REGULATION OF THE STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) BY cAMP AND TRANSFORMING GROWTH FACTOR-BETA (TGF-BETA) DEPENDENT PATHWAYS

By

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ABBREVIATIONS

3-B-HSD = 3-beta-hydroxy-steroid-dehydrogenase
ACTH = adrenal corticotrophic hormone
AhR = aryl hydrocarbon receptor
bp = base pair
cAMP = cyclic adenosine monophosphate
COUP-TF = chicken ovalbumin upstream promoter transcription factor
CRE = cAMP response element
CREBP = cAMP response element binding protein
DAX-1 = dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DHP = 17alpha, 20B-hydroxy-4-pregnen-3-one
ELISA = enzyme linked immunosorbent assay
EMSA = electromobility shift assay
ERE = estrogen response element
GFP = green fluorescence protein
GnRH = gonadotropin releasing hormone
Kb = kilobase
LCAH = lipoid congenital adrenal hyperplasia
LH = luteinizing hormone
LMB = Largemouth Bass
MALDI = matrix assisted laser desorption ionization
PBR = Peripheral Benzodiazepine Receptor
PCR = polymerase chain reaction
PKA = protein kinase A
PKC = protein kinase C
RACE = rapid amplification of cDNA ends
RAR = retinoic acid receptor
RARE = retinoic acid response element
ROR = retinoic acid related receptor
SBP = StAR binding protein
SF-1 = steroidogenic factor 1
StAR = Steroidogenic Acute Regulatory Protein
TGF-β = transforming growth factor beta
REGULATION OF THE STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) BY cAMP AND TRANSFORMING GROWTH FACTOR (TGF-BETA) DEPENDENT PATHWAYS

By

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StAR is the rate-limiting step in steroid production and is transcriptionally down regulated by toxin exposure. StAR transports cholesterol across the mitochondrial membrane for metabolism into steroids. We cloned the entire coding region of largemouth bass (LMB) StAR and used this sequence to develop a real-time PCR assay to quantify StAR mRNA levels in LMB ovarian follicle cultures. Exposure to dbcAMP and TGF-beta, two potent signaling molecules known to regulate mammalian steroidogenesis, modulate LMB StAR. TGF-beta down regulates and dbcAMP upregulates StAR mRNA. A polyclonal antibody specific to LMB StAR was developed to measure protein levels by western blot. To further analyze the regulation of LMB StAR, a 3 kb portion of the promoter was cloned. In silico analysis of this segment with other StAR promoters available in the database showed potential conserved regulatory sites that imply control by a wide range of transcription factors. The 3 kb promoter
segment was transfected into Y-1 cells, a mouse adrenal cortical cell line and tested with
dbcAMP and TGF beta. The 3 kb construct responded positively to dbcAMP but was not
significantly impacted by TGF-B exposure compared to the 1.8 kb length promoter.

Mutation of potential regulatory sites in the promoter, including ERE (estrogen response
elements), ROR (retinoic acid related receptor), and COUP-TF (chicken ovalbumin
upstream promoter) sites were tested for their role in cAMP and TGF-beta signaling.
Together, these data suggest that one way toxins may repress steroid synthesis, and more
specifically StAR, is through TGF-beta signaling.
CHAPTER 1
INTRODUCTION

The Steroidogenic Acute Regulatory Protein (StAR Protein) is the rate-limiting step in steroidogenesis and can be regulated by endogenous and exogenous agents, including environmental toxins (Walsh et al., 2000). Determining the regulation of StAR is imperative since homeostasis of steroid production is vital for various cell signaling and metabolic pathways. The aims of this project are to examine both the normal and atypical regulation of StAR in largemouth bass, with a specific focus on delineating the signaling cascades of cAMP and TGF-β, two potent molecules that can control steroid production.

Literature Review

Steroidogenic Acute Regulatory Protein (StAR)

Extensive studies in mammalian models have confirmed StAR transports cholesterol across the mitochondrial membrane. After transport, cholesterol is metabolized by the side chain cleavage enzyme to pregnenolone and then ultimately to steroid hormones (Stocco and Clark, 1996).

Identification of the StAR protein

Researchers had known for many years that de novo protein synthesis was required for steroid synthesis; however, the identity of the protein involved in facilitating the transport of cholesterol across the mitochondria eluded them until 1995. A 30 kDa protein in rat adrenal cells stimulated by ACTH, now known as StAR, was first observed when the $[^{35}\text{S}]$ methionine labeled proteins were electrophoresed through a 2D gel.
Cycloheximide, a protein synthesis inhibitor, blocked steroid production without impairing the activity of the side chain cleavage or the delivery of cholesterol to the outer mitochondrial membrane (Stocco and Clark, 1997). The 30 kDa protein was purified from ACTH stimulated MA-10 mouse leydig cells using detergent solubilization followed by separation of the proteins by 1D and 2D gels. Bands at the 30 kDa location were excised and digested with trypsin for microsequence analysis. Degenerate oligonucleotides for PCR amplification of the 30 kDa protein were designed based on the microsequencing results to obtain a 400-base pair partial product. The full coding sequence for StAR from MA-10 cells was then screened from a cDNA library using the 400-base pair product as a probe (Clark et al., 1994). Since the original identification in adrenal and leydig cells, StAR has also been located in the brain, kidney, and heart (Young et al., 2001; Pezzi et al., 2003).

**Mode of action**

StAR is a mitochondrial protein synthesized as a 37 kDa precursor protein in the cytosol of mammalian cells (Clark et al., 1995). Upon stimulation, the 37 kDa precursor is targeted via its signal sequence to the mitochondria. As the precursor protein is imported into the mitochondrial inner compartment, the protein’s signal sequence is removed by a matrix processing protease and contact sites are formed between the outer and inner mitochondrial membranes. The precursor protein is further processed by the mitochondrial intermediate processing peptide to remove the targeting sequence, forming the mature 30 kDa protein (Stocco and Clark, 1997). The cytoplasmic 37 kDa protein has a half-life of around 10-15 minutes; however, the 30 kDa inactive protein has a longer half-life of up to several hours (Christenson and Strauss, 2000).
Two current models suggest StAR transports cholesterol by either acting on the outside of the mitochondria as a molten globule (a protein with extensive secondary structure but disorganized tertiary structure, potentially allowing for hydrophobic amino acids to be exposed) or as an intermembrane shuttle. FRET (fluorescence resonance energy transfer) data indicate that StAR undergoes a conformational change to a molten globule once it interacts with the outer mitochondrial membrane, enabling it to bind cholesterol (Christenson et al., 2001). Additionally, recombinant mammalian StAR protein lacking the first 62 amino acids is localized to the surface of the outer mitochondria and is prevented from entering the intermembrane space. It remains active and steroid production continues, suggesting that StAR functions by binding to the outer membrane (Bose et al., 1999).

The second, but increasingly dubious, model shows StAR acting primarily to shuttle cholesterol between the outer and inner mitochondria membranes, across the intermembrane space (Mathieu et al., 2002). This theory was fueled by data showing contact sites between the two mitochondrial membranes when cholesterol is bound (Thomson, 1998). It is during the formation of these contact sites that cholesterol is thought to be transported across the mitochondrial membranes to be metabolized.

Cholesterol is specifically encompassed or bound by the START (StAR-related lipid-transfer) domain located towards the C-terminus of StAR. The START domain contains about 210 amino acids with a hydrophobic core where cholesterol binds (Strauss et al., 2003). Crystal structure of the START domain from the MLN64 protein, another lipid transporter protein, suggests there would have to be a conformational change in the hydrophobic core of StAR for a molecule of cholesterol to enter and bind under natively
folded conditions (Tsujishita and Hurley, 2000.) Furthermore, although the START crystal structure shows room for one molecule of cholesterol, ligand binding assays with recombinant StAR and increasing titrations of fluorescent cholesterol from 5-100 nM gave a sigmoidal-shaped binding curve (Petrescu et al., 2001). The binding studies suggest there are two cholesterol binding sites, necessitating a conformational change of StAR for sterol binding and transfer, further negating the shuttle model.

Studies show that StAR protein can be degraded by proteasomes. In the presence of a proteasome inhibitor, MG132, there is accumulation of the cytosolic, 37 kDa form of StAR in human or rat granulosa cells (Tajima et al., 2001). There is even evidence suggesting that StAR could be subjected to degradation by different proteases in a biphasic manner, ensuring removal of residual protein that escaped the initial protease (Granot et al., 2003). COS cells chased for 15 minutes with $^{35}$S methionine prior to treatment with MG132 showed that degradation of StAR was prevented for the first 2 hours. The protein, however, began degrading after two hours of MG132 treatment, suggesting that StAR is subjected to degradation by multiple proteases (Granot et al., 2003).

**Protein-protein interactions with StAR**

Studies have indicated there are a couple of putative protein interacting partners for StAR. FRET studies showed that PBR (peripheral-type benzodiazepine receptor) associates with StAR at the mitochondrial membrane. A PBR-StAR association would, in theory, make sense because PBR is necessary for cholesterol transport (West et al., 2001).

Immunoprecipitation experiments showed that StAR also interacts, *in vivo*, with HSL (hormone-sensitive lipase). HSL mediates the availability of unesterified
cholesterol for steroid synthesis (Shen et al., 2003). Rats were injected with ACTH to induce StAR expression and anti-HSL antibodies were used to immunoprecipitate HSL from the adrenal glands. The HSL-immunoprecipitate complexes were separated on an SDS-PAGE gel and the presence of StAR in the complex was detected with anti-StAR antibodies.

Additionally, a recent study used a yeast two-hybrid system to identify another protein that binds StAR, which they named SBP (StAR binding Protein). Binding of StAR to SBP results in increased steroidogenesis (Sugawara et al., 2003). A recombinant form of StAR (N-62 StAR) lacking the first 62 amino acids in which the mitochondrial import signals are located was used as the bait for the yeast assay. Interaction of SBP with N-62 StAR suggests the binding occurs in the cytoplasm or at the outer mitochondrial membrane. Northern blot analysis suggests that SBP may be found in several tissues, including the gonads, liver, lung, and kidney. It is possible that identifying protein interacting partners will help to elucidate the mode of action for StAR mediated cholesterol transport.

Mutations and associated diseases

StAR knockout mice and humans with LCAH (lipoid congenital adrenal hyperplasia) disease exhibit similar symptoms of severely repressed steroid synthesis (Hasegawa et al., 2000). Characteristic of LCAH patients is the presence of large adrenal glands with high levels of cholesterol or cholesterol esters. Death can result in infancy if patients are not treated with hormone replacement. A series of various nucleotide insertions and deletions in StAR DNA are attributed to LCAH and these can vary among the afflicted individuals. This disease just emphasizes the importance of StAR (Stocco and Clark, 1996).
Promoter

Characterizing the response elements in the StAR promoter is important for determining specific proteins and protein-DNA interactions used to regulate its transcriptional activity. The promoter for StAR has been sequenced for several mammalian species, including human, rat, mouse, pig, sheep and cow (Sugawara et al., 1997; Reinhart et al., 1999; Rust et al., 1998). In these systems, the StAR promoter contains a TATA box and several recognizable response elements for transcription factors. Some of the most prevalent response elements identified in mammalian species bind C/EBPs, GATA-4, SF-1, DAX-1, AP-1, and AhR (Manna et al., 2004; Sugawara et al., 2001; Sandhoff and McLean, 1999) and are conserved across species (Figure 1-1). Interestingly, although StAR is a cAMP-dependent regulated gene, a perfect consensus site for CRE (cAMP response element) has not been recognized in any of the StAR promoters sequenced. However, studies have shown that when wild type CREB (cAMP response element binding protein) is transfected into several mammalian cell lines, there is an increase in StAR promoter activity and mRNA expression (Stocco et al., 2001). It is suggested that CREB may play a role in the absence of a consensus CRE by binding indirectly to a non-consensus site. It is also possible that CREB regulates an activator of StAR expression such as SF-1.

One of the most abundant response elements in the mammalian StAR promoter is SF-1 (steroidogenic factor-1), a known inducer of steroidogenic transcriptional activity. SF-1 is an orphan nuclear receptor transcription factor that has been sequenced from many species, including mammalian and fish systems (Yaron et al., 2003). SF-1 also is called AD4-BP (adrenal 4-binding protein) and is the mammalian homolog of the Drosophila factor FTZ-F1. FTZ-F1 regulates transcription of the fushi tarazu homeobox
gene in fly embryos. Four transcripts are encoded by the SF-1 gene, including ELP1, ELP2, ELP3, and SF-1, partly generated through alternate splicing (Nawata et al., 1999). SF-1 is expressed in all steroidogenic tissues such as the adrenal, gonads, and the placenta. SF-1 knockout mice implicate the importance of SF-1 in cellular functions. The knockout mice lack adrenal glands and gonads, which leads to lethal adrenocortical insufficiency (Hammer and Ingraham, 1999.)

It is not exactly known how ligands lead to the activation of SF-1; however, there are a lot of studies showing post-translational modification of SF-1 occurs. SF-1 does have a consensus site for protein kinase A (PKA) phosphorylation (Bertherat, 1998). Further studies have shown that inhibition of mitogen activated protein kinase (MAPK) can decrease SF-1 responsive genes (Hammer and Ingraham, 1999).

SF-1 binds as a monomer to its response element and has two zinc fingers which helps it bind to its consensus site (Ito et al., 2000). Up to 6 SF-1 binding sites have been identified in the mammalian StAR promoter, with the nearest element typically located only about 40 base pairs away from the transcription start site. The first two SF-1 sites closest to the start site appear to be conserved across species (Reinhart et al., 1999). SF-1 sites have been found in the promoter of aromatase for several fish species, including goldfish, medaka, and zebrafish (Callard et al., 2001; Tchoudakova et al., 2001). Aromatase is the final protein in the steroidogenic pathway involved in the conversion of testosterone to estradiol (Callard, 2001). SF-1 sites in promoters of genes in fish studied to date have the general consensus sequence of PyCAAGGPyPyPur, with the exception that zebrafish have a purine instead of a pyrimidine for the first nucleotide (Kazeto et al., 2001; Honda et al., 1993). It appears that one way in which SF-1 regulates StAR
transcriptional activity is by interacting with other transcription factors, including C/EBP, AP-1, and SP1 (Reinhart et al., 1999; Shea-Eaton et al., 2002). Promoter regulation can, therefore, be very complex and involve transcription factors acting individually or cooperatively to exert their actions.

RAR and RXRs are part of the steroid-thyroid hormone receptor subfamily and each are encoded by three genes, α, β, and γ. RAR mediates activation by heterodimerizing with RXR and it is the heterodimer which binds RARE (retinoic acid response element) (Pfahl, 1993). Typically, the RARE has a direct repeat of an AGGTCA core motif which is separated by 2 or 5 nucleotides (Bastien and Rochette-Egly, 2004).

ROR (retinoic acid receptor-related orphan receptor) is also a member of the nuclear hormone receptor superfamily. ROR can bind to the RORE (ROR response element) as a monomer or homodimer. If ROR binds as a monomer, it recognizes a 6 base pair A/T rich region followed by an AGGTCA motif. To bind as a homodimer, a direct repeat of the RORE separated by 2 nucleotides is necessary (Boukhtouche et al., 2004). ROR has been well characterized to activate gene transcription in the absence of a ligand; however, a recent study shows that cholesterol is an ROR ligand (Kallen, 2002). ROR alpha transcriptional activity is repressed in U20S osteosarcoma cells depleted of cholesterol with statins, a family of drugs that inhibit cholesterol synthesis (Boukhtouche et al., 2004). Although RORs are known to be involved in tissue development or differentiation like some other nuclear receptors, there is still much to be known about the genes that it regulates (Jarvis et al., 2002).
To date, only DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) is well documented to repress StAR transcription (Jo and Stocco, 2004). When Y-1 mouse adrenal cells were co-transfected with 2 Kb of the rat StAR promoter and a vector encoding DAX-1, basal and 1mM dbcAMP stimulated luciferase activity were repressed by at least 40% (Sandhoff and McLean, 1999). Although the exact mechanism is still being elucidated, it has been shown in mammalian systems that DAX-1 binds to a hairpin structure in the promoter rather than at a consensus site (Stocco et al., 2001).

COUP-TF (chicken ovalbumin upstream promoter-transcription factor) is also known to downregulate steroidogenesis; however, its role in the regulation of StAR is just beginning to be investigated (Buholzer et al., 2005; Shibata et al., 2004). A recent study showed that mRNA induction of StAR by angiotensin II was completely suppressed by overexpression of COUP-TF in bovine adrenal glomerulasa cells (Buholzer et al., 2005). COUP-TF are nuclear orphan receptors that can form homodimers and bind to response elements with variations of an AGGTCA core motif, which includes the RARE (Tran et al., 1992). COUP-TF can also silence the activity of other transcription factors like RXR by heterodimerizing with them and thereby limiting their availability for other binding partners (Berrodin et al., 1992). COUP-TF can also interact synergistically with corepressors like N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor) (Shibata et al., 1997).

Endogenous regulators and signaling pathways

StAR has been shown to be upregulated by cAMP, forskolin, GnRH, ACTH, and cholesterol containing lipoproteins, including both LDL and HDL (Clark and Stocco,
The signaling cascade triggered by cAMP and other related inducers is complex and encompasses many different proteins and crosstalk between various pathways.

