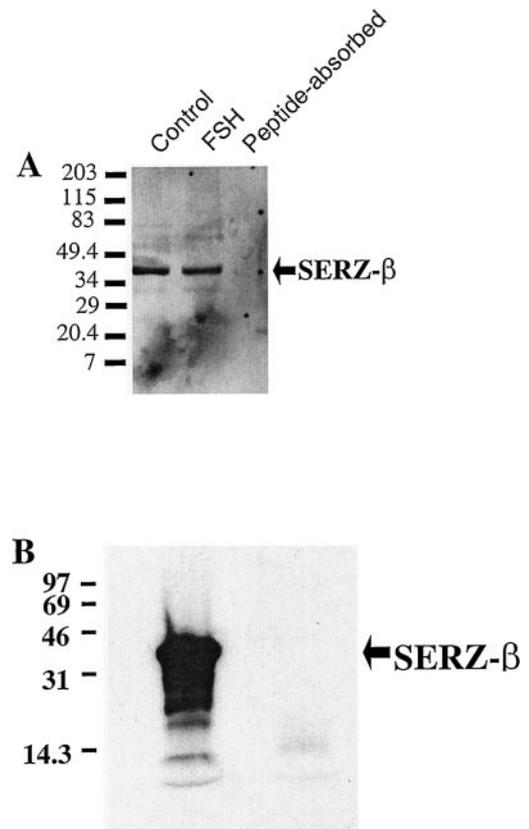

FIG. 7. A, Western blot analysis of total Sertoli cell proteins using rabbit polyclonal antibodies to SERZ-β. Sertoli cell extracts from control and FSH-treated cells were analyzed along with a SERZ-β peptide-preabsorbed antibody control. A representative of three different experiments is shown. B, *In vitro* translation of SERZ. [^{35}S]Methionine-labeled products obtained from an *in vitro* translation reaction using SERZ plasmid as template, then immunoprecipitated with SERZ-β antibody, run on a denaturing gel, and fluorographed. The *left* margin markers are in kilodaltons. A representative of three different experiments is shown.

vitro using [^{35}S]methionine. The translated products were subjected to denaturing gel electrophoresis and fluorographed. A single band at approximately 40 kDa was observed (Fig. 7B). The 40-kDa molecular mass of the SERZ-β protein was observed in both the *in vitro* translation reaction and the Western blot of total Sertoli cell proteins. This correlates well with the SERZ-β calculated molecular mass of 34 kDa.

The observations that SERZ-α may be an alternate or second product of SERZ prompted an investigation of the possibility that the SERZ mRNA may code for an additional protein SERZ-α. A 15-amino acid long peptide fragment within the SERZ-α polypeptide (amino acid, CLPHTCRAGPSRTEL; Fig. 1) was synthesized and used as an antigen to raise antibodies in rabbits. The purified rabbit anti-SERZ-α IgG was used to probe total Sertoli cell proteins. As shown in Fig. 8, a protein migrating at approximately 25 kDa was observed. The blot had a high background, possibly because the SERZ-α peptide used to generate the antibody was coupled to BSA. Since the 25-kDa protein band was consistently observed, the SERZ-α antibody was preabsorbed with BSA. Results demonstrate that the SERZ-α antibody recognized a

Sertoli cell protein at 25 kDa. Analysis of the SERZ *in vitro* translation product with the SERZ-α antibody showed no detectable SERZ-α protein (data not shown). Therefore, the SERZ-α protein was detected in Sertoli cell extracts, but was not translated by the full-length SERZ clone *in vitro*.

