Regulation of Steroidogenic Acute Regulatory Protein Transcription in Largemouth Bass by Orphan Nuclear Receptor Signaling Pathways

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The steroidogenic acute regulatory (StAR) protein mediates the rate-limiting step of mitochondrial transport of cholesterol for steroid biosynthesis. To investigate the regulation of this protein in lower vertebrates, we cloned the *StAR* coding region from large-mouth bass for analysis. Induction of the mRNA corresponded with increasing levels of plasma sex steroids *in vivo.* Cultures of largemouth bass ovarian follicles were exposed to dibutyryl cAMP (dbcAMP), a potent signaling molecule for steroidogenesis. *StAR* mRNA expression was significantly up-regulated by dbcAMP signaling, suggesting that the 5′ regulatory region of the gene is functionally conserved. To further analyze its transcriptional regulation, a 2.9-kb portion of the promoter was cloned and transfected into Y-1 cells, a steroidogenic mouse adrenocortical cell line. The promoter activity was induced in a dose-responsive manner upon stimulation with dbcAMP; however, deletion of 1 kb from the 5′ end of the promoter segment significantly diminished the transcriptional activation. A putative retinoic acid-related receptor-α/rev-erbα element was identified between the -1.86- and -2.9-kb region and mutated to assess its potential role in dbcAMP regulation of the promoter. Mutation of the rev-erbα element significantly impeded dbcAMP-induced activation. Chromatin immunoprecipitation and EMSA results revealed rev-erbα and retinoic acid-related receptor-α enrichment at the site under basal and dbcAMP-induced conditions, respectively. These results implicate important roles for these proteins previously uncharacterized for the *StAR* promoter. Altogether these data suggest novel regulatory mechanisms for dbcAMP up-regulation of *StAR* transcription in the distal part of the largemouth bass promoter. **(*Endocrinology* 151: 341–349, 2010)**

Identification of the steroidogenic acute regulatory (StAR) protein in 1994 significantly advanced the field of cholesterol metabolism (1). It has now been well characterized in multiple mammalian species that StAR transports cholesterol across the mitochondrial membrane and controls the rate-limiting step for steroidogenesis (2–6). Regulation of steroidogenesis occurs in a tissue-specific manner and involves multiple signaling pathways, including protein kinase A (PKA) and protein kinase C (PKC), among others, and this appears to be conserved across most vertebrate species (2, 7–10). It is known that ACTH, an upstream regulator of cAMP production, induces *StAR* mRNA expression in rainbow trout and eel (11–13). It has also been shown that exogenous exposure of Atlantic Croaker ovarian follicles to human chorionic gonadotropin (hCG; a potent LH receptor agonist) robustly stimulates gonadal *StAR* mRNA expression (14). Because *StAR* gene expression is highly responsive to cAMP and hCG in both higher and lower vertebrates, mammals and lower vertebrates such as fish are likely to

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Abbreviations: AP, Activator protein; ChIP, chromatin immunoprecipitation; dbcAMP, dibutyryl cAMP; E_2, 17β-estradiol; hCG, human chorionic gonadotropin; LMB, largemouth bass; PKA, protein kinase A; PKC, protein kinase C; Q-PCR, quantitative real-time RT-PCR; RACE, rapid amplification of cDNA ends; SF, steroidogenic factor; ROR, retinoic acid-related receptor; RORE, ROR element; StAR, steroidogenic acute regulatory; UTR, untranslated region.

exhibit similar transcriptional regulatory mechanisms for the *StAR* gene.

It is well established that the *StAR* gene is highly regulated by the cAMP/PKA pathway across multiple species and that this pathway is important in reproduction. Binding elements for transcription factors known to mediate cAMP responses, such as steroidogenic factor (SF)-1, activator protein (AP)-1, cAMP response element-binding protein (CREB), and others, have been extensively characterized in mammalian *StAR* gene promoters (15–24); however, there are no previous publications citing *in silico* or functional promoter analysis of the *StAR* gene in any fish model. In all vertebrates studied, the promoter for *StAR* is very complex with many transcriptional elements for which the functions are still unknown (25). The transcriptional mechanisms controlling the *StAR* gene in lower vertebrate animals such as fish have not been investigated and could provide much needed insight into the complex networks involved in the regulation of steroidogenesis.

Circadian rhythm plays an important role in reproduction in vertebrates, and it exists centrally, in peripheral tissues, and even within individual cells (26). Control of gene expression at the cellular level is important in regulating reproductive processes, including steroid hormone production. It is known that retinoic acid-related receptor (ROR)-α and rev-erbα are two signaling proteins that play integral roles in controlling genes central to the circadian cascade (27–29). RORα and rev-erbα both bind to similar core sequences [ROR element (RORE)]; however, they induce opposing effects on the transcription of target genes (30).

The goals of this study were to clone and characterize the largemouth bass (LMB) *StAR* gene and promoter at the tissue and cellular levels, respectively, and characterize a functional regulatory role for a RORE in LMB *StAR* transcription. Our results show that RORα and rev-erbα are both capable of binding a core sequence in the LMB *StAR* promoter and, interestingly, that rev-erbα also binds to a core sequence in the mouse *StAR* promoter. Altogether our study presents a novel mechanism through which *StAR* promoter activity is controlled.