It has been confirmed that cAMP modulates StAR through a PKA (protein kinase A) mediated event. PKA activation can regulate StAR directly and indirectly. Indirectly, PKA can induce transcriptional activation by phosphorylating SF-1, a transcription factor commonly known to regulate StAR (Aesoy et al., 2002). There are multiple SF-1 sites throughout the mammalian StAR promoter (Sandhoff et al., 1998).

Additionally, there are PKA sites within the protein sequence of StAR. In vitro studies in mammalian cells showed specifically that mutation of a serine in a protein kinase A (PKA) site at amino acid position 195 resulted in 40% less steroidogenic capacity (Arakane et al., 1997). Studies with a normal and PKA mutant cell line showed that phosphorylation of StAR most likely stabilizes the protein and therefore results in a dose response increase to dbcAMP as seen by western blot (Clark et al., 2001). [\(^{35}\)S]methionine incorporation into StAR was shown in the PKA mutant cell line (Kin-8), suggesting that PKA can act post-translationally.

Less is known about endogenous downregulators of StAR. Some studies have shown PGF-2 (prostaglandin factor), TGF-\(\beta\) (transforming growth factor beta), and glucocorticoids can repress StAR transcription (Sandhoff and McLean, 1999; Brand et al., 2000; Huang and Shirley, 2001). When dexamethasone, a glucocorticoid, was added to follicles cultured from LH (luteinizing hormone) treated rats, StAR activity was impaired but side chain cleavage was not (Huang and Shirley, 2001). This is potentially
one example of how steroids can regulate themselves and that StAR may be subject to feedback inhibition by downstream steroids.

**Regulation by environmental contaminants**

Several recent studies have shown that StAR expression can be downregulated by environmental contaminants, including phthalates, which are plasticizers (Shultz et al., 2001; Barlow et al., 2003), the pesticide Roundup, the herbicide Lindane (Walsh et al., 2000), and the insecticide Dimethoate (Walsh et al., 2000). Studies have shown that these toxins could impact transcription or post-transcription activity. Experiments using MA-10, mouse Leydig cells, showed Lindane and Dimethoate inhibit both mRNA and protein expression while not repressing overall protein synthesis or inhibiting PKA activity (Walsh et al., 2000). Also, both microarray and real-time PCR analysis showed di-butyl phthalate to repress StAR in fetal rat testes, further suggesting a negative regulation of transcription by environmental contaminants (Shultz et al., 2001; Barlow et al., 2003).

The regulation of StAR by paper mill contaminants, which is known to cause repression of steroid levels, has not been investigated (McMaster et al., 1996). Several studies have linked the altered steroid levels in fish to β-sitosterol (Lehtinin et al., 1999; MacLatchy et al., 1995). Interestingly, StAR can transport β-sitosterol as efficiently as cholesterol under *in vitro* conditions (Kallen et al., 1998). It is currently unknown how these contaminants could specifically regulate the promoter, even though several of the toxins listed above decreased StAR mRNA expression.

**Endocrine Disruption**

There is substantial evidence that humans and wildlife exposed to chemicals in the environment can exhibit alterations in steroidogenic capacity, leading to changes in
secondary sex characteristics, gonad weight, and production and size of eggs (McMaster et al., 1995; Sepulveda et al., 2001.). The consequences of steroid imbalance are so significant and universal that the Environmental Protection Agency (EPA) Office of Research and Development decided to incorporate endocrine disruption as one of its top six research priorities. The EPA established several long-term goals, which included providing a better understanding of the science underlying the effects, exposure, assessment, and risk management of endocrine disruptors; determining the extent of the impact on humans, wildlife, and the environment; and finally, supporting the EPA’s screening and testing program.

Environmental contaminants can impact reproduction and steroidogenesis by mimicking the actions of endogenous androgens or estrogens, or by affecting their synthesis or metabolism. Cellular signaling pathways can converge to mediate the response to the endocrine disruptors, which includes endogenous signaling molecules like cAMP and TGF-β as well as critical transcription factors like SF-1, ER, COUP-TF, and RAR.

Some environmental toxins that have been shown to impact steroidogenesis in fish include PCB (Spies and Rice, 1988), polycyclic aromatic hydrocarbons (PAH) (Spies and Rice, 1988), phthalates (Barlow et al., 2003), Roundup (Walsh et al., 2000), and paper mill effluents (McMaster et al., 1996), among others. Extensive studies on several fish species exposed to paper mill toxins, including the white sucker in Lake Superior and largemouth bass (LMB) in Florida, show males have decreased testosterone and females have decreased estrogen levels (McMaster et al., 1995; Sepulveda et al., 2001; MacLatchy and Van Der Kraak, 1995). Several of the compounds present in paper mill
effluent have been identified, including resin acids, dioxins, abietic acid, and phytosterols (MacLatchy and Van Der Kraak, 1995; McMaster et al., 1996; Sepulveda et al., 2001). While paper mill effluent is a complex mixture of chemicals, attention has been focused on β-sitosterol. β-sitosterol is a phytosterol released by paper mills into rivers and lakes. Although published results suggest β-sitosterol acts through the estrogen receptor (Gutendorf and Westendorf, 2001), other studies suggest β-sitosterol affects the transport of cholesterol across the mitochondrial membrane (MacLatchy and Van Der Kraak, 1995; MacLatchy et al., 1997). Studies show that goldfish injected with β-sitosterol exhibit decreased steroid levels and expression of the P450 side chain cleavage enzyme, an enzyme involved in the conversion of cholesterol to pregnenolone (MacLatchy et al., 1997). However, when ovarian tissue cultures from β-sitosterol exposed fish are treated with a membrane permeable form of cholesterol, steroid production is recovered (MacLatchy et al., 1997). This study implicates StAR as a site of regulation by β-sitosterol since it binds and transports cholesterol to the inner mitochondrial space for steroid production.

Ovarian follicles were cultured from goldfish that were exposed in vivo to β-sitosterol to test whether their steroidogenic ability was impaired by the toxic treatment (MacLatchy and Van Der Kraak, 1995.) Culturing follicles has also been used to study mammalian StAR function (Huang and Shirley, 2001) and it is a very relevant and physiological assay to study regulation of steroidogenic proteins for many different types of model species.

**Largemouth Bass as a Model**

Fish are often surrounded by many environmental contaminants in the water, therefore, are a good model system to study the effects of toxins on reproduction in
vertebrates. Valuable information on the specific mode of cellular regulation by these toxins can be gathered. In particular, LMB exposed to chemical contaminants discharged from the Palatka paper mill into the St John’s River have already been studied extensively and have been shown to exhibit decreased steroid levels of 17-β-estradiol (E₂) and 11-ketotestosterone as well as other reproductive anomalies (Sepulveda et al., 2001; Sepulveda et al., 2003). Additionally, the gonads were smaller in weight and underdeveloped for fish closer to the source of contamination (Sepulveda et al., 2001). LMB can bioaccumulate the environmental toxins in their tissues and organs since they survive on a diet of other fish, crabs, frogs, snakes, mice, turtles, and birds (http://www.go4bass.com/largemouth.html.) LMB are particularly useful for studying exposure to environmental contaminants because they are a freshwater species found across the country.

Their reproductive cycle extends for several months, usually from November through April, which allows for an extended period for experiments. Because LMB are annual spawners, their reproductive cycles are fairly synchronous making controlled experiments more feasible. Some fish, such as zebrafish, spawn several times in a year and do not have synchronized cycles. Our laboratory has preliminary data indicating LMB exhibit seasonal changes in E₂ and testosterone. Steroid levels were correlated with the corresponding stage of ovarian follicle maturation during a one year time span. LMB can release up to 100,000 eggs from the matured ovarian follicles per year (http://www.go4bass.com/largemouth.html.)
Ovarian Follicles

Ovarian follicles provide an essential source of steroid biosynthesis in females. The term follicle refers to the oocyte surrounded by an internal granulosa cell layer and external thecal cell layer, which contains fibroblasts, collagen fibers, and thecal cells (Figure 1-2). The thecal and granulosa layers are separated by a basal lamina (Babin, 1986) and both cell types are able to produce steroids. The thecal cells, in fish, are what primarily have been found to form testosterone since biochemical and ultrastructural studies show these cells contain 3-beta-hydroxy-steroid-dehydrogenase (3-β-HSD) (Kusakabe et al., 2003). 3-β-HSD is an enzyme involved in the conversion of pregnenolone to progesterone, and progesterone can then be metabolized to testosterone. Granulosa cells contain the P450 aromatase enzyme, which converts testosterone to E₂ (Nagahama et al., 1995). It has been shown in fish that synthesis of testosterone by thecal cells increases during vitellogenesis (Nagahama et al., 1995).

Vitellogenesis is defined as the hepatic synthesis and secretion of vitellogenin (VTG), an egg yolk precursor, followed by uptake of VTG into the oocyte from the bloodstream by receptor-mediated endocytosis. After endocytosis, VTG is cleaved by specific cathepsins to form yolk proteins (Wallace and Selman, 1990). The production of VTG is stimulated by E₂ (Skipper and Hamilton, 1977).

Ovarian follicles go through several stages of development which involves many complex processes. At the most immature stage, oocyte cell cycles are arrested at prophase of meiosis and are called primordial follicles (Wallace and Selman, 1990). The phase during which the oocyte grows and granulosa cells proliferate is called the primary-follicle stage. During this stage, the theca cells begin to differentiate and continued follicle development becomes reliant on gonadotrophins, with FSH (follicle
stimulating hormone, also called GTHI in fish) levels being very elevated (Kagawa et al., 2003).

Upon completion of follicle growth, LH (luteinizing hormone, also called GTHII in fish) levels rise for the final maturation and ovulation of the oocyte. Gonadotropin stimulates the production of a steroid, DHP (17α, 20ß-dihydroxy-4-pregnen-3-one), which is involved in the maturation by binding directly to a receptor on the oocyte (Pang and Ge, 2002). DHP is made in the granulosa cells from 17α-hydroxyprogesterone, which is made in the thecal cells, showing the interconnection between the two cells. Following maturation, the oocyte is released and is either fertilized or undergoes atresia, a process shown to involve apoptosis (Manabe et al., 2004). Completely developed ovarian follicles in LMB are about 1.4 to 1.5 mm in diameter, but that diameter range can vary across species.

Ovarian follicles have been cultured from various species, including mammals and fish, to study the regulation of steroidogenic enzymes by endogenous and exogenous substances (Petrino and Shuetz, 1986; Babin, 1986). Follicle diameter, stage of maturation, and quantity of cultured follicles are all parameters that can be selected in this assay. Another benefit of using cultured ovarian follicles is that the contact between granulosa and thecal cells remains intact, which more closely resembles an in vivo system (figure1-2). It has been shown that there is increased steroidogenesis in co-cultures of granulosa and theca cells than when either cell type is cultured alone (Shores et al., 2000).

**Cholesterol and Steroidogenesis**

Cholesterol is the backbone for steroid hormones and can be derived exogenously from dietary sources or endogenously synthesized in the small intestine or liver.
 Typically, hepatic or intestinal cholesterol production supplies 2 to 3 times the amount that is absorbed from food (Lu et al., 2001). In addition to playing a vital role in metabolic homeostasis, cholesterol is a major component of the plasma membrane, helping to ensure the integrity of cellular structure. That same cholesterol can also be extracted from the membranes for steroid production (Pichler and Riezman, 2004).

Multiple fates exist for cholesterol after being synthesized or absorbed, but it is primarily packaged into lipoproteins for intracellular transport. Lipoproteins are categorized as chylomicrons, LDL (low density lipoprotein), VLDL (very low density lipoprotein), and HDL (high density lipoprotein) based on their roles in sterol transport. Chylomicrons shuttle dietary cholesterol from the small intestine to the peripheral tissues; VLDL and LDL transfer endogenously derived sterols from the liver to tissues. HDL provides one of the only ways to clear cholesterol from the body by returning cholesterol from the tissues back to the liver (Ginsberg, 1998). The liver metabolizes cholesterol into bile acids that can either be further broken down by microorganisms in the large intestine and excreted in urine, or re-used by the body to aid in fat digestion.

There are a couple of proteins which are crucial to maintaining appropriate levels of cholesterol. HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis and is therefore a target of many pharmaceuticals to alleviate symptoms of high blood pressure, atherosclerosis, as well as other diseases (Rosanoff and Seelig, 2004). The steroidogenic acute regulatory protein (StAR Protein) is the rate-limiting step in cholesterol metabolism and it is just beginning to be investigated (Figure 1-3).

\( \beta \)-Sitosterol

\( \beta \)-sitosterol is a phytosterol that shares structural similarity with cholesterol (Figure 1-4). It is discharged from paper mills into waterways upon processing of paper.
products. β-sitosterol makes up about 65% of the phytosterols present in paper mill effluent. The other phytosterols include campesterol, stigmasterol, and sitostanol (MacLatchy and Van Der Kraak, 1995; McMaster et al., 1996; Sepulveda et al., 2001). Unlike cholesterol, there is no endogenous production of the phytosterols in vertebrates and they can only be obtained through dietary sources (Salen et al., 1970).

β-sitosterol is metabolized to pregnenolone and steroid hormones; however, studies suggest β-sitosterol metabolism is less efficient than cholesterol (Bennett et al., 1969; Aringer et al., 1979; Werbin et al., 1960). Absorbed phytosterols circulate in lipoprotein particles but the rates of absorption for the different phytosterols vary. There is evidence that phytosterols may accumulate in steroidogenic tissue, including the ovary, testis, and adrenal gland of animals (Moghadasian and Frohlich, 1999). Absorption into steroidogenic tissues suggests that phytosterols, like cholesterol, can serve as precursors to steroid hormone synthesis.

β-sitosterol is used clinically to lower cholesterol. Studies have shown that plasma cholesterol levels are significantly lowered by 10 days in humans fed a diet of 20µg/g body weight of phytosterols (Jones et al., 1998). Although only about 5% of phytosterols are absorbed, studies have shown that β-sitosterol competes with cholesterol for uptake into bile acid micelles (Compassi et al., 1997). In cells incubated with micelles containing either β-sitosterol, cholesterol, or both, β-sitosterol was shown to decrease the movement of cholesterol from the plasma membrane into the cell (Field et al., 1997). It is thought that β-sitosterol displaces cholesterol from the bile acid micelles. Bile acids, metabolites of cholesterol, are necessary for cholesterol absorption (Sirtori et al., 1991). If cholesterol is displaced from the bile acid micelles by sitosterol, the absorbability of
cholesterol by cells is diminished (Field et al., 1997). Phytosterols lower overall plasma cholesterol levels by inhibiting intestinal cholesterol absorption or by preventing recirculation of bile acids.

Although β-sitosterol is used to treat patients with high cholesterol levels, high plasma concentrations of phytosterols in animals may have deleterious effects on reproductive organs. Rats injected with 0.5 to 5 mg/kg body weight per day of β-sitosterol exhibited a diminished sperm count and testes weight (Moghadasian and Frohlich, 1999). In addition studies have shown that steroid levels are decreased in goldfish when given a single injection of 5, 10, or 100 µg/g β-sitosterol (MacLatchy and Van der Kraak, 1995). Pregnenolone, a metabolite in the conversion of cholesterol to steroid hormones, is also decreased with β-sitosterol exposure.

Transforming Growth Factor-Beta (TGF-β)

Although the exact mechanism of how β-sitosterol represses steroidogenesis is still unknown, it has been shown that β-sitosterol upregulates TGF-β in prostate cells (Kassen et al., 2000). There is also evidence that TGF-β may be regulated by dioxins, also found in the environmental (Dohr et al., 1994), suggesting a possible signaling pathway used by toxins to repress steroidogenesis. TGF-β, through SMAD proteins, has even been shown to modulate the arylhydrocarbon receptor, a transcription factor which is regulated by dioxins and for which a response element has been found in the human StAR promoter (Wolff et al., 2001).

TGF-β is a powerful cytokine involved in cell signaling and has been shown to inhibit steroidogenesis in adrenocortical, trophoblast, and testicular cells (Liakos et al., 2003; Luo et al., 2002; Gautier et al., 1997). Several recent studies have even shown the
StAR Protein to be a major target for TGF-β regulation. Transfection studies in a human adrenocortical cell line, H295R, showed that 1.3 Kb of the human StAR promoter is inhibited by about 25% when cells are treated with 1ng/ml TGF-β for 24 hours (Brand et al., 1998, 2000). The authors then tested several deletions of the 1.3 Kb promoter with TGF-β and showed that all the deletions were downregulated except the 0.085 Kb construct. The study suggests the regulation of StAR by TGF-β is mediated by elements located between 0.085 Kb and 0.15 Kb upstream of the transcriptional start site in the mammalian promoter. It was also shown that mutating the various SF-1 sites did not alleviate the downregulation of the promoter, however, no other transcription factors were examined.

There is substantial research outlining the specific signaling cascades that TGF-β triggers. Two serine/threonine receptor tyrosine kinases, Type I and Type II, are assembled and dimerized when TGF-β or other related ligands are bound. The dimerization leads to Type I receptor activation by phosphorylation in a glycine/serine rich domain. The active receptor can then phosphorylate and activate SMAD proteins (Figure 1-5). SMAD proteins are the essential link in TGF-β signaling (de Caestecker, 2004).

Research has specifically shown SMAD3 involvement in TGF-β inhibition of human StAR transcriptional activity in adrenocortical cells. Overexpression of a wild-type SMAD3 protein in the cells potentiated the inhibitory action of TGF-β on StAR mRNA levels, whereas, expression of a mutant SMAD3 alleviated some of that repression (Brand et al., 1998). SMADS encompass a large and diverse family of proteins that can either activate or repress gene expression. A primary way in which
TGF-β signaling can be terminated is by ubiquitinylation of SMADS in the nucleus, which targets the proteins for proteosome-mediated degradation (Lee et al., 2003).