An antisense oligonucleotide approach was used to determine the functional significance of SERZ. Cultured Sertoli cells from 20-d-old rats were transfected with a CAT reporter construct driven by the proximal 600 bp of the mouse transferrin promoter. A significant increase in the CAT activity was observed when the Sertoli cells were treated with FSH (Fig. 9). In the presence of antisense oligonucleotide to SERZ, FSH-stimulated CAT activity was significantly decreased (Fig. 9). Similar results were obtained when Sertoli cells were treated with the cAMP analog (Bu)$_2$cAMP (cAMP; Fig. 9). No decrease in the CAT activity was observed in the presence of scrambled SERZ oligonucleotide, suggesting the effect of SERZ antisense oligonucleotide on FSH or (Bu)$_2$cAMP-stimulated CAT activity was specific. The presence of the antisense oligonucleotide had no significant effect on the basal CAT activity in Sertoli cells. The inhibitory effect of SERZ
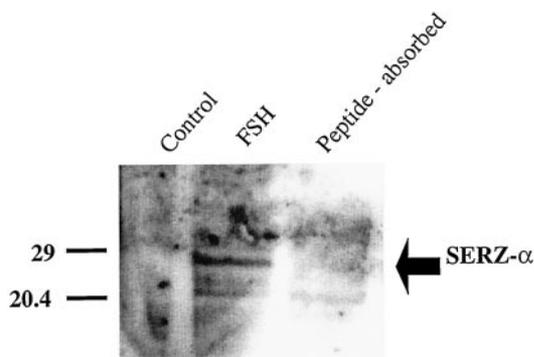
FIG. 8. Western blot analysis of total Sertoli cell proteins using rabbit polyclonal antibodies to SERZ-α. An approximately 25-kDa peptide was detected in the protein extract from Sertoli cells stimulated with FSH. No band was detected in the lane in which SERZ-α antibody was preabsorbed with the corresponding peptide or in the control lane with BSA alone.
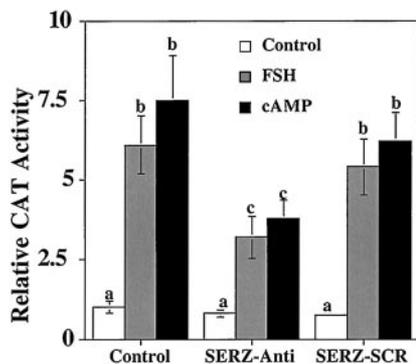
FIG. 9. Regulation of the 600-bp Tf promoter-CAT construct in Sertoli cells by antisense oligonucleotides to SERZ (SERZ-Anti) and scrambled SERZ (SERZ-SCR). Cells were untreated (control) or treated with FSH and (Bu)$_2$cAMP. The data are expressed as relative CAT expression with respect to the activity of the untreated control set at 1 and presented as the mean ± SEM from three different experiments performed in duplicate. *Different letter superscripts* indicate a statistical difference by ANOVA ($P < 0.001$).

antisense oligonucleotide on the transferrin promoter activation was further confirmed by treating the Sertoli cells transfected with the transferrin promoter with an oligonucleotide complementary (*i.e.* sense) to the SERZ antisense oligonucleotide. The presence of the complementary SERZ sense oligonucleotide had no significant effect on either FSH- or (Bu)$_2$cAMP-stimulated activation of the transferrin promoter (data not shown). Therefore, the presence of SERZ appears to be necessary for FSH- and (Bu)$_2$cAMP-induced Sertoli cell function (*i.e.* transferrin promoter activation).

Discussion

The present study involves the identification of a new gene termed SERZ. SERZ was identified by screening a rat Sertoli cell cDNA phage library with a 170-bp sequence of the bHLH transcription factor REB α within the highly conserved bHLH domain (20, 22). The absence of any similarity between the SERZ sequence and the REB α gene sequence suggested that the identification of SERZ was possibly due to a bHLH motif sequence interaction in the SERZ transcript.

The nucleotide sequence of SERZ revealed an insert of 2698 bp that corresponds to the estimated transcript size of approximately 2.7 kb from Northern blot analysis. The presence of a consensus poly(A) site followed by a poly(A) region at the 3′-end of the SERZ sequence was identified. The longest open reading frame of 924 bp coding for a 308-amino acid protein was observed in the first reading frame, followed by 550 bp of 3′-untranslated region. The ATG start site for this open reading frame is at 304 bp. This open reading frame and its product were termed SERZ-β. 5′ to this SERZ-β open reading frame is an alternate open reading frame that starts at the SERZ 5′-end and has a stop codon at 271 bp. No start site was found for this open reading frame that is termed SERZ-α. 5′-RACE analysis demonstrated the SERZ sequence is complete. These two products of the SERZ transcript, SERZ-α and SERZ-β, are further characterized in the current study.