Materials and Methods

Cloning of LMB *StAR* cDNA coding region

LMB *StAR* was PCR amplified from LMB ovarian cDNA using primers designed with the web program CODEHOP (http://blocks.fhcrc.org/codehop.html) and are listed in supplemental Table S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The cDNA was amplified using 10 pmol of each primer and 1 U Amplitaq (PerkinElmer, Waltham, MA) according to the manufacturer's protocol. The PCRs used a primer annealing temperature of 60.9 C for 45 cycles (PerkinElmer 9600 thermocycler). The remaining cDNA sequence for LMB *StAR* was obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE protocol (CLONTECH, Mountain View, CA). Primers used for both 5′ and 3′ RACE are listed in listed in supplemental Table S1. The nucleotide sequence for the LMB *StAR* coding sequence has been deposited in the GenBank database under GenBank accession no. DQ166820.

In vivo LMB study

Adult LMB were purchased from American Sport Fish Hatchery (Montgomery, AL) and housed in fresh water ponds at the U.S. Geological Survey (Gainesville, FL) under ambient conditions. Females were collected every 2 wk by electroshock from October 1999 to April 2000. Fish were killed and the ovaries were stored in RNA*later* solution (Ambion, Austin, TX) at −20 C until RNA isolation.

Quantitative real-time RT-PCR (Q-PCR)

LMB *StAR* mRNA levels were quantified using Q-PCR. In all experiments, total RNA was extracted using RNA STAT-60 reagent according to the manufacturer's protocol (TEL-TEST, Friendswood, TX). Three μg of each total RNA sample was reverse transcribed into cDNA using 25 U of Stratascript reverse transcriptase according to the manufacturer's protocol (Stratagene, La Jolla, CA). All Q-PCRs used 2× SYBR Green iQ Supermix (Applied Biosystems, Foster City, CA) and 10 pmol of forward and reverse primers (supplemental Table S1) to amplify 0.12 μg of cDNA using thermocycler parameters as recommended by Applied Biosystems. All Q-PCRs were normalized to 18S rRNA (Applied Biosystems). A standard curve for real-time PCR quantitation was developed using dilutions of a plasmid incorporated with a 300-bp fragment of the LMB *StAR* cDNA. *StAR* mRNA was quantified by extrapolation to the standard curve.

Ovarian follicle cultures

Adult LMB between 2 and 3 yr old were maintained at the Aquatic Toxicology Facility at the University of Florida. Ten ovarian follicles between 0.68 and 0.76 mm in diameter were cultured in 1 ml of DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 1.2 g of sodium bicarbonate and 1% antibiotic/antimycotic solution. Cultures were equilibrated in a chilled incubator at 21–22 C with 5% CO_2 for 24 h before exposure to dibutyryl cAMP (dbcAMP) for 14 h. After exposure, tissues were snap frozen in liquid nitrogen and stored at −80 C until RNA was extracted using the STAT-60 protocol mentioned above (TEL-TEST).

Cloning of the LMB *StAR* promoter

Genomic DNA was isolated from LMB ovarian tissue using the Wizard genomic DNA isolation kit (Promega, Madison, WI). The LMB *StAR* promoter was cloned using the GenomeWalker kit (CLONTECH) according to the manufacturer's protocol. All primer sequences for promoter cloning are listed in supplemental Table S1. PCR products were initially cloned into the $TOPO_2$.1 vector (named pSP1) and then into the pGL3 basic plasmid (Promega) for transfection experiments. The promoter for the LMB *StAR* gene has been deposited in the GenBank database under GenBank accession no. DQ166819.

Transcriptional start site identification and *in silico* promoter analysis

The transcriptional start site for the LMB *StAR* promoter was identified by aligning the promoter sequence with the end of the 5′ untranslated region (UTR) obtained with RACE. Sequence upstream of the transcription start site was then analyzed with two different web search engines, MatInspector version 2.2 (http://searchlauncher.bcm.tmc.edu/gene-finder/examples/mat.html), and Genomatix MatInspector (http://www.genomatix.de/online_help/help_matinspector_help.html) to identify putative consensus binding sites.

Generation of deletion and site-directed mutagenesis promoter constructs

The 2.9-kb LMB *StAR* promoter vector was digested with *Eco*RV and *Bst*EII to eliminate 1 kb of the distal sequence, producing a 1.86-kb promoter construct extending to the transcriptional start site. Additionally, the putative binding site for the ROR/−1969 in the 2.9-kb promoter was mutated using the QuikChange XL site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene). The primer sequences for the mutagenesis constructs are listed in supplemental Table S1.