**Research Objectives**

The goals of this project were to examine cellular signaling mechanisms involved in regulation of LMB StAR by environmental toxins. cAMP and TGF-β are two potent signaling molecules known to regulate steroidogenesis in mammalian species, therefore, their role in LMB StAR regulation was examined. The overall hypothesis of this project was that LMB StAR transcription and post-translation activity are upregulated by cAMP and downregulated by TGF-β.

The project was divided into three specific aims to meet the overall objective. Specific aim 1 was cloning the LMB StAR cDNA and development of a specific polyclonal antibody. Specific aim 2 was to develop LMB ovarian follicle cultures. The follicle assays were used to measure changes in mRNA by real-time PCR and protein by western blot detection with the anti-StAR antibody upon exposure to cAMP or TGF-β. Specific aim 3 was to comprehensively examine the transcriptional regulation of LMB StAR. For aim 3, the promoter was cloned and used in transfection assays in conjunction with promoter deletion and site-directed mutagenesis experiments for functional transcriptional response element analysis.
Figure 1-1. Alignment of mammalian StAR promoters. Line-up of the first hundred base pairs for the sheep, pig, human, and rat showed conservation of several critical transcriptional binding sites, including SF-1, GATA, and C/EBP.
Figure 1-2. Fish ovarian follicle. The ovary of a fish is composed of many individual follicles, where steroids are produced by the theca and granulosa cells, located below the epithelial membrane. Vitellogenin, made in the liver, is the egg yolk precursor for the growing follicle.
Figure 1-3. General pathway for steroidogenesis. Cholesterol is the backbone for all steroid hormones. Cholesterol is metabolized in the mitochondria to pregnenolone. The various steroids are then formed from pregnenolone.

- Cholesterol
- StAR Protein
- Mitochondria
- Pregnenolone → Progesterone → Mineralocorticosteroids
- 17-Hydroxyprogesterone → Glucocorticosteroids
- Androstenedione → Steroid hormones: Estrogens, Androgens
Figure 1-4. Structures of β-sitosterol and cholesterol. β-sitosterol is the plant equivalent of cholesterol with the only structural difference being an additional ethyl group (outlined in red) for β-sitosterol.
Figure 1-5. TGF-β signaling pathway. Binding of TGF-β to the Type II receptor kinase induces dimerization of the Type II and Type I receptor. A cascade of phosphorylation and signaling events mediated by SMAD proteins occurs following the receptor dimerization, ultimately activating transcription of many different genes.
CHAPTER 2
MATERIALS AND METHODS

The overall goals of this project were to characterize the expression of the largemouth bass (LMB) Steroidogenic Acute Regulatory Protein (StAR) and then specifically examine its regulation at the transcriptional level. Cloning the LMB StAR coding region and a portion of the promoter region were the initial steps in achieving the goals of this project. The sequence information was used for development of assays, including real-time PCR, transfections, and western blots, to quantitate changes in LMB StAR transcription and translation. StAR expression was examined in response to various endogenous and exogenous chemicals, including cAMP and transforming growth factor beta (TGF-β), central molecules involved in cell signaling pathways.

Animals

Largemouth bass (*Micropterus Salmoides*) were used for all tissue and ovarian follicle cultures and were purchased from American Sport Fish Hatchery (Montgomery, AL). All fish were housed at the Center for Environmental and Human Toxicology at University of Florida in accordance with the National Institute for Health (NIH) Guide for the Care and Use of Laboratory Animals.

**LMB StAR mRNA Expression**

**Cloning of StAR**

Total RNA was isolated from largemouth bass ovarian tissue using the RNaseasy Kit with spin columns (Qiagen). The quality of RNA was verified by examining 3-5 µg on a formaldehyde based gel and looking for the presence of two bands, with the 28S rRNA...
band being twice the intensity of the 18S rRNA band. 3 µg of RNA was reverse transcribed into cDNA with 200 units of SuperscriptII enzyme (Invitrogen). Basically, the reverse transcription reaction involved heating the RNA and 150 ng random hexamers at 70 °C for 10 minutes. A master mix of 10 mM DTT, 0.2 mM dNTP mix (stock has 2.5 mM each nucleotide), and 5X buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂] was diluted to 1X in a 50 µl final reaction volume. After adding the master mix, the reaction was heated at 42 °C for 2 minutes. The reaction was then stopped to add 200 units of SuperscriptII which was then followed by continued heating at 42 °C for 50 minutes. A final extension at 70 °C for 15 minutes completed the reaction.

LMB StAR was PCR amplified from the ovarian cDNA using partially degenerate primers designed with the web program CODEHOP (http://blocks.fhcrc.org/codehop.html) and an alignment of various mammalian sequences in the database, including human, pig, horse, and cow. Sequences for the forward and reverse primers were 5’TGGAGCAGATGGGCGANTGGAAYCC3’ and 5’TGATGATGGTCTTGGGCADCCANCCYTT3’, respectively. The cDNA was amplified using 10 picomoles (pmol) of each primer, 1 Unit of amplitaq, and 1.5 mM MgCl₂, with 10X PCR buffer [500 mM KCl and 100 mM Tris-HCl (pH 8.3)] diluted to 1X in a 20 µl final volume. The PCR reactions used a primer annealing temperature of 60.9 °C for 45 cycles in a Perkin Elmer 9600 model thermocycler. The PCR reaction was run out on a 1% agarose gel and the predicted 350 base pair band was gel purified by excising the ethidium bromide stained bands from two 20 µl loaded lanes under low UV light and then using the gel purification kit and protocol from Qiagen.
The gel purified DNA was ligated into a pGEM-T cloning vector. The amount of DNA to ligate into the vector was calculated by multiplying 50 ng of vector by the size of the DNA to be inserted and then dividing that number by the size of the cloning vector, which was 3 Kb for pGEM-T. The concentration of the gel purified product was quantified using a DNA mass ladder. A molar ratio of both 1:1 and 3:1 for insert to vector was used. Ligation was done at 16 °C overnight with a reaction containing the gel purified product (see calculation above), 3 units T4 DNA ligase (Promega), 50 ng pGEM-T vector (Promega), and 2X ligation buffer (Promega-proprietary) diluted to 1X in a final volume of 10 µl.

The ligation product (3 µl) was transformed into 50 µl of DH5α cells (Invitrogen). Basically, the transformation involved incubating the cells and ligation product on ice for 20 minutes, then heat shocking the cells for 45 seconds at 42 °C followed by immediate cooling on ice for 2 minutes. The cells were then shaken in 950 µl of LB broth for 1 ½ hours at 230 rpm before being plated on ampicillin (100 µg/µl) LB/agar plates for overnight incubation at 37 °C. Colonies were screened for ligation by PCR with M13 primers. Positive clones were minipreped using the Qiagen spin column kit and sent for sequencing at the University of Florida ICBR DNA Sequencing Core.

RACE (rapid amplification of cDNA ends) were used to sequence the remaining coding regions of LMB StAR using the SMART RACE protocol from Clontech/BD Biosciences (Figure 2-1). Briefly, first-strand cDNA for 5’ RACE was prepared by reverse transcribing 1 µg of total RNA isolated from LMB ovary using 1 µl of Powerscript Reverse Transcriptase (Clontech, proprietary), 1 µl of 5’ RACE primer (proprietary concentration; 5’ (T)25VN3’), 1 µl SMART oligo
(5’AAGCAGTGGTATCAACGCAGAGTACGCGGG-3’), 1.3 mM DTT, and 2 µl of 5X First Strand Buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 30 mM MgCl2) in a final volume of 15 µl for a 1.5 hour incubation at 42 º C. The reverse transcriptase reaches the 5’ end of the mRNA and adds several dC residues, which serves as a template on the opposite strand for an oligo that contains 3 G residues (Smart II oligo) at its 3’ end.

To make cDNA for 3’ RACE, the same protocol was followed for that of 5’RACE, however, the following primer [5’AAGCAGTGGTATCAACGCAGAGTAC(T)30VN3’] in proprietary concentrations was used and the SMART oligo was not added to the reaction mix.

The 5’RACE and 3’ RACE cDNA that was synthesized was then used in PCR reactions with gene specific primers and primers to the Smart II oligo. The primers and thermocycler conditions used for 5’ and 3’ RACE are listed in Table 2-1 and Table 2-2. All gene specific primers, designed using the Oligo program, were between 23 and 28 nucleotides in length, 50-70% GC content, and a melting temperature of greater than 65 º C. Nested gene specific primers were also designed to help eliminate non-specific PCR amplification. All PCR reactions contained 1 µl of 50X Advantage 2 Polymerase Mix (Invitrogen) and 0.2 mM dNTP stock mix in a final volume of 50 µl. Initial PCR reactions were diluted 1:50 in Tricine-EDTA (10 mM Tricine-KOH-pH 8.5; 1 mM EDTA) and 1 µl of the diluted DNA was used for nested/secondary PCR amplifications. For 3’RACE, a gene specific primer and primer to the poly A adaptor oligo (Invitrogen) that was attached during reverse transcription was used for PCR amplification. 5’RACE amplification used a primer (Invitrogen) to the 5’cDNA adaptor oligo and gene specific primers. Products were cloned into the pGEM-T vector and plated onto LB/AMP agar
plates as described in the above section. All sequence information was verified by at least 3 different clones.

**Development of LMB Real-Time PCR Assay for mRNA Quantitation**

Quantitation of LMB StAR mRNA levels in ovarian tissue and follicle cultures was done by real-time PCR using Taqman technology. RNA for all Taqman reactions was extracted from about 5 mg (10 follicles) of bass ovarian follicles using RNA STAT-60 reagent (Tel-TEST). The ovarian follicles were homogenized in 500 µl of RNA STAT-60 using a polytron followed by addition of 150 µl of chloroform. After centrifugation at 12,000 x g for 15 minutes, the upper aqueous RNA containing layer was removed for an additional RNA STAT-60/chloroform extraction. The RNA was then precipitated with 250 µl of 100% isopropanol overnight at -20 º C and then pelleted by a 30 minute centrifugation at 12,000 x g at 4 º C. The RNA pellets were washed twice with 75% ethanol (made with DEPC treated water) and then resuspended in 25 µl of RNAsecure, a reagent that helps minimize RNase activity (Ambion). All of the RNA (all samples contained less than 10 µg total RNA) was treated with 2 units of DNA-Free (Ambion) for an hour to remove traces of contaminating chromosomal DNA. Quality of RNA was checked on an agarose gel for the presence of 28S and 18S ribosomal bands.

3 µg of each RNA sample was reverse-transcribed into cDNA using 25 units of Stratascript enzyme (Stratagene), 1500 ng random primers, 1mM of each dNTP (4 mM total dNTP), along with 10X Stratascript buffer (Stratagene) diluted to be 1X in a final reaction volume of 25 µl. In short, RNA, random primers, and water were added first, then all tubes were heated at 65 º C for 5 minutes followed by cooling at room temperature. Then, 4 µl of a master mix of dNTPs, buffer, and Stratascript enzyme was
added to each reaction followed by incubation at 42 °C for 1 hour. The enzyme was inactivated by heating the reactions at 90 °C for 5 minutes.

The primers for real-time PCR using Taqman technology were designed using the Primer Express program (Applied Biosystems) based on the original sequence obtained for LMB StAR. All PCR reactions used 10 pmoles of the forward primer, 5’ACCCCTCTGCTCAGGCATTT3’, and 10 pmoles of the reverse primer, 5’GGGCTCCACCTGCTTCTTG3’, to amplify 0.12 µg of reverse-transcribed RNA using universal thermocycler parameters as recommended by Applied Biosystems. For the real-time PCR assays, 2X Sybr Green reagent (Applied Biosystems) was used at a concentration of 1X in the final reaction volume. Sybr Green is a fluorescent dye that binds to double stranded DNA. Therefore, as more amplification occurs, more fluorescence is detected. A dissociation curve was run with the LMB StAR Taqman primers to check for amplification specificity after the PCR cycles are completed (Figure 2-2). A dissociation curve is generated by raising the temperature and obtaining a specific melting point for separation of double stranded amplified StAR DNA, which is represented by a steep loss of fluorescence signal.

A standard curve for real-time PCR quantitation was developed with known amounts of the StAR plasmid. Standard curves typically were performed in 10X dilutions from 8.1 X 10^6 to 8.1 X 10^1 copy numbers of plasmid and samples with unknown amounts of StAR were quantitated by extrapolation to the standard curve. A sample standard curve and amplification curve is shown in Figure 2-2. The slope for the standard curve should be around -3.3, which indicates perfect doubling per cycle.
All Taqman reactions were normalized to 18S rRNA using universal thermocycler conditions as recommended by Applied Biosystems. Each 25 µl reaction contained 1.25 µl of 18S rRNA master mix containing proprietary amounts of primers and probes (Applied Biosystems, catalog #4310893E), 0.12 µg of reverse transcribed RNA, and 2X Taqman universal primer mix (Applied Biosystems, catalog # 4304437) diluted down to 1X in the final reaction volume.

All real-time PCR calculations were based on converting the concentration of the StAR plasmid used in standard curve to copy number. The conversion to copy number was done since the concentration of the plasmid DNA includes the cloning vector plus StAR DNA, which would not accurately reflect the concentrations for StAR. Converting to copy number ensures that the plasmid is considered as one unit, including both the vector and insert, and amplification is therefore a reflection of that unit. All quantities from real-time PCR were measured as copy number of plasmids amplified. The calculation for determining amount of copy number used to develop the standard curve is:

\[
\frac{6 \times 10^{23} \text{ (copies/mol)} \times \text{plasmid concentration (g/ul)}}{\text{molecular weight of the plasmid (g/mol)}} = \text{copies} / \text{ul}
\]

Seasonal Study

Adult LMB between 2 and 3 years old were maintained in freshwater ponds at the USGS facility in Gainesville, FL. Female fish were caught by electroshock bi-weekly over a five month time span. Ovarian tissue was carefully removed and immediately flash frozen with liquid nitrogen for long-term storage at -80 °C.
LMB Ovarian Tissue Cultures

Ovarian tissue cultures from LMB were cultured to detect changes in mRNA levels after exposure to various chemicals. The ovarian tissue was carefully dissected into 20-30 mg pieces, rinsed with culture media, and immediately placed in 1 ml of Dulbecco’s Modified Eagle’s Medium Nutrient Mixture (DMEM) with F-12 Ham containing L-Glutamine and 15mM HEPES (Sigma) supplemented with 1.2 grams of sodium bicarbonate and 1% antibiotic/antimycotic solution (ABAM). This media has the same osmolality as LMB plasma, about 295 mOsmol/kg (Bowman thesis, 2001). Cultures were equilibrated in a chilled incubator at 21-22 °C with 5% CO2 for 24 hours prior to exposure with appropriate chemical to be studied. All experiments were carried out in 24 well culture plates, which were placed on a slow moving shaker during the exposures.

LMB Ovarian Follicle Cultures

Ovarian follicles were isolated from the ovary and cultured for further examination of LMB StAR mRNA expression. Follicles were individually dissected and measured with a micrometer to control size and stage of follicles incubated. Follicles were incubated in 500 µl of the DMEM culture media and equilibrated for 24 hours prior to exposure. Ten follicles were incubated per well of a 24 well cell culture plate. Follicles and culture were removed from the culture plate post exposure by using a BSA (bovine serum albumin) coated wide boar 1 ml pipet tip and placed in a 1.5 ml microcentrifuge tube. Follicles were gently pelleted to the bottom of the tube by centrifugation for 5 minutes at less than 3000 RPM. Follicles were washed once with 1X PBS (phosphate buffered saline) and then frozen at -80 °C until RNA isolation.

The viability of follicles was tested by adding 10% Alamar Blue reagent (Biosource) to the cultures and looking for reduction of Resazurin (blue and
nonfluorescent) to resorufin (pink and highly fluorescent). Changes in the color from blue to pink for metabolically active cells can be detected with spectrophotometry readings at $A_{570}$ and $A_{600}$.

**LMB StAR Protein Quantitation**

**Protein Expression Vector**

There is no commercial antibody available for StAR that cross reacts with any fish species, therefore, we developed a polyclonal antibody for western blot detection. First, a protein expression vector was constructed by amplifying the entire coding region in one piece using 10 pmol of ATGCTACCTGCAAACCTTCAAACTGTG as the forward primer and 10 pmol of TCAGCAGCGTGAGCCATCTCCATA as the reverse primer. The annealing temperature was 72 °C for 7 cycles followed by 67 °C for 42 cycles. The PCR reaction contained the primers 1 µl of 10 mM dNTP mix, 2 µl of 25 mM Mg(OAc)$_2$, 0.15 µg of LMB ovarian cDNA, 50X Advantage Genomic Polymerase Mix diluted to 1X [Clontech: 5-6 units/µl Tth DNA polymerase, 0.5µg/µl TthStart antibody, 50% glycerol, 10 mM Tris-HCl (pH 7.5), 230 mM KCl], and 10X PCR reaction buffer [400 mM Tris-HCl (pH 9.3), 150 mM KOAc, 0.2% Triton X-100] diluted to 1X in a final volume of 50 µl.