Sequence analysis of the two potential SERZ products revealed interesting amino acid motifs. SERZ-α has a potential bHLH domain that clarifies why the SERZ transcript was isolated from the cloning strategy and suggests that this protein product may be a bHLH transcription factor (35). The SERZ-β sequence has a consensus zinc finger motif (32) as well as phosphorylation, glycosylation (33), and myristolation (34) sites. These SERZ products appear to have the potential to influence signaling pathways and transcriptional events.

The expression pattern of SERZ as observed by Northern blot analysis suggests that SERZ is ubiquitously expressed with maximum expression being in the kidney. The Northern blot data and its quantitation in Sertoli cells suggest SERZ expression is not regulated by the gonadotropin FSH that is known to regulate many Sertoli cell functions (4, 5). Therefore, SERZ mRNA is widely expressed and may be important for a variety of cell types and tissues.

An antisense oligonucleotide approach was used to investigate the functional significance of SERZ in Sertoli cells. It was previously shown that the activity of the mouse and human transferrin promoter is regulated by FSH in Sertoli

cells (8, 11, 36). A significant reduction in the FSH-induced activation of the transferrin promoter was observed with the SERZ antisense oligonucleotide. Observations suggest that SERZ has a role in maintaining Sertoli cell differentiated function and influences FSH activation of Sertoli cell function.

Based on Western blotting data, it is speculated that the SERZ gene may generate two translation products, SERZ-α and SERZ-β. The identification of two translational products from a single gene is a relatively novel observation and suggests a possible mechanism by which genetic information can be conserved to generate diverse functional proteins. Similar mechanisms have been previously reported in viral systems (37–39) and more recently in mammalian systems (40, 41). The data from *in vitro* studies partially support this observation. A distinct SERZ-β antibody-reactive product was identified when SERZ was translated *in vitro*, but no SERZ-α product was observed. However, addition of an ATG start codon site at the 5′-end of the SERZ sequence resulted in the identification of a SERZ-α antibody-reactive product in the *in vitro* translation product (data not shown). These observations support the potential that SERZ may code for at least two different proteins. Whether SERZ-α is a functional bHLH protein remains to be determined. The presence of the highly conserved Cys[4] zinc finger-like DHHC domain in the SERZ-β sequence suggests that it may have an important function in the cell, possibly related to protein-protein or protein-DNA interactions. The down-regulation of the transferrin promoter by the SERZ antisense oligonucleotide further confirms the significance of these proteins in Sertoli cells. The SERZ antisense oligonucleotide inhibited FSH activation of the transferrin promoter, but did not affect basal activity. FSH is critical for maintenance of optimal Sertoli cell differentiation and function. FSH also regulates a wide variety of Sertoli cell functions. SERZ appears to have a role in the regulation of Sertoli cell function in mediating in part FSH actions.

Sequence analysis and comparisons identified a partial restriction digest Sau3A fragment of the mouse genome C (GenBank accession no. AF059580) with a strong homology to SERZ. The alignment of the SERZ sequence with the Sau3A mouse genomic fragment spanned approximately 15 kb and included 10 exons. A detailed comparison of the sequence demonstrated strong conservation of SERZ-β in mouse, rat, and human. However, no conservation in SERZ-α and the bHLH domain was found between rat SERZ-α and mouse SERZ. This suggests that SERZ-β is probably the principal product of the SERZ gene, and this zinc finger-containing protein may influence the functions of a wide variety of cells.

In summary, we report the isolation and sequence of a novel gene, SERZ, with a principal product with strong homology to the zinc finger-like DHHC domain that may be involved in maintaining Sertoli cell function and FSH actions. Our observation that the rat SERZ may produce two translation products is intriguing and requires further investigation. The potential that a single gene transcript may generate multiple protein translation products through alternate open reading frames needs to be considered as an efficient utilization of genomic sequences. This phenomenon has been found in viral and bacterial transcripts. This provides a useful process for energy conservation and evolutionary strategy to link gene functions. The impact this would have in our analysis and understanding of the current genomic database suggests that serious consideration of multiple open reading frame use on individual transcripts should be further investigated.

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