Culturing of Y-1 and MA-10 cells

Y-1 mouse adrenocortical cells purchased from American Type Culture Collection (Manassas, VA) were cultured in Ham's F12K culture medium containing 2 mM L-glutamine, supplemented with 1.5 g/liter sodium bicarbonate, 15% horse serum, 2.5% fetal bovine serum, and 1% penicillin-streptomycin mix. The MA-10 mouse Leydig tumor cell line was generously provided by Dr. Mario Ascoli (Department of Biochemistry, Vanderbilt University, Nashville, TN). MA-10 cells were cultured in RPMI 1640 culture medium supplemented with 15% horse serum, 20 mM HEPES (pH 7.2), and 50 μg/ml gentamicin (pH 7.7). All cells were grown at 37 C in a humidified 5% CO_2 cell culture incubator.

Transient transfection assays

In transfections with Y-1 cells, 150,000 cells/well were plated in 24-well culture plates and allowed to grow for 24 h. Transfection reactions consisted of a 6:1 ratio of FuGENE 6 (Roche Diagnostics, Indianapolis, IN) to plasmid DNA (1.2 μl FuGENE 6 per 0.2 μg total DNA) suspended in 20 μl media with no serum or antibiotics. Twenty-four hours after transfection, Y-1 cells were exposed to dbcAMP for 20 h. In transfections done in MA-10 cells, 100,000 cells/well were plated in 24-well culture plates coated with 0.1% gelatin (dissolved in 1× PBS) and allowed to grow for 24 h. Transfection reactions consisted of a 4:1 ratio of FuGENE HD (Roche Diagnostics) to plasmid DNA (2 μl FuGENE HD/ 0.5 μg total DNA) suspended in 25 μl media with no serum or antibiotics. Thirty hours after transfection, MA-10 cells were exposed to 0.1% vehicle or 10 U/ml hCG in growth medium for 6 h. All transfections were normalized to Renilla luciferase. All Firefly and Renilla luciferase reactions were quantified using reagents from the dual luciferase kit (Promega).

Oligonucleotide annealing reactions

Sense and antisense oligonucleotides designed against the RORE (supplemental Table S1) were based on bioinformatic analysis of the LMB *StAR* promoter. Positive control probes were generated from published sequences. All probes were prepared and obtained commercially (Eurofins MWG Operon, Huntsville, AL). The probes were biotinylated at one end of each sense strand; cold probes lacked biotinylation. Sense and antisense oligos were annealed by adding equimolar amounts of each probe to a reaction containing 10 mM Tris (pH 8.0), 1 mM EDTA, 20 mM NaCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol. The reactions were boiled for 10 min at 95 C followed by gradual cooling to room temperature.

EMSA

Fluorescence-based EMSAs were conducted using a modification of the commercially available EMSA gel shift kit protocol (Panomics, Fremont, CA) and an RORα antibody designed against human RORα4 supplied from Steinhilber and colleagues (31). One microgram of RORα protein was used per reaction, and in the instances where antibody was added, 1 μg of RORα antibody was used. Ten nanograms of probe were added to each reaction, and if a competition assay was performed, cold probe was added in 100× excess. The binding reaction conditions are outlined in the manufacturer's instructions. The binding reactions were incubated at 17.5 C for 30 min. A 6% nondenaturing Tris-borate EDTA polyacrylamide gel was then prepared and prerun in prechilled 0.5× Tris-borate EDTA for 10 min at 120 V at 4 C. Once loaded, gels were electrophoresed for 20 min at 60 V at 4 C, followed by 80 min at 100 V at 4 C. The EMSA gel-shift kit protocol and reagents from the manufacturer were used for the remainder of the EMSA.

Chromatin immunoprecipitation (ChIP) assay

Y-1 cells were transfected with the 2.9-kb LMB *StAR* promoter plasmid using FuGENE HD (Roche Diagnostics). After transfecting overnight, medium was changed and half of the transfected plates were treated with 1 mM dbcAMP for 20 h. Samples were processed for ChIP analysis using a modified version of the ChIP-IT kit protocol (Active Motif, Carlsbad, CA). Once cells were fixed and scraped, chromatin was isolated and sheared using a Fisher sonic dismembrator. Cross-linked sheared chromatin obtained from both treatments was immunoprecipitated at 4 C overnight with antibodies against normal IgG, RORα (Santa Cruz Biotechnology, Santa Cruz, CA), or rev-erbα (Cell Signaling Technology, Beverly, MA). After immunoprecipitation and protein G collection, cross-links were reversed and protein and RNA were digested from each sample. DNA was column purified and Q-PCR was run on each sample as described above and data were normalized to 1:10 input control. The primers used in Q-PCR are listed in supplemental Table S1. The mouse primers were designed against a putative RORE (mRORE/−634) identified in the mouse *StAR* promoter using the Genomatix MatInspector software as described in the *in silico* analysis section above. The putative site was located between bp −619 and −641 relative to the transcriptional start site of the gene.

Statistical testing

Student paired *t* test was used for statistical analysis of control *vs.* treatments, and significant differences of $P < 0.05$ between the groups are noted by an asterisk in the figure.

Results

Characterization of the coding sequence for LMB StAR protein

To assess how *StAR* is regulated in LMB, a partial 345-bp sequence was amplified using degenerate primer-

based PCR and then completed with 5′ and 3′ RACE. Sequence alignment of LMB *StAR* with other species, including, human, pig, horse, zebrafish, brook and rainbow trout, revealed 52% similarity with mammals and 72% similarity with other fish species (supplemental Fig. S1).