The entire coding region was ligated into the pET-28b vector (Novagen) (Figure 2-3) and transformed into DH5α cells (Invitrogen) using similar procedures as outlined in the section for cloning of StAR. Expression with the pET-28b vector produces proteins with a 6 histidine tag on to N-terminus to ultimately allow for purification with an appropriate affinity column.

Amplification of the full length StAR resulted in 8 amino acid mistakes that were fixed using the QuikChange Kit (Stratagene). Primers to change the appropriate
nucleotides were designed using Stratagene’s website program for site directed mutagenesis (Table 2-3). The primers were designed to have the targeted mutation near the middle, a minimum of 40% GC content, and a 3’ end that terminates with one or more G or C bases. Complementary reverse primers were designed against each forward primer. The QuikChange protocol basically involved a PCR reaction using 125 ng of each relevant forward and reverse primer, 38 ng of StAR plasmid template, 1 µl of a proprietary dNTP mix, and 2.5 U/µl of Pfu Turbo DNA polymerase in a 50 µl final volume. Reactions were amplified in a thermocycler for 12 cycles of 95 °C at 30 seconds, 55 °C for 1 minute, and 68 °C for 7 minutes. The parent strand is digested by DpnI, leaving the corrected product to be transformed in DH5α cells. The transformed constructs were minipreped with the Qiagen miniprep spin kit and DNA was sent for sequencing to verify that the relevant nucleotides were fixed. Since pET-28b is a low-copy number plasmid, concentration yields from minipreps were maximized by using 3 ml of bacterial culture and by eluting the purified DNA from the spin column with 70 °C water. The final, completely corrected construct was transformed into BL21 (DE3) cells (Novagen) for bacterial expression.

**Bacterial Protein Induction**

The LMB StAR expression construct was grown in 30 ml of LB broth with 24 µg/ml kanamycin for 4 hours at 37 °C with constant shaking at 280 rpm. The bacterial cultures reached a desired density after the 4 hours with $A_{600}$ spectrophotometry readings between 0.6 to 0.8. StAR protein expression was then induced in the cultures with 1 mM or 3 mM IPTG for an additional 4 hours with 1ml aliquots taken hourly. Bacterial cells were pelleted by spinning down the 1 ml of culture at 10,000 x g for 1 minute, discarding the supernatant. The pellet was resuspended in 100 µl of 1X phosphate buffered saline
(PBS) along with 1 µl of benzonase to lessen the viscosity. 100 µl of 4X SDS buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 10% 2-mercaptoethanol, 300 mM DTT, 40% glycerol, and 0.02% bromophenol blue) was then added to the protein sample and heated at 85 °C for 3 minutes for denaturation. The denatured protein samples were run on a 4-12% Bis-Tris NuPAGE gel (Novex) with MES running buffer for 30 minutes. The gel was stained with Colloidal Coomassie Blue Stain (Genomic Solutions) overnight followed by de-staining for 2 hours.

The bands on the protein expression gel that were induced by IPTG compared to the controls were excised and ultimately subjected to digestion with trypsin for definitive identification of largemouth bass StAR peptide fragments in the gel. The in-gel trypsin digestion protocol involved washing the gel pieces with 50% acetonitrile (ACN) 3 times while vortexing for 15 minutes followed by dehydration of the gel with 100% ACN until gel piece turns white. The gel was then rehydrated with 100 mM ammonium bicarbonate (ABC) for 5 minutes. The proteins in the gel piece were reduced with 45 mM DTT for 30 minutes at 55 °C. DTT is a reducing agent that separates proteins which are linked by disulfide bonds for more effective analysis by mass spectrometry. To prevent the cysteine residues in the separated peptides from recombining, they are alkylated with 100 mM iodoacetatamide for 30 minutes in the dark at room temp. The gel piece was then washed for 15 minutes 3 times with 50% ACN/50 mM ABC while vortexing. The gel was completely dried in a speed vac prior to digestion with 12.5 ng/µl Trypsin (Promega) prepared in 50 mM ABC pH 8.4, 5 mM CaCl₂ on ice for 45 minutes. The enzyme solution was then removed and replaced with just the buffer for incubation overnight at 37 °C prior to analysis by mass spectrometry.
Protein Purification

Purified LMB StAR was obtained by inducing 10 ml of bacterial culture for 4 hours with the StAR expression plasmid (see section on bacterial protein induction for details). The bacteria were pelleted at 4000 x g for 15 minutes and stored at -80 °C until ready for purification.

A nickel affinity column (Ni-NTA spin column kit, Qiagen) was used to purify histidine-tagged StAR from the bacterial pellet under denatured conditions. The bacterial cells were lysed by thawing the pellet for 15 minutes and then resuspending them in a buffer of: 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 8.0. The cells were shaken for 1 hour at room temperature. Cellular debris was removed from the lysate by centrifuging at 10,000 X g for 20 minutes. Spin columns were equilibrated with 600 µl of the pH 8 buffer followed by centrifugation at 700 x g for 2 minutes. Histidine tagged proteins were bound to the nickel affinity column by flowing 600 µl of the bacterial lysate through the equilibrated column at 700 x g for 2 minutes. The columns were washed 3 times with 600 µl of a buffer containing: 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 6.3. Finally, the bound proteins were eluted from the spin column with 200 µl of the following buffer: 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 4.5.

The purified protein was quantitated by protein assay using Coomassie Plus Reagent. Two micrograms of the purified protein was run out on a 4-12% Bis-Tris NuPAGE gel (Novex) and stained overnight in Colloidal Coomassie Blue followed by a 2 hour de-stain.

Development of StAR Antibody

A polyclonal antibody was made against the largemouth bass StAR Protein. The antibody was produced by injecting two different rabbits (Cocalico company) with a
synthetic peptide designed to a part of the coding sequence. The antigenic peptide used for rabbit injections, CFLAGMSTQHPKMPEQRGVVR (Figure 2-4), was constructed in an area of the coding region where prolines were present so the antibodies are able to recognize relatively exposed areas of StAR. The peptide was conjugated to a carrier protein, KLH (keyhole limpet hemocyanin), at the University of Florida Protein Core to help ensure an immunogenic response to the StAR peptide.

For the rabbit injections, the following schedule was followed: Day 0 = Prebleed/Initial Inoculation, Day 14 = Boost, Day 21 = Boost, Day 35 = Test Bleed, Day 49 = Boost, Day 56 = Test Bleed. Monthly boosts were subsequently continued for about ½ year to obtain more antiserum.

**Western Blots**

The specificity and reactivity of the LMB StAR antibody was tested by western blot using the purified StAR protein as a positive control. 1µg of purified protein was run on a 4-12% Bis-Tris NuPAGE gel followed by transfer to nitrocellulose membrane at 100 volts for 1 hour. The transfer buffer contained 20 mM Tris, 144 mM glycine, and 20 % methanol. The nitrocellulose membrane was blocked for 3 hours, while shaking, with 5% Carnation non-fat dry milk diluted in 1X TBST (25 mM Tris, 0.15 M NaCl, 0.05 % Tween-20, pH 7.6) to help reduce non-specific antibody binding. The membranes were then washed 4 times, at 15 minutes each, with 1X TBST.

Washed membranes were then exposed to the primary antibody for 3 hours with vigorous shaking. Dilutions of 1:5000 and 1: 15000 primary antibody were tested. Following the incubation, primary antibody was removed by washing 4 times with TBST before incubation with concentrations of 1:20000 and 1:40000 secondary antibody for one hour. The secondary antibody used was a mouse anti-rabbit IgG conjugated to
horseradish peroxidase (Pierce). Membranes were again thoroughly washed with TBST before antibody binding was detected using chemiluminescence after exposing for 3-4 minutes to 4 ml of each chemiluminescent reagent (Super Signal West Pico Chemiluminescence Kit, Pierce.) Presence of luminescence was captured on Kodak film.

**Transcriptional Regulation of LMB StAR**

**Cloning of the Promoter**

Most transcriptional regulation occurs in the promoter, therefore, the first goal for this part of the project was to clone the promoter for LMB StAR using the GenomeWalker Kit (Clontech) (Figure 2-5). The promoter cloning started with isolating high quality genomic DNA that was phenol/chloroform purified twice from 20 mg of LMB ovarian tissue using the Wizard Kit (Promega.). Basically, the genomic isolation involved lysing the tissue with 600 µl of a lysis solution (proprietary, Promega) for 20 minutes at 65 °C. Contaminating RNA was degraded by incubation of the lysate for 20 minutes at 37 °C with 3 µl of an RNase solution (proprietary, Promega). The solution was chilled on ice with 200 µl of a Protein Precipitation Solution (proprietary) and the protein was removed by centrifuging at 14,000 X g for 4 minutes. The supernatant was put into a new tube. Genomic DNA was precipitated from the remaining supernatant with 600 µl of isopropanol and pelleted by centrifuging at 15000 X g for 1 minute. The pellet was washed with 70% ethanol and then air dried for 12 minutes. The pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH7.3; 1 mM EDTA, pH 8.0) by incubating at 65 °C for 1 hour. Residual protein was removed from the genomic DNA with a phenol/chloroform extraction followed again by resuspension in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.5).
Genomic DNA (2.5 µg) was digested for 20 hours with 4 different restriction enzymes; StuI, EcoRV, DraI, and PvuII to create digested libraries for PCR amplification. Complete digestion of the genomic DNA was checked by running 5 µl on a 0.5% agarose gel stained with ethidium bromide at 70 volts for one hour. The restriction libraries were purified with 2 phenol/chloroform extractions. An adaptor oligo (Clontech) was ligated onto the various digested pieces of genomic DNA by incubating with 3 units of T4 DNA ligase for 20 hours at 16 °C.

PCR amplification of the LMB StAR promoter from the restriction libraries was done using a primer to the ligated adaptor oligo and a gene specific primer. Two different sets of gene specific primers were used, one set of primers started closer to the 5’end of the coding sequence than the other, which gave overlapping sequences and provided for some sequence verification. One set of gene specific primers used was 5’CAGGCAACATCTTACTCAGGACTTTGTC3’ (promoter jk1). It was followed by 5’TACCTTGCTTCACATAAGACATCTCT3’ (promoter jk2) for the nested reaction. The second construct used 5’TTCCTGGCTTCACATAAGACATCTCT3’ (promoter jk3) for the primary PCR reaction and 5’CAGGCAACATCTTACTCAGGACTTTGTC3’ (promoter jk1) for the nested reaction. All primers were designed using the Oligo Program and were between 26 and 30 nucleotides with a 40-60% GC content, with no more than 3 G and C’s in the last 6 bases of the 3’ end. Nested gene specific primers were used to help reduce non-specific amplification. All secondary PCR reactions using the nested primers were done using a 1:50 dilution of the primary PCR product in a final volume of 50 µl. Thermocycler conditions for the promoter cloning are listed in Table 2-4. PCR products were gel
purified with spin columns and protocol from Qiagen and ligated into the pCR2.1-TOPO vector (Invitrogen) using a similar protocol as outlined under the section for StAR cloning. The sequence for the 2.9 Kb promoter fragment (named pSP1 plasmid) was verified with two independent PCR reactions.

The promoter was cloned into the pGL3 basic vector (Promega) (Figure 2-6) for transfections. The cloning of the promoter involved two phases, first, both the pGL3 and pSP1 plasmids were double digested with XhoI and HindIII promega enzymes and Buffer B (6 mM Tris-HCl, 6 mM MgCl₂, 50 mM NaCl, 1 mM DTT, pH 7.5) for 13 hour digestion at 37 °C. The XhoI/HindIII fragment vector was ligated into the cut pGL3 vector by protocol outlined under StAR cloning section and the plasmid was named pLUCStAR1. The pLUCStAR1 construct, however, still had the translational start site from the LMB StAR sequence since the primers used for promoter cloning originated in the coding region. Removing the ATG start site from the plasmid was essential since the pGL3 plasmid contains its own ATG site for luciferase protein synthesis. The ATG start site was removed from the pLUCStAR1 with a double digest of HindIII (Stratagene) and BlpI (NEB labs) in buffer #2 (NEB labs = 20 mM Tris-OAc, 50 mM KOAc, 10 mM Mg(OAc)₂, 1 mM DTT; pH 7.90) @ 25°C.

All promoter constructs were obtained using endotoxin–free maxiprep kits (Qiagen). The maxiprep protocol basically involved growing up a single colony of the plasmid in DH5α cells in a 3 ml LB/Ampicillin (100 µg/ml AMP) starter culture for 8 hours. The starter culture was then added to 100 ml of LB/Ampicillin (100 µg/ml AMP) for overnight growth at 37 °C. The plasmid was then purified under endo-toxin free conditions from the DH5α cells by binding of the DNA to an anion-exchange resin. The
plasmid was eluted from the resin under high salt conditions (1.6 M NaCl; 50 mM MOPS; 15% isopropanol). The DNA was then concentrated and rid of salt with isopropanol precipitation. All maxipreps were suspended with 2 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

Promoter Analysis

The transcription start site for the LMB StAR promoter was identified by sequencing to the end of the 5′UTR (untranslated region) using 5′RACE. The 5′UTR information was then matched up with genomic sequence to determine where the UTR ends and the promoter begins. Sequence upstream of the transcription start site was then analyzed with three different web search engines, MatInspector V2.2, Professional-MatInspector V7.3, and TFSearch, to identify putative consensus binding sites.

LMB SF-1 Cloning

A 246 base pair portion of LMB SF-1 was cloned from ovarian tissue by PCR with a forward primer 5′CCAACCGCACCATCAAGTCNGARTAYCCNG 3′ and reverse primer 5′GAAGACCATGCAGCGGCKNGCCCANTC 3′. The PCR reactions consisted of 10 pmol each primer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 1 unit of amplitaq (Perkin Elmer/Applied Biosystems), 0.45 µg cDNA, and 10X PCR buffer [500 mM KCl and 100 mM Tris-HCl (pH 8.3)] diluted to 1X in a 20 µl final volume. PCR amplification conditions using a Perkin Elmer 9600 thermocycler are listed in Table 2-5.

Promoter Deletion

One deletion of the 2.9 Kb LMB StAR promoter was made. The deletion was made via a double digest with EcoRV and BstEII. The EcoRV restriction site is at the 5′end of the promoter and the BstEII is about 1000 bp from the 5′ end, leaving a 1.86 Kb promoter when digested. The digested promoter construct was analyzed on a 1% agarose
gel and the bands corresponding to the proper deletion pieces were purified using spin columns from a Qiagen kit. Additionally, unlike EcoRV, BstEII is not a blunt-end cutting enzyme, which left incompatible ends, requiring filling in the ends with Klenow enzyme (NEB labs). For the Klenow reactions, 1 unit of Klenow was incubated with 1µg DNA and 0.2 µl of a 10 mM dNTP mix in a final volume of 20 µl. The Klenow reactions were stopped immediately after 15 minutes by flowing the DNA through spin columns from a PCR purification kit (Qiagen). Then, 60 ng of purified, digested DNA was re-ligated with 2000 units of concentrated T4 DNA ligase (NEB labs) in a final volume of 20 µl for 40 minutes at room temp. The ligation was then transformed into DH5α cells using protocol outlined under section for StAR cloning. All promoter constructs were verified by sequencing and restriction digested with DraI.

**Mutagenesis of Putative Transcription Factor Binding Sites**

Several transcription factor binding elements in the 2.9 Kb promoter were mutated to a NotI restriction site. These were constructed using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene.) The protocol combined 125 ng of each relevant forward and reverse primer (Table 2-5), 10 ng of the 2.9 Kb StAR promoter construct, 1 µl of a proprietary dNTP mix, and 2.5 units of Pfu Turbo DNA polymerase in a final volume of 50 µl. PCR amplification conditions for all QuikChange reactions are listed in Table 2-6 and Table 2-7. Following PCR amplification to create mutagenized promoter constructs, the parent, unmutagenized strands were digested with 10 units of DpnI at 37 ºC for 1 hour. 1 µl of the DpnI digested PCR product was transformed into DH5α cells using the protocol outlined under the section for StAR cloning. The transformation was plated on LB/AMP agar plates (100 µg/ml AMP). Several colonies were minipreped for
each QuikChange reaction and were screened for creation of the desired mutation by digesting 2 µg of DNA with NotI for 3 hours.

**Culturing of Y-1 Cells**

Y-1 mouse adrenal cells (passage 1) were purchased from ATCC (American Type Culture Company). The cells were cultured in media containing: Ham's F12K medium with 2 mM L-glutamine supplemented with 1.5 g/L sodium bicarbonate; 15% horse serum; 2.5% FBS; and 1% penicillin-streptomycin mix. The cells were grown in T-75 flasks at 37 °C for normal propagation with a media change every other day to retain the endogenous steroidogenic activity (following the recommendation from ATCC). After 4 days in culture, the cells typically reached about 70 to 80% confluency and were then split 1:3 after trypsinization. Each T-75 flask received 2 ml of trypsin for 4 minutes at 37 °C followed by inactivation of trypsin with 4 ml of media with serum. Lastly, 2 ml of the trypsinized cells where then added to one of the three pre-equilibrated flasks with 13 ml of media. To help alleviate clumping, cells were gently pipetted up and down with a 2 ml glass pipet about 10 times.