A sequence comparison of the LMB StAR protein with StAR protein sequences from several species revealed several important residues, including a glutamic acid residue at amino acid 170 within the START domain, the hydrophobic region in which cholesterol binds. Analysis with the ScanProsite web program (http://www.expasy.ch/tools/scanprosite) revealed multiple putative PKA and PKC phosphorylation sites, which are known to be critical for mammalian StAR function. A potential PKA site was identified at amino acids 193–196 as well as four potential PKC sites at amino acids 5–7, 13–15, 60–62, and 187–189. Collectively the protein sequence information suggested that LMB StAR protein may be regulated by similar mechanisms and signal transduction pathways as seen in higher vertebrates and mammals (25), such as regulation by the cAMP/PKA signaling cascade.

In vivo expression of *StAR* mRNA and serum 17β-estradiol (E_2) levels

To determine the basal expression of LMB *StAR* mRNA *in vivo*, tissue samples were collected from pond-raised fish throughout the months of October to April, which spans the typical reproductive cycle for LMB. Quantitation of *StAR* message by Q-PCR revealed that *StAR* mRNA levels fluctuate in correlation with steroid production throughout the reproductive cycle (Fig. 1). LMB *StAR* mRNA levels started increasing in late December and then peaked between February and March before declining by April, and the message levels paralleled serum 17β-estradiol levels throughout the reproductive season. Histology of the ovarian follicles revealed that vitellogenin, the egg yolk protein necessary for the nourishment of newly hatched fry, was present in ovarian follicles with increased levels of *StAR* mRNA (Fig. 2A). These data clearly demonstrate that LMB *StAR* is acutely regulated, probably through signaling initiated by LH and FSH, in the same period as the production of steroid hormones, as in mammalian models.

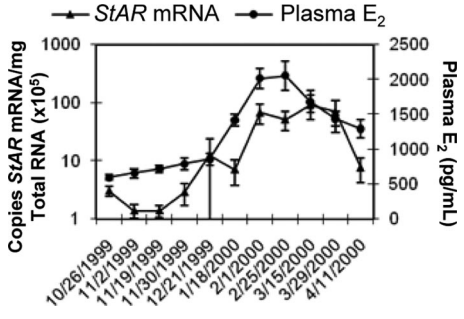

FIG. 1. Seasonal expression of LMB *StAR* mRNA and plasma E_2. RNA was isolated from ovarian tissue of LMB previously collected every few weeks through the reproductive cycle (n = 7 for the first 10 time points and n = 6 for April 11, 2000) and *in vivo* expression of *StAR* mRNA was analyzed by Q-PCR. Data are reported on a logarithmic scale as mean copy number ± mean SE. E_2 levels were quantified from the plasma of the same individuals by RIA as described previously (43).

Regulation of endogenous LMB *StAR* by dbcAMP in ovarian follicle cultures

Based on the *in vivo* pattern of *StAR* expression, vitellogenic ovarian follicles with a defined diameter range of 0.68–0.76 mm were cultured for *in vitro* analysis. This experiment was repeated with four individual fish. Ovarian follicles were exposed to 0.25, 0.5, or 1 mM dbcAMP for 14 h to investigate the potential for regulation of *StAR* in lower vertebrates by the PKA/cAMP pathway. RNA was subsequently extracted from the follicles for quantitation of *StAR* mRNA expression by Q-PCR. A 4-fold induction of *StAR* mRNA was seen with the 0.25 and 0.5 mM doses, and a 10-fold stimulation occurred with 1 mM dbcAMP (Fig. 2B). The ovarian follicle data show that LMB *StAR* transcription is responsive to cAMP signaling.

Cloning of the LMB *StAR* promoter

To ascertain the location of cAMP regulatory regions in the LMB *StAR* gene, we cloned the promoter using the GenomeWalker kit (CLONTECH) and primers designed within the coding sequence previously discussed. PCR products were obtained from all four of the restriction digested genomic libraries that were generated. 5′ RACE of the cDNA transcript was used to locate the boundaries of the 142-bp 5′ UTR and, by inference, the transcriptional start site. Additionally, the transcriptional start site identified from 5′ RACE matched the site predicted by a web-based program called Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.htm). A TATA box is located about 23 bp upstream from the start site, and putative transcriptional elements were identified via bioinformatics by the MatInspector and TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html) programs. A 2.9-kb portion (upstream from the transcriptional start site) of the LMB *StAR* promoter was cloned into the pGL3 basic vector for functional studies in Y-1 mouse adrenocortical cells.

The 2.9-kb LMB *StAR* promoter is responsive to cAMP and hCG-mediated signaling when transfected into mammalian steroidogenic cells

We next examined the response of the 2.9-kb promoter construct to dbcAMP in Y-1 cells. Transfected cells were exposed to a range of dbcAMP between 0 and 2 mM for 20 h. Maximal induction of LMB *StAR* promoter activity occurred between 0.75 and 2 mM dbcAMP, by an average of about 2.6–2.8-fold above basal control levels (Fig. 3A). Indeed, the 2.9-kb promoter could also be stimulated by about

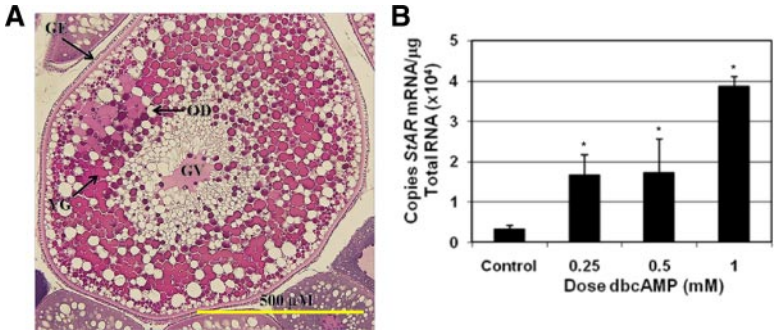

FIG. 2. dbcAMP induction of *StAR* mRNA in LMB ovarian follicle cultures. A, Hematoxylin-eosin histology of a vitellogenic LMB ovarian follicle. The following are labeled: germinal epithelium (GE), germinal vesicle (GV), yolk globules (YG), and oil droplets (OD). B, Representative ovarian follicle culture. Ten follicles were cultured per well in a 24-well culture plate and exposed to three doses of dbcAMP for 14 h. RNA was isolated from the follicles and reverse transcribed for analysis by real-time Q-PCR. This graph represents data obtained from one fish, with each treatment cultured in triplicate. *Error bars*, SE between wells. This experiment was repeated three times with follicles from three individual fishes. Student's *t* test was used to test for significance. *, $P < 0.05$.

2.8-fold with hCG in MA-10 mouse Leydig tumor cells, another mammalian steroidogenic cell line (Fig. 3B). Thus, the promoter is responsive to both cAMP and hCG, supporting that the LMB *StAR* gene is regulated by established steroidogenic signaling pathways.

Deletion of the distal region of the 2.9-kb promoter diminishes dbcAMP response

To begin to identify potentially critical regions involved in the cAMP response of the LMB *StAR* promoter, 1 kb was deleted from the 5′ end using *Eco*RV and *Bst*EII restriction sites to yield a shorter 1.86-kb sequence. The response of the transfected 1.86-kb construct to 1 mM dbcAMP was significantly diminished by greater than 80% in comparison with the response seen with the 2.9-kb construct (Fig. 3C). These data suggest the presence of transcriptional elements important for cAMP activation between the −1.86- and −2.9-kb region.

Site-directed mutagenesis reveals a novel dbcAMP-responsive RORE

The acute dbcAMP regulation of the distal region of the LMB *StAR* promoter was further examined by mutating a putative site for RORE/−1969. The nucleotide mutations yielded a *Not*I restriction site, which web-based programs predicted no transcription factors can bind. The site-specific mutations were confirmed by digestion with *Not*I and sequence verified.

The mutated RORE/−1969 construct and the intact 2.9-kb construct were exposed in parallel to 1 mM dbcAMP. Indeed, mutation of the RORE/−1969 site substantially reduced the dbcAMP induction from that observed in the 2.9-kb construct by approximately 85% (Fig. 3C). The reduced response implicates a regulatory role for the RORE in activating the promoter.

In silico analysis of the 2.9-kb LMB *StAR* promoter sequence revealed several well-characterized putative transcriptional elements that have been previously described in mammalian systems (6, 20, 21, 25). In addition, several putative ROREs were identified throughout the 2.9-kb sequence, with only one putative RORE (RORE/−1969) lying in the distal 1 kb. The *StAR* promoter sequences from multiple species were analyzed for putative ROREs and in all species examined, ROREs were present in the *StAR* promoters (Fig. 3D).

ChIP verification of RORα/rev-erbα proteins binding to the RORE/−1969 element in the LMB *StAR* promoter and to the mRORE/−634 element in the murine *StAR* promoter

To verify that RORα and rev-erbα bind to the RORE/−1969 element in the LMB *StAR* promoter, ChIP assays were run using chromatin fixed from Y-1 cells transfected with the LMB *StAR* promoter and cultured under both basal and dbcAMP-induced conditions. After immunoprecipitation with a polyclonal antibody against mouse IgG (nonspecific control), RORα, or rev-erbα, DNA was purified and Q-PCR was run on each sample using primers encompassing either the RORE/−1969 element in the LMB *StAR* promoter (Fig. 4A) or the mRORE/−634 element in the murine *StAR* promoter (Fig. 4B) present in the same cells. Note that *asterisks* in Fig. 4B indicate that DNA was below detection limits.

In both species, the DNA encompassing each of the ROREs was highly enriched under basal conditions when pulled down with the antibody for rev-erbα, implicating that rev-erbα binds to the LMB RORE/−1969 element and to the mRORE/−634 element in the *StAR* promoter of each species under basal conditions. Upon treatment with dbcAMP, the enrichment of DNA bound to rev-erbα observed under basal conditions diminished to levels observed with the nonspecific IgG antibody with both the LMB and mouse elements. Concomitantly with this decrease, a slight increase was seen for the LMB element when the RORα antibody was used, whereas there was no detectable enrichment above the nonspecific IgG control with the mRORE/−634 element.