**Transfection Assays**

Cells were trypsinized as outlined in the section above on culturing of Y-1 cells, however, after inactivation of the trypsin by media with serum, the cells were pelleted at 1500 rpm for 5 minutes and resuspended with 10 ml of fresh media. Clumping of the cells was alleviated by pipeting the cells up and down with a 2 ml pipet and a brief 1 second vortex. Cells were counted with a hemacytometer and 150,000 cells/well were plated in 500 µl of media with serum and allowed to attach and equilibrate for 24 hours. Cells received fresh media immediately prior to transfection. Transfection reactions consisted of 0.1995 µg of the appropriate StAR promoter construct, 0.0005 µg of the
control renilla luciferase vector, and Fugene6, where the ratio of µg of DNA to µl of Fugene6 was 6:1 (1.2 µl Fugene6/ 0.2 µg total DNA), suspended in 20 µl media with no serum or antibiotic. The transfection mixture sat for 30 minutes at room temperature before adding it dropwise to the cells. All transfections were done in 24 well plates from Corning CoStar.

Doses were prepared in the same manner for all experiments. dbcAMP doses were made fresh for each experiment by diluting the appropriate amount of powder into cell culture media. For TGF-β exposures, stock solutions of 1 µg/ml were kept aliquoted and frozen at -80 °C until appropriate dilutions were made fresh for each experiment.

**GFP Quantitation**

To measure the transfection efficiency, 0.1995 µg of GFP (pEGFP, Clontech) was transfected in place of the LMB StAR promoter DNA. Cells were transfected as normal and subsequently trypsinized for GFP quantitation on a hemacytometer.

**Luciferase Measurements**

After exposures were completed, cells were immediately washed once with 1X PBS (phosphate buffered saline; 0.144 g/L KH₂PO₄, 9 g/L NaCl, 0.795 g/L Na₂HPO₄, pH 7.4) and then lysed with 100 µl of 1X passive lysis buffer (Promega) for at least 15 minutes at room temperature.

Luciferase measurements were done using reagents from the Dual Luciferase Kit (Promega). Firefly luciferase was measured first by adding 20 µl of cell lysate to 100 µl of reagent that contains substrate for the firefly luciferase and luminescence was immediately measured by luminometer. Renilla luminescence was then quantitated by adding 100 µl of Stop and Glo reagent, which contains reagents to quench the firefly reaction and substrate for renilla luciferase.
**Mouse StAR Real-Time PCR Assay**

A real-time PCR assay was developed to measure endogenous levels of mouse StAR in the Y-1 cells. Total RNA was extracted from the Y-1 cells by lysing with 500 µl of RNASTAT (Tel-Test) and then following the same protocol as outlined for LMB. Primer sequences for mouse StAR had previously been designed and published (Fielden et al., 2002); forward primer 5’ TGCTAAGGATCGGGAATGT 3’; reverse primer 5’ TCTGGCCTTTTACAGGAGA 3’. The PCR reactions for the mouse StAR were set up in the same manner as the LMB StAR with the exception of having no standard curve, all quantitations were relative.

The relative calculations for the mouse StAR real-time PCR were done by subtracting the 18S rRNA Ct value (cycle threshold) from the StAR Ct values. Ct values represent the initial detection in the increase of fluorescence signal associated with an exponential increase of PCR product during the log-linear phase. An average of the normalized Ct values is obtained for each treatment and then the value for the experimental group is subtracted from that of the control group, which leaves a log number that can be calculated into a fold change.

**Statistics**

Student’s T-test was used for evaluation of significance between control and experimental groups. Results were reported as significant if $P \leq 0.05$. 
Table 2-1. Primers for 5’ and 3’ RACE.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5’ RACE</th>
<th>3’ RACE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Original primer</strong></td>
<td>5’TTTTCCGGGTGCTGAGTGGACATCCCATCCAG3’ (for 1st round of 5’ RACE)</td>
<td>5’ATCGGCCAAGACACAAATGGTTACC3’</td>
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<tr>
<td></td>
<td>5’TTCCACTCCCCCATTTGCTCCATATTT3’ (for 2nd round of 5’ RACE)</td>
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</tr>
<tr>
<td><strong>Nested primer</strong></td>
<td>5’CTTGGCCGATCTTTTGAAGGATCT3’</td>
<td>5’AGCGGAGAATGGACCTACCTGTAT3’</td>
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<tr>
<td></td>
<td>5’CAGGCAACATCTTTACTCAGGACTTTTGTC3’</td>
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Table 2-2. Thermocycler conditions for 5’ and 3’ RACE.

<table>
<thead>
<tr>
<th>Original RACE Cycle Parameters</th>
<th>Nested RACE Cycle Parameters</th>
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<tbody>
<tr>
<td><strong>Cycle 5X:</strong></td>
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<tr>
<td>94 °C / 5 seconds</td>
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</tr>
<tr>
<td>72 °C / 2:30 minutes</td>
<td></td>
</tr>
<tr>
<td><strong>Cycle 5X:</strong></td>
<td></td>
</tr>
<tr>
<td>94 °C / 5 seconds</td>
<td>94 °C / 5 seconds</td>
</tr>
<tr>
<td>70 °C / 10 seconds</td>
<td>68 °C / 10 seconds</td>
</tr>
<tr>
<td>72 °C / 2:30 minutes</td>
<td>72 °C / 2:30 minutes</td>
</tr>
<tr>
<td><strong>Cycle 27X:</strong></td>
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</tr>
<tr>
<td>68 °C / 10 seconds</td>
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</tr>
<tr>
<td>72 °C / 2:30 minutes</td>
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Table 2-3. Primers used to fix nucleotide mistakes in full length StAR cDNA sequence.

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<th>QuickChange Reaction</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>1</td>
<td>5’CATATGAGGAACATGACAGTTTGAGGAAGAATGCAA TG3’</td>
<td>5’CATTGCATTCTTCCTCAAAACCTGTCAATGTTCTCAGTATG3’</td>
</tr>
<tr>
<td>2</td>
<td>5’GCCATCAGCATCCTCAGGCACCAGGAT3’</td>
<td>5’GTCCTGGTCGCTGAGGATGCTGATGGG3’</td>
</tr>
<tr>
<td>3</td>
<td>5’AGTAACTGGGATCAACCAAACCCGAGGAAGAAGCTCCC TCCTCAG3’</td>
<td>5’CTGAGGAGGGAGCTTCTTCTCCGGGGTTGGTGATCCAGTTACTG3’</td>
</tr>
<tr>
<td>4</td>
<td>5’GCAGAGGGGGTTGTGTCAGAGCGAGGAAATG3’</td>
<td>5’CATTCTCCGCTCTGACAAACCCCCCTGTC3’</td>
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<td>5</td>
<td>5’CTAAATATAGATCTAAAGGGCTGGATCCCAAAGACAATCATAAAC3’</td>
<td>5’GTATATGATTTGCTTTTGAGATCCAGCCCTTATTAGATCTATATTAG3’</td>
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<td>6</td>
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<td>8</td>
<td>5’GTGGACTCTTGCCAACCCACTCCCGGCAAGG3’</td>
<td>5’CTTTTGCCGGAGGTTGGTGCAAGGCTCCAC3’</td>
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Table 2-4. Thermocycler conditions for LMB StAR promoter cloning.

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<td>5 cycles:</td>
</tr>
<tr>
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<td>94 ° C / 2 seconds</td>
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</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>37 cycles:</td>
<td>24 cycles:</td>
</tr>
<tr>
<td>94 ° C / 2 seconds</td>
<td>94 ° C / 2 seconds</td>
</tr>
<tr>
<td>67 ° C / 3 minutes</td>
<td>67 ° C / 3 minutes</td>
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<tr>
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<tr>
<td>67 ° C / 4 minutes</td>
<td>67 ° C / 4 minutes</td>
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Table 2-5. Thermocycler conditions for cloning of LMB SF-1.

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<th>Time</th>
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<td></td>
<td>95 ° C</td>
<td>2 minutes</td>
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<tr>
<td>40</td>
<td></td>
<td>95 ° C</td>
<td>30 seconds</td>
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<td>61.8 ° C</td>
<td>25 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 ° C</td>
<td>45 seconds</td>
</tr>
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Table 2-6. Primers for promoter mutagenesis.

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<th>Site Mutated</th>
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<th>Reverse Primer</th>
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<tr>
<td>ERE/2678</td>
<td>5'ATAGCGCCTTTTCTAGTCTTTTGCCGGGCTCAAAAGCGCGCTTTTACATG3'</td>
<td>5'CATGTAAAGCGCTTTTGAGCCCGCAAAGACTAGAAAGGCCTAT3'</td>
</tr>
<tr>
<td>COUP-TF/2027</td>
<td>5'GAATTGCAGTTTTCCCATGGCGGCCGCTCATTTAAACCTGAACAGCTGC3'</td>
<td>5'GCAGTTTTAGGTATTAATGAGCGGCCGCCATGCGGAAAAACTGCAATT3'</td>
</tr>
<tr>
<td>ROR/1969</td>
<td>5'TAGGGAGCCATTTGAAATAGGCCGCCGCCCTACTTTTGCTCTTGAAAAAAAG3'</td>
<td>5'CTTTTTTTCAAAAGAGCCAAAAGTACGGCCGCCCTATTCAAATGGCTCCCTA3'</td>
</tr>
<tr>
<td>GATA/AP-1/ERE1882</td>
<td>5'CTGTGGCTGAGTAATGCGGCCCTAGTACTAGGCCTGT3'</td>
<td>5'ACACAGGCCTAGTACTAGCGGCCGATTACTCAGCCACAG3'</td>
</tr>
<tr>
<td>COUP-TF/2304</td>
<td>5'GGTGATATTTTGCGAAGGAGCGGCCGCACAAACGTCCTTTTCCTGAA3'</td>
<td>5'TTCAGGAAAGGACGTGTTGAGGCGCCGCTCCTCTCGCAATAATACAC3'</td>
</tr>
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Table 2-7. Thermocycler conditions for promoter mutagenesis with QuikChange-XL protocol.

<table>
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<th>Cycles</th>
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<th>Time</th>
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</tr>
<tr>
<td>18</td>
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<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td>60 º C</td>
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<td>8 minutes</td>
</tr>
<tr>
<td>1</td>
<td>68 º C</td>
<td>7 minutes</td>
</tr>
</tbody>
</table>
Figure 2-1. Rapid amplification of cDNA ends (RACE). (A) 5’ RACE protocol and (B) 3’ RACE protocol to extend the cDNA sequence for LMB StAR. Both 5’ and 3’ RACE required primers specific to the original 345 bp sequence obtained for LMB StAR.
Figure 2-2. Sample standard curve for real-time PCR. A) A standard curve is generated by plotting Ct (cycle threshold) values versus the log of the copy numbers for minipreps of the StAR plasmid done in 10X serial dilutions. Two replicates were done for each standard. Unknown samples are then extrapolated to the standard curve. B) Dissociation curve for LMB StAR to check for primer specificity. The dissociation of fluorescence from the amplified, double-stranded DNA was detected with a melting curve.
The full coding sequence for StAR was cloned into the pET-28b vector (Novagen) for His-tagged protein expression in BL21(DE3) bacterial cells.
Figure 2-4. Location of peptide used for antibody development is indicated by a green box. A 21 amino acid peptide was designed to a conserved region for LMB StAR. Two different rabbits were immunized with the peptide for production of StAR antibodies.
Figure 2-5. Promoter cloning. 3 Kb of the LMB StAR promoter was cloned using the Genomewalker protocol (Clontech). One round of PCR is done using GSP1 (gene specific primer) and a primer against the adaptor, AP1. A second round of PCR is done for added specificity using a nested gene specific primer, GSP2, and nested adaptor primer, AP2.
Figure 2-6. Map of pGL3 basic vector (Promega). The 2.9 kb StAR promoter was cloned into the pGL3-basic vector for transfection experiments in Y-1 mouse adrenal cells. The promoter DNA was inserted into the MCS (multiple cloning site).
CHAPTER 3
REGULATION OF STAR IN LARGEMOUTH BASS OVARIAN FOLLICLE CULTURES

Introduction

A major discovery was made in the steroidogenic pathway within the past decade upon the identification of the Steroidogenic Acute Regulatory Protein (StAR Protein). It has now been well characterized in mammalian species that StAR transports cholesterol across the mitochondrial membrane and controls the rate-limiting step for steroidogenesis (Stocco and Clark, 1996). Humans with mutations in StAR can’t synthesize steroids from cholesterol, causing lethal accumulation of lipids in the adrenal glands and ultimately cell death, underscoring the importance of proper StAR function (Khoury et al, 2004).

It is clear, however, that steroidogenesis can be negatively impacted without the presence of mutations in StAR DNA, implicating instead steps in regulation of StAR mRNA synthesis or protein expression. TGF-β, a signaling molecule, and toxins, such as phthalates, are known to repress mammalian StAR transcription, however, a link, between the pathways they modulate has not been fully established (Brand et al., 2000; Barlow et al., 2003).

The steroid synthesis pathways of several fish species, including LMB, white sucker, zebrafish, and goldfish, have been shown in various cell culture systems to be targeted by toxins such as dioxins, pesticides, plasticizers, and paper mill effluents (Sepulveda et al., 2001; McMaster et al., 1995; Carvan et al., 2000; MacLatchy and Van
The negative impact of these toxins on fish steroid production implores the examination of how they specifically regulate StAR.

To date, there is minimal information about StAR in fish beyond the existence in the NCBI database of DNA sequences for a few lower vertebrate species, which now includes brook trout, rainbow trout, eel, cod, and zebrafish. There are 7 exons and 6 introns in the StAR gene which are conserved from mammals to fish (Goetz et al., 2004), however, the regulation of fish StAR has not been examined besides the induction of StAR mRNA in eel 1.5 hours post-injection with ACTH (adrenal corticotrophic hormone) (Li et al., 2003). The ACTH experiment did suggest a conservation in the cAMP inducibility of StAR across species.

Using largemouth bass (LMB) as a model fish since they are known to be sensitive to environmental toxins, I sequenced the entire coding region of StAR. The sequence was used to develop a real-time PCR assay for mRNA quantitation of StAR and for generation of a polyclonal antibody to detect protein changes in LMB ovarian follicle cultures. The ovarian follicle cultures were used to establish StAR mRNA expression patterns by two potent signaling molecules, cAMP and TGF-β. It is known that cAMP can stimulate mammalian StAR, partially through activation of SF-1, and that TGF-β can downregulate mammalian StAR transcription, however, these studies have never been done in fish prior to this study. My main hypothesis was that LMB StAR mRNA expression is upregulated by cAMP and downregulated by TGF-β in ovarian follicle cultures. Since a direct link between β-sitosterol exposure and TGF-β upregulation in prostate cancer cells has been shown (Kallen et al., 2000), this implies that toxins such as paper mill effluent repress steroid production through this signaling pathway.
Results

Cloning of StAR Protein

A partial, 345 base pair sequence for LMB was amplified using degenerate primer based PCR (Figure 3-1). The partial sequence was then used to obtain the full length coding and untranslated regions with 5’ and 3’ RACE. Sequence alignment of LMB StAR with other species, including brook and rainbow trout, zebrafish, pig, horse, and human, shows 52% similarity between mammalian and fish and 72% similarity amongst fish species (Figure 3-2).

Based on the sequence comparison of StAR with other species, there appears to be conservation of a couple important residues, including an important PKA phosphorylation site at nucleotides 193-196. The ScanProsite web program also putatively identified 4 PKC sites at nucleotides 5 – 7, 13 – 15, 60-62, and 187 – 189, as indicated by the number in the alignment (Figure 3-2). An important glutamic acid residue at nucleotide 170 which may bind to the hydroxyl group of cholesterol is also conserved amongst the species. The glutamic acid residue is within the START domain, the hydrophobic region for cholesterol binding, which spans from nucleotides 67 to 286.

Seasonal Expression

Changes in temporal expression of LMB StAR mRNA was quantitated from ovarian tissue samples previously collected at approximately two week intervals from October to April. RNA from seven fish at 11 time points during the year were analyzed and showed StAR mRNA levels do change in correlation with steroid production during the reproductive year (Figure 3-3). StAR levels peaked between February and March and began declining by April, therefore, suggesting a short window for maximal steroid
expression. This information was useful in understanding when LMB are steroidogenic and which months are optimal for culturing ovarian follicles for in vitro studies.

**Regulation of StAR mRNA Expression in LMB Ovarian Cultures**

Alamar blue viability assay showed that the ovarian follicles remained viable after an 18 hour equilibration in the incubator. The follicles successfully reduced the components in the alamar blue reagent as detected with A\textsubscript{570} and A\textsubscript{600} spectrophotometry readings. The follicles remained viable and responsive to dbcAMP in either the absence or presence of charcoal-stripped serum, therefore, serum was not added to culture media for experiments. Additionally, viability of the follicles was also tested by inducing with dbcAMP after a couple of equilibration timepoints, showing that basal and induction levels weren’t impacted whether exposed for 6, 12, or 24 hours post-equilibration.

**cAMP Induction of LMB StAR**

About 20-30 mg pieces of ovarian tissue were cultured and exposed to increasing doses of dbcAMP from 0 to 1 mM to characterize the regulation of LMB StAR. Exposures of the ovarian tissue cultures to dbcAMP resulted in upregulation of StAR mRNA levels as quantitated by real-time PCR. A dose response of cultured ovarian slices to dbcAMP showed a significant 3.5 fold induction from a mean of 13,529 copies of StAR mRNA/µg total RNA for controls to an average of 47,408 copies of StAR mRNA/µg total RNA after exposure to 1mM dbcAMP for 4 hours (Figure 3-4).