EMSA analysis of RORα4 binding to the RORE/−1969 site in the LMB *StAR* promoter

To assess whether recombinant human RORα4 protein could bind to the RORE/−1969 transcriptional site in the LMB *StAR* promoter, an EMSA was performed using the specific probes for this promoter site and recombinant human RORα4 protein (Fig. 4C). The recombinant protein bound to the RORE/−1969 probe, producing a single band. Addition of both the RORα antibody and the cold

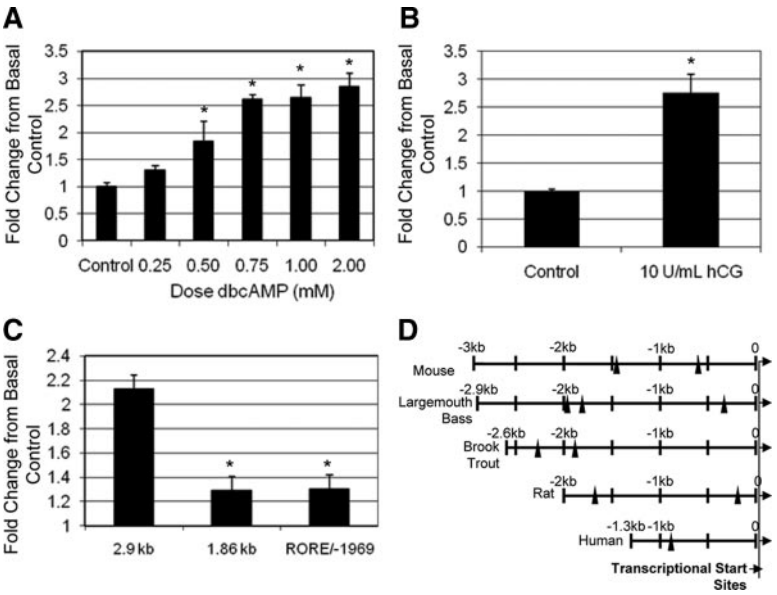

FIG. 3. A, Dose-response exposure of LMB *StAR* promoter to dbcAMP. Y-1 cells were transfected with the 2.9-kb LMB *StAR* promoter and exposed to increasing doses of dbcAMP from 0 to 2 mM dbcAMP. Graph is representative of one experiment done in triplicate that was repeated at least three individual times. B, Exposure of LMB *StAR* promoter to hCG. To further verify responses observed in Y-1 cells, MA-10 cells were transfected with the 2.9-kb LMB *StAR* promoter and exposed to 10 U/ml hCG. C, LMB *StAR* promoter constructs containing a deletion (~1 kb from distal end of promoter) or a site-directed mutation (RORE/−1969) were evaluated for basal and dbcAMP stimulation of *StAR* promoter activity. C, Data collected from three to five individual experiments. In all experiments, values are normalized to renilla luciferase (internal control) and are reported in fold change from basal control. Student's *t* test was used to determine significance. *, $P < 0.05$. D, *In silico* comparison of the *StAR* promoter across species. Promoter sequences (including the 5′ UTR) for the *StAR* gene promoters from LMB (accession no. DQ166819), brook trout (accession no. AY308064), rat (accession no. AB006007), mouse (39), and human (accession no. U29098) were aligned based on the transcriptional start sites for the gene. Putative ROREs were identified using Genomatix MatInspector online software. The sites selected for mapping had 80% or greater homology to the core mammalian transcription factor sequence.

unlabeled probe verified the specificity of the RORE/−1969-RORα4 interaction. Further verification of specificity was exhibited by the use of a scrambled probe, which did not bind the protein. A RORE probe designed against a consensus human RORE sequence was run as a positive control, yielding a band around the same size as that seen with RORE/−1969.

Discussion

We report the first in-depth study on transcriptional regulation of the *StAR* gene in a fish model, LMB. The *StAR* cDNA and a large portion of its promoter were cloned to study its regulation *in vivo* and *ex vivo* in LMB ovarian follicle cultures and then, ultimately, for more comprehensive examination of regulation of *StAR* transcription with transfection assays. Our data show that LMB *StAR* is transcriptionally regulated by dbcAMP and that rev-erbα/RORα play critical roles in the activation of the LMB *StAR* promoter.

In vivo and *ex vivo* examination of LMB *StAR* established a temporal correlation of *StAR* mRNA expression with steroidogenesis in LMB. An increase in *StAR* mRNA levels *in vivo* paralleled the levels of 17β-estradiol detected in the plasma (Fig. 1), suggesting that the connection between *StAR* mRNA synthesis and the biosynthesis of steroids from cholesterol occurs in LMB as it does in mammals. The regulation and metabolism of steroid hormones is very complex in all vertebrates, and although we associate an increase in *StAR* mRNA abundance with increased plasma 17β-estradiol, the activities of other key enzymes, such as P450 aromatase present predominantly in the gonad and brain and at lower levels in peripheral tissues, and enzymes involved in phase II metabolism in the liver, are also important factors that control plasma levels of steroid hormones. The up-regulation of LMB *StAR* mRNA by dbcAMP in *ex vivo* cultures of LMB ovarian follicles (Fig. 2B) implicates that important signaling mechanisms that transactivate *StAR* may be conserved across species.