Additionally, more controlled experiments were done where number and stage of follicles cultured was monitored. Vitellogenic follicles with a specific diameter range of 0.68 mm to 0.76 mm were induced 5.9 fold from 18,054 copies of StAR mRNA/µg total RNA for controls to 105,686 copies of StAR mRNA/µg total RNA after 4 hour exposure
to 1mM dbcAMP (Figure 3-5). This suggests that more mature LMB follicles express more StAR, which fits the mammalian model for oocyte maturation (Logan et al., 2002).

**β-sitosterol Exposures**

β-sitosterol is known to downregulate steroid production in goldfish (MacLatchy and Van Der Kraak, 1995), however, no consistent effects on LMB StAR mRNA could be seen at any given dose, timepoint, or stage of follicle growth. Attempts were made with two different batches of fish after some initially promising results in the spring of 2003 suggested a decrease in LMB StAR mRNA levels by β-sitosterol. The 2003 results, however, were never able to be reproducibly substantiated, suggesting a suboptimal delivery of β-sitosterol to the steroidogenic cells.

**TGF-β Exposures**

TGF-β, a known repressor of steroid synthesis in mammals (Brand et al., 2000; Gautier et al., 1997; Liakos, 2003), was tested in cultured follicles with diameters between 0.8 to 0.9 mm and 1 to 1.1 mm. The LMB follicle cultures showed a downregulation of StAR mRNA by about 2.3 fold after a 14 hour exposure to 1 ng/ml TGF-β (Figure 3-6).

Some interesting preliminary results, however, were obtained from mature follicles between 1.2 and 1.3 mm. Data suggests that 1 ng/ml and 10 ng/ml TGF-β stimulates LMB StAR mRNA expression by about 2 fold after a 14 hour exposure, which could indicate that the various factors which mediate the signaling response may vary with follicle development (Figure 3-6).
**Antibody Development**

Currently, no polyclonal antibody exists for StAR that cross reacts with fish species, therefore, an antigenic peptide was designed and injected into two different rabbits to produce an immunogenic response against LMB StAR.

The first step in testing and optimizing the antiserum was to create purified protein for a positive control. A protein expression vector containing the full coding region for LMB StAR was created by PCR amplification (Figure 3-7) with subsequent cloning into the pET-28b expression vector containing a 6X-histidine tag (Novagen). The construct was verified by sequencing at the University of Florida DNA Sequencing Core. Bacterial cultures were induced by 1 mM or 3 mM IPTG for 1-4 hours and were analyzed on a Coomassie stained gel alongside negative controls of either empty vector or uninduced cultures. A band around 40 kD began to be overexpressed compared to controls after just one hour incubation with either 1 mM or 3 mM IPTG (Figure 3-8). Expression of StAR continued through 4 hours and appeared to be maximally induced by that timepoint. The expressed protein was successfully purified from the total protein extract using Ni-NTA spin columns (Qiagen) with a concentration of 1.1 µg/µl (Figure 3-9).

To confirm that the bacterially expressed protein was StAR, the band was excised and in-gel digested with trypsin for analysis with two different mass spectrometers, Q-STAR and LCQ. *In-silico* digest of the sequence matched several peaks seen by Q-STAR, including peaks at 722.3833, 939.4723, 1404.7140, 1509.8019, 1601.8558, 2329.1177, and 2395.1682 (Figure 3-10). With the Q-STAR, we used a MALDI (matrix assisted laser desorption ionization) based technique for sample analysis. Additionally, at least 5 tryptic peptides were identified by LCQ mass spec using an electrospray
ionization technique (Figure 3-11). For LCQ, the peptides were separated by reverse-phase chromatography. Both the mass spec techniques are complementary to each other.

The rabbit antisera from rabbit UF408 and UF409 were tested by ELISA against the purified protein for presence of LMB StAR antibodies. After 30 minutes of exposure in an ELISA assay, 7 µg/ml of purified protein had an absorbance at 450 nm of 0.3 with the pre-bleed versus about 2.8 for the test bleed at a 1:1600 dilution. ELISA results showed strong reactivity of the protein with antiserum dilutions down to 1:51,000, suggesting the presence of very strong and specific LMB StAR antibodies (Figure 3-12). The ELISA results also helped to establish the dilution ranges of antiserum to test by western blot.

To determine whether the antibodies could also bind StAR in a western blot, I used the recombinant protein with ECL (chemiluminesence) detection. The purified protein was successfully detected by the LMB StAR antibodies at several dilutions of primary antiserum as well as at several concentrations of protein. A 1:500 antiserum dilution was too concentrated for western blot and resulted in an overexposure on the film, even after a 1 second exposure, and a similar result was still seen with up to a 1:5000 dilution when a 1:20,000 secondary was used. The most optimal conditions to detect the recombinant StAR was at a 1:15,000 antiserum concentration with a 1:40,000 secondary dilution (Figure 3-13).

**Western Blot Detection of Endogenous LMB StAR Protein**

Preliminary western blot analysis was done on LMB ovarian tissue exposed to either 0 or 1 mM dbcAMP for 24 hours. The western blot shows detection of StAR in both the control and 1 mM dbcAMP samples (Figure 3-14). The band detected for the dbcAMP sample appeared more intense compared to the control, however, the presence
of non-specific bands suggests the StAR antibody needs to be purified from the anti-sera to obtain optimal results. The non-specific bands are of about the same intensity, indicating equivalent protein was loaded into each lane.

**Discussion**

Steroid hormones are key regulators of many cellular pathways and steroid synthesis can be regulated at the level of cholesterol transport with the StAR Protein. The StAR Protein has been cloned from several different species, ranging from higher mammals to lower vertebrates like fish, however, the similarities and differences in their function and regulation have never been investigated in any depth (Stocco and Clark, 1996; Goetz et al, 2004). A main goal of this project was to clone StAR from LMB and examine the conservation of key amino acids and whether the sequence similarity corresponds with conservation of function and activity.

The ovarian tissue and follicle cultures were excellent systems for comprehensive study of LMB StAR mRNA and protein regulation by endogenous signaling molecules like cAMP and TGF-β. Exposure of the ovarian cultures to dbcAMP showed no blatant differences in magnitude of induction between species or the timepoints at which induction can be seen. There was a 6 fold induction of LMB StAR after a 4 hour exposure to dbcAMP, which compares with the 4 fold induction seen in MA10 mouse Leydig cells (Clark et al., 1995.) The cAMP data establishes that the overall regulation of transcription is very similar across species and that key response elements in the promoter which mediate the response to cAMP, including those for SF-1, must be conserved.

The exact molecular pathways are still being elucidated for regulation of StAR, therefore, it is important to detect changes in protein as well as mRNA levels since
compounds could regulate either the transcription or translation of StAR. Previously, however, there was no antibody that cross-reacted with vertebrate species to examine protein alterations in StAR. We successfully generated a polyclonal antibody that can detect LMB StAR protein by ELISA or western blot. The antibody proved to be very potent with purified protein and works optimally at very dilute concentrations of around 1:15,000. The antibody is being purified from the anti-sera for optimal detection of StAR in complex protein samples, however, preliminary results suggests protein levels are induced by cAMP.

The ovarian follicle cultures were also used to establish whether LMB StAR mRNA is regulated by TGF-β, a potent signaling molecule like cAMP. The cultures showed about a 57% reduction in StAR mRNA levels after a 14 hour exposure to 1 ng/ml TGF-β for two different diameter sizes of follicles. Follicles of the same size studied for the TGF-β exposures, such as the 0.8 – 0.9 mm, were inducible by dbcAMP. Interestingly, the stage at which follicles were cultured may play a key role in the regulation since StAR mRNA levels were activated by 1 or 10 ng/ml TGF-β in more mature follicles of 1.2 to 1.3 mm. One possible explanation for this may be that SMAD2 and SMAD3 proteins, the key signaling molecules of TGF-β, may not be present at sufficient levels in the more developed LMB follicles. It has been reported that both SMAD2 and SMAD3 levels are very low or non-existent in the large antral or pre-ovulatory follicles of rats (Xu et al., 2002). SMAD3 has been shown to mediate the TGF-β repression of mammalian StAR (Brand et al., 1998).

TGF-β, unlike cAMP, significantly represses mammalian StAR mRNA expression. The repression can range from 40% in unstimulated cells treated with 2 ng/ml TGF-β for
12 hours to greater than 60% downregulation when induced with cAMP in H295 cells, a human adrenocortical cell line (Brand et al., 1998). The LMB ovarian follicle data suggests that, in addition to the cAMP pathway, TGF-β signaling is not only conserved between mammals and lower vertebrates but that the percent of inhibition for developing oocytes by TGF-β is also relatively comparable. Preservation of TGF-β signaling between species underscores the importance of this pathway and the chemicals that modulate it.

Interestingly, a study showed TGF-β protein levels are upregulated by at least 50% in prostate stromal cells after a 6 day exposure to β-sitosterol, one major chemical found in paper mill contaminants (Kassen et al., 2000). Fish exposed to paper mill toxins, and more specifically, β-sitosterol, exhibit decreased steroidogenic activity (MacLatchy and Van Der Kraak, 1995). The ovarian follicle cultures were used to determine if β-sitosterol exposure mimics TGF-β downregulation of LMB StAR mRNA levels, thereby elucidating a possible signaling pathway activated by environmental toxins. Overall, direct β-sitosterol exposures at several doses and timepoints in the follicle cultures did not significantly impact StAR mRNA expression, contradicting my original hypothesis that β-sitosterol represses steroidogenesis by downregulating StAR.

A possible explanation for the β-sitosterol results might be that ethanol was used as the solvent. Ethanol was chosen based on several published in vivo and in vitro experiments (Kassen et al, 2000; MacLatchy and Van Der Kraak, 1995). When administered in vivo, β-sitosterol is able to enter the bloodstream and be packaged into lipoproteins for delivery to steroidogenic cells (Carter and Karpen, 2001). Although it is not definitively known which signaling pathways are modulated by β-sitosterol, it is
possible that downstream signaling requires activation through the lipoprotein receptors. Using ethanol as a solvent might have bypassed the use of lipoprotein receptors or cellular lipid carriers and could explain the lack of significant response I saw on StAR mRNA expression by β-sitosterol. A recent publication reported that 15 µg/ml cholesterol delivered via ethanol to Y-1 mouse adrenal cells did not significantly impact StAR protein levels (King et al., 2004), however, cholesterol incorporated in either HDL or LDL does increase mammalian StAR protein production in Y-1 cells (Reyland et al., 2000). The ethanol study with cholesterol further supported the potential importance of using the lipoprotein receptors as a route of entry for StAR regulation.

Overall, the sequence, mRNA, and protein data suggests a similarity in signaling pathways and regulation across species for StAR. Induction of mRNA and protein levels by cAMP suggests that LMB StAR is subject to both transcriptional and post-transcriptional regulation by critical signaling molecules such as cAMP. The combined mRNA and protein results can provide valuable insight into the mechanism by which environmental toxins, such as β-sitosterol, can disrupt the normal regulation of StAR.
Figure 3-1. PCR amplification of LMB StAR. A 345 bp partial cDNA sequence for LMB StAR was PCR amplified and run out on a 1% agarose gel. (A) PCR marker (Promega) (B and C) PCR amplifications from gonadal RNA of two different fish. (D) negative control with no reverse transcriptase added. The amplified band for StAR is indicated by arrow.
Figure 3-2. Alignment of LMB StAR cDNA with other species. Alignment of the cDNA sequence for LMB StAR with other species shows about a 53% similarity across all species and 73% similarity between fish and lower vertebrates. Several important regulatory sites were putatively identified in the LMB StAR cDNA, including: 4 PKC sites at amino acid positions (as indicated on alignment), 5 – 7 (TFK), 13 – 15 (SYR), 60-62 (SSR), and 187 – 189 (SVR). There is one conserved PKA site at nucleotides 193 – 196 (RRGS). Additionally, an important glutamic acid residue at nucleotide 170, which may bind to the hydroxyl group of cholesterol, is conserved within the START domain (amino acids 67-286).
brook trout       MLPAFKLCAIGISYHRNMTGLKNAVMAIHELNLMA--GFNPSSWISHVRRRSSLLS
rainbow trout    MLPAFKLCAIGISYHRNMTGLKNAVMAIHELNLMA--GFNPSSWISHVRRRSSLLS
LMB             MLPAFKLCAIGISYHRNMTGLKNAVMAIHELNLMA--GFNPSSWISHVRRRSSLLS
zebrafish       MLLATFKLCAGISYRHMRNMTGLRKNAMVAIHHELNMLA--GPNPSWISHVRRRSSLLS
pig             MLLATFKLCAGISYRHMRNMTGLRKNAMVAIHHELNMLA--GPNPSWISHVRRRSSLLS
horse           MLLATFKLCAGISYRHMRNMTGLRKNAMVAIHHELNMLA--GPNPSWISHVRRRSSLLS
human           MLLATFKLCAGISYRHMRNMTGLRKNAMVAIHHELNMLA--GPNPSWISHVRRRSSLLS

Prim.cons.      MLPATFKLCAGISYHRNMTGLKNAVMAIHELNLMA--GFNPSSWISHVRRRSSLLS

70   80   90   100   110   120
|     |     |     |     |     |
brook trout   SRIEEEQGYNEAEVSYVKQGEEALQKSISILGDQDGWTTEIIAAANGDKVLSKVLPDVGK
rainbow trout SRIEEEQGYNEAEVSYVKQGEEALQKSISILGDQDGWTTEIIAAANGDKVLSKVLPDVGK
LMB           SRIEEEQGYNEAEVSYVKQGEEALQKSISILGDQDGWTTEIIAAANGDKVLSKVLPDVG
zebrafish     SPIAES-TYSEADCVQYQQQALQKSIISILGDQDGQTEIIEIANGDKVLSKVLPDVG
pig            SQLEDTF-YSDQELAYIQQGWEAMQKDILSNQEGKKSQENGDKVLSKVLPDVG
horse          SQLEDTF-YSDQELAYIQQGWEAMQKDILSNQEGKKSQENGDKVLSKVLPDVG
human          SRLEETL-YSDQELAYIQQGWEAMQKDILSNQEGKKSQENGDKVLSKVLPDVG

Prim.cons.    SRIEEE2QYNGEYSD2EL2YQQGWEAMQKDILSNQEGKKSQENGDKVLSKVLPDVG

130  140  150  160  170  180
|     |     |     |     |     |
brook trout   FKLEVLQSIDLKGWLPIKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
rainbow trout FKLEVLQSIDLKGWLPIKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
LMB           FKLEVLQSIDLKGWLPIKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
zebrafish     FKLEVTLEQLTGDLYSEDLNVEQMGDNWPKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
pig            FKLEVTLEQLTGDLYSEDLNVEQMGDNWPKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
horse          FKLEVTLEQLTGDLYSEDLNVEQMGDNWPKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
human          FKLEVTLEQLTGDLYSEDLNVEQMGDNWPKVLSQTQVDFANHLRQRMADNSVSKEMAPAC

Prim.cons.    FKLEV2LQ2M22LYEELV2M2QGDEMNWPKVLSQTQVDFANHLRQRMADNSVSKEMAPAC

190  200  210  220  230  240
|     |     |     |     |     |
brook trout   GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT
rainbow trout GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT
LMB           GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT
zebrafish     GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT
pig            GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT
horse          GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT
human          GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT

Prim.cons.    GPRDFSVRCAKRGRGTSCLAGMSTQHFMPEQGVVRAENGPTC1VMRPSSADDPNKT

250  260  270  280
|     |     |     |     |
brook trout   TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
rainbow trout TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
LMB           TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
zebrafish     TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
pig            TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
horse          TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
human          TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC

Prim.cons.    TWLLSIDLKWIPKTIINKVLSQTQVDFANHLRQRMADNSVSKEMAPAC
Figure 3-3. Seasonal expression of LMB StAR. RNA was isolated from ovarian tissue of largemouth bass previously collected every few weeks for most of a year throughout a year. Typical seasonal expression of StAR mRNA was determined by real-time PCR. RNA from seven fish at 11 time points during the year were analyzed. T-test shows a p< 0.05 between timepoints marked with red versus black stars.
Figure 3-4. Dose response of LMB ovarian tissue cultures to dbcAMP. Cultures of 20-30 mg pieces of ovarian tissue were exposed to a dose response of dbcAMP from 0 mM to 1mM for 4 hours. RNA was then isolated from the tissues and reverse transcribed to cDNA for real-time PCR analysis. T-test shows a p<0.05 as indicated with red star. Results are from 2 different fish with triplicate assays done for each fish.
Figure 3-5. cAMP induction of ovarian follicles. Thirty follicles between 0.4 and 0.88 mm were cultured in 24 well culture plates and exposed to 0 or 1 mM dbcAMP for 4 hours. RNA was isolated from the follicles and reverse transcribed for analysis by real-time PCR. This experiment represents data obtained from one fish.
Figure 3-6. Dose response exposure of ovarian follicles to TGF-β. Follicles with diameter ranges between 0.8 – 0.9 mm, 1.0 – 1.1 mm, and 1.2 - 1.3 mm were exposed to 0 ng/ml or 1 ng/ml TGF-β for 14 hours. Ten follicles were cultured in each well of a 24-well culture plate. RNA was isolated from the follicles and reverse transcribed for analysis of StAR mRNA expression by real-time PCR. T-test shows a p< 0.05 indicated with red star.
Figure 3-7. PCR amplification of entire LMB StAR cDNA. Specific primers were used to amplify 858 nucleotides of LMB StAR coding region. A) Low DNA mass ladder (Invitrogen). B and C) BamHI digest of two separate clones to confirm ligation of LMB StAR into the pET-28b vector.
Figure 3-8. Bacterial expression of LMB StAR. The entire cDNA sequence for LMB StAR was cloned into the pET-28b vector (Novagen). Bacterial expression of StAR protein was induced by 1 mM and 3 mM IPTG from 1 to 4 hours in BL21(DE3) bacterial cells. Total protein for each exposure was run out on a 4-12% Bis-Tris NuPAGE gel (Novex) and stained with Colloidal Coomassie Blue. Induction of StAR is indicated by an arrow.
Figure 3-9. Purification of StAR. His-tagged StAR was purified from the bacterial expression using Ni-NTA spin columns (Qiagen). Lane 1 contains 2 µg of purified protein and lane 2 contains See Blue Plus 2 marker run out on a 4-12% Bis-tris gel (Novex) and stained with colloidal comassie blue stain. The band for purified StAR is indicated by an arrow.
Figure 3-10. Identification of bacterially expressed StAR with Q-STAR mass spectrometry. The trypsin-digested StAR was analyzed with Q-STAR mass spectrometry for identification of bacterial LMB StAR expression. The coomassie blue stained band was excised and in-gel trypsin digested prior to a MALDI based mass spec analysis. The black arrows indicate which peaks were specific for predicted LMB StAR peptides.
Figure 3-11. Identification of bacterially expressed StAR by LCQ mass spectrometry. The coomassie blue stained band for bacterially expressed LMB StAR was cut out and in-gel trypsin digested for analysis by LCQ mass spec. Predicted tryptic fragments were identified by Prospector and the red arrows indicate which peptides were seen by LCQ.
Figure 3-12. ELISA with LMB StAR anti-serum and purified StAR protein. The antiserum from two different rabbits injected with the StAR peptide were tested by ELISA with 7 µg/ml purified protein.
Figure 3-13. Western blot detection with LMB StAR antibody and purified StAR protein. 13 ug of purified protein was run out on a 4-12% bis-tris gel, transferred to a nitrocellulose membrane. The membrane was divided into strips for probing with several combinations of primary antibody (LMB StAR antiserum) and secondary (mouse anti-rabbit IgG) conjugated to horseradish peroxidase). Primary antibody incubations were done for 3 hours and secondary incubations done for 1 hour followed by chemiluminescence detection on kodak film.
Figure 3-14. Western blot detection of StAR in dbcAMP exposed LMB tissue cultures. 60 mg pieces of LMB ovarian tissue were cultured and exposed to 0 or 1 mM dbcAMP for 24 hours. Total protein was extracted and run on a 4-12% bis-tris for detection of StAR with a 1: 5,000 dilution of the polyclonal antibody and 1: 40,000 secondary antibody.
CHAPTER 4
TRANSCRIPTIONAL REGULATION OF THE LMB STAR PROMOTER

Introduction

Several transcription factor binding sites have been identified in the mammalian StAR promoter, including SF-1, AP-1, GATA-4, C/EBP, involved in the activation of transcription, and DAX-1, a repressor of mRNA synthesis (Sugawara et al., 2004; Jo and Stocco, 2004; Manna et al., 2004; Silverman et al., 2004). An alignment of the first 200 base pairs of mammalian promoters for mouse, rabbit, sheep, pig, and horse shows the locations are conserved for 2 SF-1, 1 GATA, and 2 C/EBP response elements. One of the SF-1 sites, in fact, is only about 12 base pairs upstream from the TATA box. The promoter alignments indicate how important those binding sites are in mediating the transcriptional activity of StAR, however, more studies are necessary before the function or even the identification of many of the response elements is determined.

It is well characterized in the mammalian system that dbcAMP upregulates StAR transcription via PKA phosphorylation of SF-1 (Aesoy et al., 2002). The magnitude of activation of StAR by SF-1 is partially contingent on surrounding response elements and the proteins that they bind. C/EBP interaction with SF-1 can amplify the cAMP induction of StAR (Reinhart et al., 1999), however, when DAX-1 and SF-1 were co-transfected in HTB9 cells, a bladder carcinoma cell line devoid of endogenous steroid production, SF-1 could no longer stimulate StAR in response to cAMP (Sandhoff and McLean, 1999). The SF-1 data underscores the importance and complexity of protein-protein and protein-DNA interactions in StAR transcriptional regulation.
StAR transcription can be modulated by both endogenous and exogenous factors, which includes environmental toxins. The AhR element in the StAR promoter is a ligand for dioxins, a very toxic chemical found in water supplies and other locations. AhR co-transfection in Y-1 cells with 1.3 Kb of the human StAR promoter increased its transcriptional activity (Sugawara et al., 2001). Additionally, when the Y-1 cells were treated with β-napthoflavone, a ligand for AhR, StAR promoter activity was also increased (Sugawara et al., 2001). Induction of the promoter by cAMP, however, was not changed by co-transfection with the AhR construct. Although the AhR study suggested a pathway for StAR regulation that is cAMP/PKA independent and could be targeted by toxins, it is important to also characterize pathways which use alternative signaling pathways and response elements.

The mammalian StAR promoters were re-examined using the professional based web program and sites for COUP-TF, ER, RAR, and ROR were putatively identified. Their specific roles in StAR regulation were previously uncharacterized. COUP-TF can repress steroidogenesis and antagonize the induction of steroids by retinoic acids through competitive binding with the RAR/RXR response element (Barger and Kelly, 1997; Butler and Parker, 1995), a possible new model to be examined in StAR downregulation. The ROR is a relatively novel class of proteins related to the RAR and their interactions with COUP-TF or ER are unclear and open for investigation (Jarvis et al., 2002). Estrogen receptors are significant transcriptional regulators partly because they can bind not only to their own ERE but also to AP-1 sites (Bjornstrom and Sjoberg, 2005), which are present in the StAR promoter.
A major goal of this project was to clone the promoter for LMB StAR and develop a transfection assay with Y-1 cells for in-depth transcriptional analysis. The transfections were used to examine how deletions in promoter length or site-directed mutations in key response elements impact StAR transcription. To expand on the cAMP and TGF-β ovarian follicle exposure data in chapter 3, the role of those same signaling molecules in modulating transcriptional regulation of LMB StAR was examined. My main hypothesis was that cAMP activates LMB StAR transcription through sites independent of SF-1, such as the ROR. Additionally, there are critical regulatory sites for direct regulation of the StAR promoter by TGF-β since LMB StAR mRNA is modulated by the pathway.

**Results**

**Cloning of the StAR Promoter**

A 2.9 Kb portion of the LMB StAR promoter was cloned to examine its transcriptional regulation in transfection assays. The genomic DNA isolated for promoter sequencing was intact and of good quality with minimal if any protein contamination as evidenced by digestion with EcoRV, StuI, PvuII, and DraI compared to an undigested control (Figure 4-1). The digested genomic DNA was used to PCR amplify 2.9 Kb of the LMB StAR promoter with gene specific primers starting from the coding region that was already sequenced.

PCR products greater than 2 Kb were obtained from all four restriction libraries, as well as a shorter piece around 400 nucleotides from the EcoRV library. The amplified product from the PvuII library using promoter jk1 and promoter jk2 primers was ligated into a TOPO cloning vector. Ligation was confirmed by a double digest with EcoRV and DraI (Figure 4-2) and sent for sequencing at the DNA Sequencing Core at University of
Florida.  This PCR product was subsequently cloned into the pGL3 vector for transfection into Y-1 cells.

**Identification of the Transcriptional Start Site**

5’ RACE of the transcript was used to locate where the LMB StAR 5’UTR ends, which is followed immediately by the transcriptional start site and the beginning of the promoter (Figure 4-3). The length of the 142 base pair LMB StAR 5’UTR was similar to sequences reported for brook trout (180 bp), rainbow trout (160 bp), and zebrafish (40 bp) sequence in the NCBI database. Additionally, the transcriptional start site identified from 5’ RACE matched the site predicted by a web-based program called Neural Network Promoter Prediction. A TATA box is located about 23 base pairs upstream from the start site.

**Identifying Transcriptional Response Elements**

Sequences upstream from the transcriptional site were analyzed by 3 different web search engines, MatInspector V2.2, Professional-MatInspector V7.3, and TFSearch, for identification of putatively important response elements (Figure 4-4). Many important sites appeared to be conserved in the promoter between mammals and fish, although the specific position in the sequence didn’t always correspond.

Sites were only considered putatively functional if the same response element was identified by at least 2 of the 3 search programs, and if the matrix similarity score for the sequence was greater that 0.8 using the Professional-MatInspector. A perfect match for a response element gets a matrix similarity score of 1.00, where each nucleotide in the sequence being analyzed matches the most conserved sequence known for a given response element. The matrix similarity score can be decreased by mismatches in highly conserved positions of the binding site. The professional web-based program identified
several response elements in the LMB StAR promoter; 2 RAR, 1 ROR, 4 COUP-TF, 2 SF-1, 6 CREB, 8 GATA-1, 1 AhR/ARNT complex, 1 Smad3, and 1 full ERE site. Additional SF-1 sites were also identified by comparing mammalian EMSA data with LMB sequence information.

**Cloning of LMB Steroidogenic Factor -1 (SF-1)**

SF-1 has been extensively shown in mammalian species to upregulate StAR, therefore, a 280 bp sequence of LMB SF-1 was obtained to examine the sequence similarity across species. LMB SF-1 was amplified using specific primers designed to the ligand binding domain (Figure 4-5). Alignment of SF-1 with other species, including rainbow trout, sheep, and human, revealed greater than 40% similarity within the region amplified.

**Optimization of Transfection Assays**

The StAR promoter was cloned into the basic pGL3 luciferase vector and transfected into the Y-1 mouse adrenal cell line to examine its regulation by cAMP and TGF-β. Y-1 cells were chosen since they have endogenous steroidogenic activity and therefore, likely produce the critical transcription factors necessary for StAR regulation. The transfections were optimized with 3 different ratios of volume of transfection reagent to mass of promoter DNA (µl/µg), 3:1, 3:2, and 3:2, under control and 1 mM dbcAMP stimulated conditions. Under basal conditions, the greatest amount of DNA was transfected using the 6:1 ratio and with 1 mM dbcAMP stimulation resulted in a 2.2 fold induction. Since the fold stimulation with 1 mM dbcAMP using the 6:1 ratio was about the same as the 3:1 ratio, and slightly more than the 1.9 fold stimulation obtained with the 3:2 ratio, the 6:1 ratio was used for all future experiments (Figure 4-6).
Transfection time was also optimized. Y-1 cells were transfected for either 6 or 24 hours followed by exposure to either 0 or 1 mM dbcAMP for 20 hours (Figure 4-7). Both timepoints produced a similar fold induction by 1mM dbcAMP, however, the basal luciferase levels were higher at 24 hours and that timepoint was used for all subsequent transfections.

The efficiency and percent of transfected cells was quantitated by GFP co-transfection into the Y-1 cells (Figure 4-8). Transfection efficiency, using trypsinized cells on a hemacytometer, was calculated to be about 18%.

**dbcAMP Exposures**

Y-1 cells were transfected with the LMB StAR promoter and exposed to a range of dbcAMP between 0 mM and 2 mM for 20 hours. cAMP is known to induce mammalian StAR transcription, however, previously only SF-1 sites in the promoter were well characterized to mediate the response of StAR through this pathway (Brand et al., 2000). The 2.9 Kb LMB promoter was maximally induced between 0.75 mM and 1 mM dbcAMP by an average of about 2.4 fold (Figure 4-9). The transfections were done in both the presence and absence of 17.5% serum to determine whether factors present in the serum impact transcriptional activity. No impact was observed on the induction of StAR, therefore, all further transfections were done completely in the presence of serum for maximal viability of the cells. The Y-1 cells and other steroidogenic cells undergo a well characterized physical change in response to dbcAMP by rounding up. The exposures must be carried out in the presence of serum since the cells appear to round up and eventually detach from the plate much more quickly without serum. It is possible that the sources for modulation of the transcription factors involved in the dbcAMP signaling pathway, such as SF-1, are depleted more rapidly in the absence of serum.
Also, since dbcAMP can be broken down by phosphodiesterases, a phosphodiesterase inhibitor, IBMX, was added, to determine its impact on StAR induction. IBMX did not significantly impact the upregulation of StAR transcription, either at 0.1 mM or 0.5 mM IBMX, however, all experiments were carried out in the presence of IBMX to minimize dbcAMP breakdown during the 20 hour exposures.

**Promoter Deletion Experiments**

A deletion of the promoter was made to examine regulation of the shorter promoter by cAMP or TGF-β. Deletion of the 2.9 Kb promoter to 1.86 Kb was accomplished by using EcoRV and BstEII restriction sites already present in the sequence (Figure 4-10).

The 1.86 Kb promoter deletion construct was exposed to dbcAMP for comparison in activity to the 2.9 Kb construct. The deletion appeared to significantly diminish the dbcAMP activation of LMB StAR by 70-80%, suggesting an important activation site was eliminated (Figure 4-11).

The impact of TGF-β exposure on LMB StAR transcriptional activity was examined using the transfection assays since TGF-β is known to decrease steroid synthesis. Surprisingly, the 2.9 Kb construct was less susceptible to repression by 20 ng/ml TGF-β than the 1.86 Kb construct after a 40 hour exposure (Figure 4-11). The 2.9 Kb promoter was downregulated about 20% versus a 40% repression for the 1.86 Kb construct, suggesting there are elements within that region which mediate TGF-β signaling.

**Site-Directed Mutagenesis Experiments**

The role of individual transcription factors in regulating the StAR promoter was studied by mutating five different sites. Potentially important response elements involved in StAR regulation were identified using web based search programs, including
Professional-MatInspector V7.3 and TFSearch. Mutation to a NotI restriction site was made for the following putative sites (location for all mutation sites are noted in reference to the LMB StAR transcriptional start site): ERE at nucleotide 2678; COUP-TF site at nucleotide 2027; ROR at nucleotide 1969; a combined GATA/AP-1/ERE site at nucleotide 1882; and another COUP-TF site at nucleotide 2304. The web-based programs predicted no transcription factors would bind to the mutated sites. All mutations were confirmed by digestion with NotI (Figure 4-10).

All of the site-mutagenesis constructs were exposed to 1 mM dbcAMP, however, none of the mutated promoters were as inducible as the unmutated 2.9 Kb construct (Figure 4-12). Altering the ERE/2678 site resulted in 30% loss of response to cAMP, however, the data was not significant according to T-test with a P value greater than 0.05. However, mutation of the COUP-TF/2027, combined GATA/AP-1/ERE/1882, or ROR/1969 sites reduced the cAMP induction by 80%. The site-directed mutations suggest that the COUP-TF, ROR, and GATA/AP1/ERE sites are critical for dbcAMP regulation.

Another goal of the site-directed mutagenesis experiments was to evaluate the role of critical response elements, specifically between the 1.86 and 2.9 Kb region, in TGF-β regulation of the StAR promoter. All of the site-mutagenesis constructs were exposed to 20 ng/ml TGF-β for 40 hours (Figure 4-13). Mutation of the ERE/2678 and Coup/2027 sites diminished the repression by about 16%, suggesting these sites could mediate the inhibitory response to TGF-β. Mutation of COUP-TF/2304, GATA/AP-1/ERE/1882, or ROR/1969 resulted in no significant change in TGF-β regulation, suggesting these elements are not involved in TGF-β signaling.
TGF-β Regulation of Y-1 Cell Endogenous Mouse StAR mRNA

Y-1 mouse adrenal cells used for transfections have endogenous steroidogenic activity, therefore, TGF-β impact on mRNA expression of mouse StAR in Y-1 cells was examined by real-time PCR. A 40 hour exposure to 10 ng/ml TGF-β resulted in a 60% reduction of mouse StAR mRNA compared to controls (Figure 4-14). This set of experiments served as a reference point since TGF-β regulation of the entire mouse StAR gene was examined as opposed to either a partial 2.9 Kb or 1.86 Kb promoter piece in the transfection studies.

Discussion

Mammalian StAR is regulated by a diverse array of proteins, including SF-1, Dax-1, and AP-1 (Stocco et al., 2001; Buholzer et al., 2005; Sandhoff and McLean, 1999; Shea-Eaton et al., 2002). A few of the response elements putatively identified in mammalian StAR have been examined in more depth by transfection experiments or EMSA for functional capabilities. The binding sites for SF-1 in the StAR promoter are probably, to date, the most well researched of all the sites identified (Sandhoff et al., 1998; Sugawara et al., 1997). Although SF-1 is very critical to the upregulation of StAR, there are other potential response elements that have not even begun to be analyzed, such as the ERE, COUP-TF, and ROR. A main goal of this project was to develop assays to examine the LMB StAR promoter and to make inferences on factors which might modulate the response to dbcAMP and TGF-β, two critical and separate signaling pathways.

dbcAMP upregulates LMB StAR transcriptional activity in a similar manner as published for mammalian species. In chapter 3, real-time PCR with ovarian follicle
cultures showed a dose responsive induction of LMB StAR mRNA with dbcAMP. The mRNA data provided a benchmark for how the promoter should respond in transfection assays if functional and in a compatible cell line. The amount of induction of the LMB StAR promoter by 1mM dbcAMP was comparable to that of mammalian species. 1.3 Kb of human StAR promoter is upregulated about 2 fold after 24 hours in Y-1 cells (Sugawara et al., 2000), which parallels with the LMB results. The cAMP transfection data implies that critical transcription factors and their corresponding protein sequences are conserved across species. This was additionally confirmed by the cloning of LMB SF-1. The amplified ligand binding domain for LMB SF-1 was about 42% similar with mammals and greater than 90% homologous to other fish. A conserved alanine at residue 288 on the alignment was published to be critical for transcriptional activity of SF-1 (Wang et al., 2005), suggesting SF-1 is regulated in a similar fashion for different species.