To identify the transcriptional elements involved in the regulation of *StAR* transcription in lower vertebrates, a 2.9-kb portion of the LMB *StAR* promoter was cloned for sequence analysis and used in transfection assays. *In silico* analysis revealed multiple putative ROREs, which appeared to be conserved in the promoters of fish and mammals, although the specific positions in the sequences did not always correspond. The putative elements predicted by the bioinformatic programs are not necessarily biological regulators of the promoter; therefore, determination of their functionality requires further experimentation.

Critical elements involved in dbcAMP regulation of LMB *StAR* transcription are located in the distal portion of the promoter. The 2.9-kb LMB *StAR* promoter was induced greater than 2.7-fold in response to both 1 mM dbcAMP (Fig. 3A) and 10 U/ml hCG (Fig. 3B), which mirrored results published for the human *StAR* promoter in Y-1 cells (32). However, deletion of 1 kb from the 5′ end of the LMB 2.9-kb promoter significantly diminished the induction by dbcAMP (Fig. 3C). There are putative AP-1, SF-1, ROR/rev-erb, and estrogen response element sites as well as others within this region, which could be critically involved in regulating *StAR* transcription. Functional roles of SF-1- and AP-1-mediating *StAR* promoter trans-

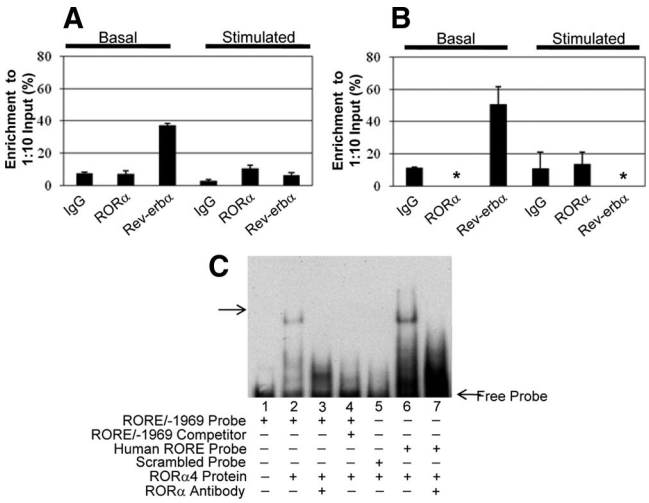

FIG. 4. Functional analysis of RORα and rev-erbα by ChIP and EMSA. ChIP assays were run using chromatin fixed from Y-1 cells transfected with the LMB *StAR* promoter and cultured under both basal and dbcAMP-induced conditions. ChIP was run on each sample with antibodies specific to mouse IgG (nonspecific control), RORα, or rev-erbα. Immunoprecipitation was accomplished by the addition of protein G agarose beads. The pull-downs were washed, complexes were eluted, and DNA was purified. Q-PCR was run on each sample and results are reported graphically as percent enrichment to a 1:10 dilution of each input control. Primers used in the Q-PCR encompassed the RORE/−1969 element in the LMB *StAR* promoter (A) or the RORE/−634 element in the mouse promoter (B), immunoprecipitated from the same transfected cells. Each figure is representative of one of three replicated experiments. *Asterisks* (B) indicate that DNA levels were below detection limits. To further investigate the capacity for the LMB RORE/−1969 element to bind RORα, an EMSA was run using recombinant human RORα4 protein (C). A probe encompassing the RORE/−1969 sequence was added to recombinant human RORα4 protein in 1× binding buffer. The reactions were separated on a native gel and transferred to a membrane for EMSA analysis using chemiluminescence. Addition of an antibody specific to RORα to the reactions diminished the banding pattern observed in the lanes containing the probe and protein only, verifying specificity of the protein-DNA interaction *in vitro*. The following are the sequences for the probes used: RORE/−1969 probe (5′-AAT AGG CAT ATG ACC TAC TTT GGC TC); perfect (human consensus) RORE probe (5′-TCG AGT CGT ATA ACT AGG TCA AGC GCT GGA C-3′); scrambled probe (5′-CCT CTA TAA CGG GTC GGA TAC TAT TA-3′). Lane 1, RORE/−1969 probe only; lane 2, RORE/−1969 probe with RORα4 protein; lane 3, RORE/−1969 probe, RORα4 protein, and RORα4 antibody; lane 4, RORE/−1696 probe, RORE/−1969 cold unlabeled probe, and RORα4 protein; lane 5, scrambled probe and RORα4 protein; lane 6, perfect (human consensus) RORE probe and RORα4 protein; and lane 7, perfect (human consensus) RORE probe, RORα4 protein, and RORα4 antibody.

activation have previously been reported (17, 32, 33), and these sites were not tested in this study. Instead our research was focused on the RORE site.

Recent studies implicated RORα and rev-erbα in regulating the activity of genes involved in circadian rhythm (34). Because steroidogenesis in fish is seasonally regulated, we focused our investigation on characterizing the potential roles of RORα and rev-erbα in transcriptional regulation of the *StAR* promoter.

Site-directed mutation of the putative RORE/−1969 element (located between the −1.86- and −2.9-kb region of the LMB *StAR* promoter) robustly diminished the response of the promoter to dbcAMP (Fig. 3C), suggesting potentially new signaling mechanisms for dbcAMP regulation of *StAR* transcription. RORα is an important nuclear factor involved in the transcriptional activation of genes that control multiple physiological processes, including those central to controlling peripheral circadian rhythm (35). There are four isoforms of RORα that arise from alternative promoter usage and alternative splicing. RORα1 and -4 are ubiquitously expressed. It is notable that cholesterol has been identified as a ligand for RORα in recent studies (36, 37), suggesting that it may be a key sensor for steroid production. In addition, rev-erbα has been reported to competitively bind the same element as RORα, disallowing the activation of target genes by RORα (38). The possibility that RORα and rev-erbα regulate steroidogenesis is strongly supported by the presence of multiple ROR elements in the LMB *StAR* promoter, in particular the RORE at −1969 bp upstream from the transcriptional start site located in the 5′ distal promoter segment. Indeed, bioinformatic analysis also revealed high-affinity sites for RORα/rev-erbα in several mammalian species, including human, rat, and mouse, but the roles that these proteins may play in regulating the mammalian *StAR* gene have not yet been investigated.

Noting that RORα and rev-erbα bind to the same core sequence, mutation of the RORE/−1969 site in the LMB *StAR* promoter could attenuate the binding of the two proteins under dbcAMP-stimulated and basal conditions, respectively. This may account for the loss of response to dbcAMP in the LMB *StAR* promoter observed upon mutation, particularly if RORα mediates dbcAMP activation of the promoter. This site, with both positive and negative regulatory potential (*e.g.* through RORα/rev-erbα signaling pathways), may be especially important in regulating fish steroidogenesis and may be the link to circadian control of this process.

Endogenous binding of RORα and rev-erbα to the LMB RORE/−1969 element (Fig. 4A) and also of rev-erbα to the mouse mRORE/−634 element (Fig. 4B) were verified by ChIP, implicating that these proteins play significant roles in controlling the transcriptional activation of the promoters in both species. The mouse *StAR* promoter has been very well characterized (20), and it has been reported that negative regulatory elements may lie between base pairs −966 and −254 relative to the transcriptional start site (39, 40). Our studies have shown that rev-erbα binds to the mouse RORE/−634 element (located ~−634 bp upstream of the transcriptional start site) under basal conditions and that the protein does not associate with the element under dbcAMP-stimulated conditions, signifying that rev-erbα may play an important role in the basal control of the promoters in LMB and mice.

Because the enrichment of RORα in the ChIP studies on the LMB RORE/−1969 element was not as robust when compared with the results with rev-erbα, we chose to conduct EMSA experiments to further investigate the binding of RORα to the LMB promoter. Indeed, the human RORα4 protein is capable of binding to the LMB RORE/−1969 site (Fig. 4C). Interestingly, one study investigated gene expression profiles in staggerer mice (which do not express the *RORα* gene) and reported that key enzymes in the steroid biosynthetic pathway were down-regulated in the mutant phenotype, indicating that RORα may play a role in controlling genes involved in steroid hormone biosynthesis (41). These studies further suggest that the ROR and rev-erb families of orphan nuclear receptors may play an integral role in modulating steroidogenesis.

Several studies have shown that the proximal several hundred base pair segment of the mammalian *StAR* promoter is sufficient in mediating maximal cAMP-induced transactivation (19, 39). The results observed in the current study suggest that the functional RORE found in the distal segment of the LMB *StAR* promoter is important in mediating cAMP-induced promoter transactivation. It has been reported that RORα/rev-erbα elements located nearly −7 kb upstream of the transcriptional start site of a gene can play critical roles in enhancing or repressing gene transcription (42). This phenomenon could be due to the recruitment of essential cofactors, the presence of enhancers in distal regions, or altered DNA folding and structure. It is possible that the distal segment of the LMB *StAR* promoter functions in one of these ways to regulate the LMB *StAR* gene, although further studies are warranted to investigate this hypothesis.

The combination of ovarian follicle, promoter deletion, and site-directed mutation data implies that transcriptional elements between −1.86 and −2.9 kb of the LMB *StAR* promoter are required for cAMP-induced activation. The mutation data revealed that more than 80% loss in transcriptional activity of the LMB *StAR* promoter can be attributed to an RORE upstream from −2 kb of this region. ChIP and EMSA analyses in our studies reveal that both rev-erbα and RORα may play integral roles in the activation of the LMB *StAR* promoter. Additionally, it is likely that regulation of *StAR* transcription is conserved from mammals to lower vertebrates and that nonclassical species such as LMB are increasingly pertinent model systems for comparative studies.

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