Deletion of 1000 base pairs from the 2.9 Kb construct, leaving a 1.86 Kb construct, diminished the inducing effect of cAMP treatment. One explanation for the difference in response of the two constructs may be the removal of critical elements involved in StAR activation. There are AP-1, SF-1, RAR, ROR, as well as other sites within this region which could be crucial for induction of steroid synthesis.

Directed mutations of several putative response elements provided valuable insight into the upregulation of StAR by the cAMP pathway. Mutation of either the COUP-TF/2027 or ROR/1969 diminished the cAMP induction compared to the control. It is known that COUP-TF is an inhibitor of steroid synthesis (Shibata et al., 2004; Tran et al., 1992), however, this study is the first to implicate a putative role for COUP-TF in StAR.
transcriptional activity. The LMB StAR cAMP data with the various mutations suggests that COUP-TF does not repress StAR or steroid synthesis by binding to its own response element but rather by competitively binding to other transcription sites. The mutation data suggests the response element for COUP-TF actually participates in the activation of StAR. The antagonistic effect of mutations in the COUP-TF binding region on cAMP induction of the LMB promoter could be explained by its protein interacting partners or the response elements it can bind. COUP-TF is known to homodimerize with itself or heterodimerize with RAR or ROR (Tran et al., 1992; Schrader et al., 1996; Berrodin et al., 1992). COUP-TF can also bind to an ERE, even an ERE half-site (Klinge et al., 1997). More specifically, COUP-TF is known to downregulate steroid production through competitive binding with the RARE as opposed to binding to the response element for COUP-TF itself, thereby preventing RAR from activating transcription (Butler and Parker, 1995; Tran et al., 1992). Additionally, COUP-TF can also bind response elements for ROR, further indicating the arbitrary response elements it binds (Schrader et al., 1996). Most nuclear receptors, unlike COUP-TF, have clear and defined binding sites, and the arbitrary sites COUP-TF binds leads to several potential avenues for downregulation of the StAR promoter. This is the first study to show that the response element for COUP-TF may be involved in the upregulation of steroidogenesis via StAR since mutation experiments rendered the promoter significantly less responsive to cAMP activation.

Little is known about the role of ER and ROR in StAR regulation. The interesting interactions between COUP-TF and RAR, RARE, ROR, or ERE further warranted the examination of the ROR and ERE function in StAR. We found RAR and ROR sites in
both the mammalian and LMB promoters using public and professional web-based servers and examined the function of the ROR more closely. The transfection data suggests the ROR plays a critical role in the induction of StAR transcription and it modulates the activity through a cAMP dependent pathway. Interestingly, recent published results show that cholesterol is a ligand for the previously “orphaned” ROR (Kallen et al., 2002), and the LMB transfection data suggests this site could be one mechanism for feedback control of cholesterol metabolism. Mutating the ERE/2678 site, however, had no significant impact on cAMP activation.

Deletion and mutagenesis experiments also suggested putative transcription elements which mediate the TGF-β response exists between the 2.9 and 1.86 Kb region. The 1.86 Kb construct was more repressed by 20 ng/ml TGF-β compared to the 2.9 KB promoter. The site-specific analysis showed that the ERE/2678, COUP-TF/2027, and relieved the 20% repression seen with the 2.9 Kb promoter by about 16%, suggesting that these sites might bind proteins involved in the TGF-β pathway. It has been published that both COUP-TF and ER can interact with the TGF-β signaling pathway by binding to Smad complexes (Calonge et al., 2004). Furthermore, ER has been shown to bind to Smad3, the primary Smad found to mediate the inhibitory response of StAR to TGF-β (Matsuda et al., 2001). It is possible there are other sites within the 1.8 Kb to 2.9 Kb region or upstream from this which mediate the inhibitory response since none of the mutations restored the level of the repression to that seen with the 1.86 Kb length promoter.

Overall, the transfection results suggest that response elements far upstream in the promoter, at least 5’ of the 1.86 region, are just as important or potentially even more
important than sites present within the first 1000 to 1500 base pairs. Previous studies with mammalian StAR have focused on relatively short pieces of the StAR promoter, however, the LMB data implies that critical regulatory sites and mechanisms may be resolved by examining much longer constructs.
Figure 4-1. Digested LMB ovarian genomic DNA for promoter cloning. The quality of genomic DNA isolated from LMB ovarian tissue was tested before use in promoter cloning. 2.5 µg of the DNA was digested for 20 hours with one of 4 restrictions enzymes, (B) DraI, (C) EcoRV, (D) PVUII, and (E) Stul and 5 µLs was run out on a 0.5% agarose gel along with a DNA marker (A). (F) An undigested control was also run to confirm DNA integrity. Arrow indicates where undigested DNA would run. The gel was stained with ethidium bromide for visualization of bands.
Figure 4-2. Cloning of LMB StAR promoter. The GenomeWalker protocol was used to amplify the promoter for LMB StAR. The 2.9 Kb length promoter was sequenced and ligated into the pCR2.1-TOPO cloning vector (Invitrogen). Creation of the promoter construct was confirmed with a double digest using EcoRV and Dral restriction enzymes. A) Low DNA mass ladder (Promega). B) EcoRV/ Dral double digest of the LMB StAR promoter ligated into the TOPO vector.
Figure 4-3. 5' and 3' untranslated region (UTR) for LMB StAR and identification of transcription start site. The overall structure for the LMB StAR gene is outlined and the DNA sequence for the 5' and 3' UTR's are shown. Both the 5' and 3' UTR's were obtained using RACE. 5' RACE was also used to identify the transcription start site, which is the first nucleotide following the end of the 5'UTR.
Figure 4-4. Putative transcription response elements identified in the LMB StAR promoter. Sequence for 2.9 Kb of the LMB StAR promoter was analyzed by a professional web-based program, MatInspector, for putative transcription sites. Several potential sites identified are highlighted:

- CREBP
- AP-1
- GATA
- SF-1
- AhRE
- ER
- SF-1
- ROR
- RAR
- COUP-TF

CCG 3′
Figure 4-5. Cloning of LMB SF-1. A) A 246 base pair sequence for SF-1 was amplified from LMB ovarian tissue using specific primers and gel purified using spin columns from Qiagen (gel purified PCR product indicated by arrow). A DNA ladder was run to approximate the size of the amplified gene. B) Alignment of two different clones sequenced for LMB SF-1 with rainbow trout, sheep, and human revealed a 42% similarity with mammals and greater than 90% similarity with other fish for the fragment cloned within the ligand binding domain.
Figure 4-6. Optimization of ratio for transfection reagent (Fugene6) and promoter DNA concentration. Y-1 cells were transfected for 6 hours with either a 3:1, 3:2, or 6:1 ratio of Fugene6 (µl)/promoter DNA (µg) and exposed to 0 or 1mM dbcAMP for 20 hours. All wells were normalized to Renilla. Student’s t-test was used to determine significance and a * indicates a P < 0.05.
Figure 4-7. Optimization of transfection timepoint. Y-1 cells were transfected for 6 or 24 hours prior to a 20 hour exposure of 0 or 1 mM dbcAMP. Student’s t-test was used to determine significance and a * indicates a P < 0.05.
Figure 4-8. Quantitation of DNA transtection with GFP. GFP (0.1995 µg) was transfected into Y-1 cells to measure the amount of DNA getting transfected into the cells using the Fugene6 protocol. A) GFP fluorescence in a 1 mm² square for one well of a 24-well plate. B) GFP fluorescence in 1 mm² square of a hemacytometer using cells that were trypsinized and diluted 1/10. C) Picture of 1 mm² hemacytometer square in figure B with no fluorescence. Cells that fluoresced in figure B are indicated by arrows in figure C.
Figure 4-9. Dose response exposure of Y-1 cells to dbcAMP. Y-1 cells were transfected with the 2.9 Kb LMB StAR promoter and exposed to increasing doses of cAMP from 0 to 2 mM dbcAMP. Represented is one experiment done in triplicates which has been repeated 4 times. All values are normalized to Renilla. Student’s t-test was used to determine significance and a * indicates a P < 0.05.
Figure 4-10. Creation of promoter deletion and site-mutagenesis constructs. A deletion of the 2.9 Kb LMB StAR promoter to 1.86 Kb was created by digestion with EcoRV and StuI to 1.86 Kb. The deletion was verified by digestion with DraI. A and F) DNA mass ladder B) uncut 2.9 Kb C) DraI cut 2.9 Kb D) uncut 1.86 Kb E) DraI cut 1.86 Kb. Site-directed mutations of potentially important transcription elements were made using the Stratagene QuikChange system. Primers were designed to mutate 5 different putative response elements to a NotI restriction site. Creation of the constructs was verified by restriction digest with NotI; H) ERE/2678 construct I) COUP-TF/2027 construct J) GATA/AP-1/ERE/1882 construct K) COUP-TF/2304 construct L) ROR/1969 construct.
Figure 4-11. Promoter deletion analysis. The 2.9 Kb and 1.86 Kb constructs were exposed to cAMP for 20 hours and TGF-β for 40 hours to examine differences in transcriptional activity between the two promoter lengths.
Figure 4-12. Exposures of promoter site-mutagenesis constructs to dbcAMP. Mutation of five putative transcriptional elements between the 1.86 and 2.9 Kb region were made and individually evaluated for induction by 1 mM dbcAMP after 20 hours via transfection in Y-1 cells. All values were normalized to renilla luciferase. All experiments were done in triplicates and repeated at least 3 times. Student’s test was used to test for significance and a * indicated a P < 0.05.
Figure 4-13. StAR promoter mutation analysis with TGF-β regulation. Various mutations of putative response elements in the 2.9 Kb promoter were transfected into Y-1 cells and the regulation by 20 ng/ml TGF-β was compared with the 1.86 Kb construct. The regulation of the endogenous mouse StAR mRNA in the Y-1 cells was also tested to help interpret the transfection results. All wells were normalized to renilla.
Figure 4-14. Endogenous mRNA regulation of StAR in Y-1 cells by TGF-β. Y-1 cells were cultured in 24-well plates and exposed to 10 ng/ml TGF-β for 40 hours. RNA was extracted from the cells and reverse transcribed to cDNA for analysis by real-time PCR with primers that probe for mouse StAR. All samples were normalized to 18S rRNA. Student’s test was used to test for significance and a * indicated a P < 0.05.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Regulation of the StAR Protein is not fully understood for any species, primarily since it was only identified in the last decade. StAR is involved in the critical step of cholesterol metabolism (Clark and Stocco, 1996). Homeostasis of cholesterol metabolism is applicable to so many different diseases, including heart disease, hypertension, LCAH (lipoid congenital adrenal hyperplasia), among others (Khoury et al., 2004; Moghadasian and Frohlich, 1999). Understanding the regulation of StAR at the level of transcription, translation, or post-translation are all crucial to pinpointing differences in StAR function under normal versus atypical conditions. Additionally, the regulatory elements can be modulated by endogenous molecules or by exogenous ligands such as environmental toxins (Walsh et al., 2000; Barlow et al., 2003). This project primarily examines the transcriptional control of StAR using ovarian follicle cultures and transfection assays.

The promoter for StAR is very complex with many transcriptional elements for which the function is still unknown. SF-1, DAX-1, GATA, AP-1, AhR, and CRE elements are consistently found in the mammalian StAR promoters (Stocco et al., 2001; Sugawara et al., 2001), however, professional web-based programs identify many other potentially critical elements such as ER, COUP-TF, ROR, RAR, GR, TTF1, or SMAD sites. Although the genome project is now complete for many species, the length of many of the StAR promoters used in past publications is typically around 1000 base pairs
or less, leaving many of the sites upstream from this either unidentified or uncharacterized.

A main objective of this dissertation was to clone a large portion of the StAR promoter and to study its regulation by powerful signaling molecules such as cAMP and TGF-β. It is important to characterize signaling pathways that activate or repress StAR since aberrant regulation on either side of this spectrum could have severe impact on steroidogenesis. In fact, publications show steroids are downregulated through modulation of StAR by environmental toxins such as insecticides and herbicides, however, the signaling pathways triggered are unknown (Walsh et al., 2000; Barlow et al., 2003).

LMB was the model used for all studies in this project since their reproductive capabilities are severely repressed by downregulation of steroids upon paper mill contaminant exposure (Sepulveda, 2001). Additionally, there are no published reports of transcriptional regulation of StAR in lower vertebrates, therefore, conservation or deviation from transcriptional elements in the mammalian promoters could provide valuable information.

A large portion of the LMB StAR promoter, 2.9 Kb, was cloned and analyzed. Web based analysis showed numerous putative response elements, many of which are conserved with those published for mammalian species, including SF-1, AP-1, AhR, and GATA sites. Additionally, RAR, ROR, and full ERE sites were found in both the LMB and mammalian promoters, however, nothing had been previously published about either their identification or function in StAR regulation.
The presence of SF-1, AP-1, and GATA sites suggested that the LMB StAR promoter is regulated by cAMP in spite of differences in the DNA sequence. Transfection of the 2.9 Kb promoter confirmed that LMB StAR is induced in a dose-responsive manner with dbcAMP, suggesting that the function of StAR is maintained across species.

Most of the transcriptional activation for mammalian StAR was reported to be in the first couple hundred base pairs. The transfections with LMB StAR deletion and mutation constructs suggests that regions further upstream from this region, between the 1.86 Kb and 2.9 Kb region, are extremely important, at least for cAMP regulation. Mutation of no more than 5 nucleotides in the COUP-TF, ROR, or GATA/AP-1/ERE sites minimized the cAMP induction compared to unmutated controls. The GATA/AP-1/ERE mutation served somewhat as a control for the COUP-TF and ROR data since GATA and AP-1 are necessary for cAMP induction of mammalian StAR (Manna et al., 2004; Stocco et al., 2001).

The transfection data actually suggests a novel mechanism for COUP-TF functioning. Previous publications show COUP-TF is involved in the downregulation of steroid synthesis via competitive binding to other sites such as RAR (Zhang and Pfahl, 1993). The LMB data shows that mutation of the COUP-TF site diminishes cAMP induction, suggesting that the response element for COUP-TF binds a protein which activates transcription. The only study that analyzed StAR regulation by COUP-TF involved overexpression of COUP-TF in a cell line and subsequent observation of StAR transcriptional activity, however, the function of the actual response element has never been examined prior to this study (Buholzer et al., 2005).
The same mutations in the LMB promoter were tested for regulation by TGF-β since a major goal of this project was to elucidate a signaling pathway for repression of StAR by environmental toxins. TGF-β has been shown to downregulate steroidogenesis and also StAR itself in mammalian species, however, the mechanism is unclear (Brand et al., 2000). Mutation of COUP-TF/2304, GATA/AP-1/ERE/1882, or ROR/1969 sites did not significantly change the regulation of LMB StAR promoter transcription by TGF-β, suggesting these response elements examined are modulated primarily by cAMP dependent signaling. The ERE/2678 and Coup/2027 sites might be responsive to TGF-β, however, their exact role is still unclear.

LMB ovarian follicle cultures were very useful to study the effects of cAMP and TGF-β on expression of the endogenous LMB StAR mRNA and to correlate that with the transfection data. The follicle data showed that cAMP upregulates LMB StAR in a dose-responsive manner, matching the transfection results. When follicles between 0.8 and 1.1 mm were exposed to TGF-β, the mRNA expression of StAR was significantly repressed, substantiating the results seen with the 1.86 Kb StAR promoter.

The combination of ovarian follicle, promoter deletion, and promoter mutation data implies some very interesting regulation by cAMP and TGF-β in the 1.86 Kb to 2.9 Kb region which needs to be fully examined. To date, almost all of the studies have focused on the first 1000 to 1500 base pairs of the StAR promoter, however, the LMB data shows that over 80% loss in transcriptional activity can be attributed to one site, including a COUP-TF, GATA/AP-1/ERE, or ROR site, upstream of 2000 base pairs of promoter.
We identified critical response elements for cAMP induction of LMB that were not subjected to regulation by TGF-β. There are numerous transcriptional elements in the 1.86 Kb to 2.9 Kb region that could provide valuable information for the protein-protein or protein-DNA interactions which TGF-β modulates. There are SMAD3 and FAST-1 sites in the StAR promoter which had previously not been identified or characterized and may help further elucidate the TGF-β pathway. The SMAD3 site located at about 1.9 Kb in the LMB StAR promoter may not have been previously identified for any other species since it is somewhat far upstream. Additionally, there are at least 6 of the FAST-1 sites, an activin regulated SMAD interacting partner, within the 2.9 Kb LMB promoter. Coincidentally, several of the FAST-1 sites are located within 70 nucleotides of a COUP-TF site for the LMB, brook trout and zebrafish StAR promoters, which could be another possible interaction to examine.
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BIOGRAPHICAL SKETCH

The author was born and raised primarily in Indiana. After high school, she received her bachelor’s degree in chemistry from Indiana University in Bloomington. She continued her education by completing her master’s degree in biology at Purdue University in Indianapolis.

Following her master’s degree, she worked at Covance Central Laboratories in Indianapolis in the microbiology department. At Covance, she implemented tests for experimental antibiotics and medicines. She then moved to Florida and pursued her degree in the department of biochemistry.

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