Chapter 2
The Steroidogenic Acute Regulatory Protein (StAR)

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Abstract StAR, the Steroidogenic Acute Regulatory protein, was named for its critical role in the acute regulation of steroid hormone biosynthesis in the adrenal and gonads following tropic hormone stimulation. StAR synthesis is required for the first and rate-limiting step in steroid hormone biosynthesis, cholesterol transport into mitochondria. It was a long journey to finding the acute regulator of steroidogenesis, and this chapter provides a historical and personal account of this journey from the perspective of Dr. Douglas M. Stocco. Over the past two decades, we have gained significant insight into the mechanisms that regulate StAR expression, and into StAR structure and function. This chapter also provides a summary of the literature that has led to our current understanding of the cyclic adenosine-3′,5′-monophosphate (cAMP)-protein kinase A-dependent mechanisms that control StAR expression at the transcriptional and post-transcriptional levels in steroidogenic tissues.

Abbreviations

AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
AMP	adenosine-3′,5′-monophosphate
AP-1	activator protein 1
AURE	adenosine-uridine-rich destabilizing element
Bt_2cAMP	N6,2′-O-*dibutyryl*-adenosine-3′,5′-monophosphate
bZIP	basic leucine zipper
cAMP	cyclic AMP
CBP/p300	CREB-binding protein

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C/EBPβ	CCAAT enhancer-binding protein-beta
COUP-TF	chicken ovalbumin upstream promoter transcription factor I and II
CREB	cyclic-AMP responsive element-binding protein
CREM	CREB modulator protein
CRH	corticotropin hormone
DAG	diacylglycerol
DAX-1	dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome gene 1
ER	endoplasmic reticulum
FSH	follicule stimulating hormone
GnRH	gonadotropin-releasing hormone
IP3	inositol 1, 4, 5 trisphosphate
LH	luteinizing hormone
NPC	Niemann Pick type C
NUR77/NGFI-B	nerve growth factor induced-B
PAP7	TSPO associated protein 7 (ACBD3)
PIC	preinitiation complex
PKA	protein kinase A
PKC	protein kinase C
Poly(A)	polyadenylation site
RNAPII	ribonucleic acid polymerase II
SF1	steroidogenic factor 1
StAR	steroidogenic acute regulatory protein
START	StAR-related lipid transfer domain
TSPO	18 kDa translocator protein
TSS	transcription start site
UTR	untranslated region
VDAC1	voltage-dependent anion channel

Introduction

StAR Perspectives: A History of the Discovery of StAR by Douglas M. Stocco

I would first of all like to point out that this narrative is being written from strictly a personal viewpoint. I cannot speak for what others were thinking during the past two to three decades with regards to the search for the putative regulator of acute steroidogenesis, so I will not try to do so. I came into the search for this regulator from a rather circuitous route and I will try to provide you with the events of how that took place in our laboratory. As such, I hope that you will understand that my position is certainly not to slight the accomplishments of others but merely to point

out that I am not speaking for the entire field, but rather, only for myself and the members of our laboratory.

Some of the first observations on the production of steroids in response to trophic hormone treatment occurred in the laboratory of Oscar Hechter who studied the production of steroids in response to ACTH stimulation in perfused adrenal glands [1–4]. Following those early observations, James Ferguson's laboratory became engaged in similar studies [5, 6] and wrote in an article in *The Journal of Biological Chemistry* in 1963 "*of the several possible explanations for the observed effects of puromycin, the most provocative, but the most difficult to prove is the idea that a specific protein must be synthesized in order for the adrenal to increase steroid output.*" It was these words that seemed to sum up the problem at hand in understanding how the acute biosynthesis of steroids was regulated. Between Ferguson's early experiments and observations and those that followed by Garren [7–9]; Davis [10, 11]; Hall [12–15]; Brownie [16, 17]; Jefcoate [18–21]; Farese [22–26]; Boyd [27–31]; Koritz [32, 33]; Cooke [34–37]; and others, a generalized description of the characteristics of the protein predicted by Ferguson began to arise. It was thought that the putative protein was a trophic hormone-induced, rapidly synthesized, cycloheximide-sensitive protein that had a short half life and was thought to function by mediating the transfer of the substrate for all steroid hormones, cholesterol, from the outer to the inner mitochondrial membrane where it would be converted to pregnenolone by the cholesterol side-chain cleavage enzyme system that resided in the mitochondria.

Personally, I was not trained in the field of steroidogenesis nor was I trained in reproductive biology, but would soon have to learn something about both of these disciplines. I began working in the field of reproductive biology while engaging in collaborative studies with my colleague, Jim Hutson. Together, Jim and I were able to secure funding from the National Institutes of Health (NIH) and National Science Foundation (NSF), something that I had not been able to do on my own. I was interested in mitochondrial biogenesis but had difficulty in convincing the NIH of the virtues of studying how this fascinating organelle replicated itself in cells. In reading the literature on adrenal and gonadal steroidogenesis, I learned that a key step in the biosynthesis of steroids occurred in the mitochondria. In reading the works of those individuals named above, I thought that I might be able to parlay my keen interest in mitochondria into a new direction in which I might have better luck. During the course of my reading, I learned that new protein synthesis played a very important role in the regulation of the biosynthesis of steroids and that mitochondria were intimately involved in this regulation as well. I also learned that a number of investigators had sought this putative newly synthesized regulator, but that no definitive proof of its identity existed. Having run many 1-D electrophoresis gels and staining them with coomassie blue, I felt that finding a protein that might be present in trace amounts probably could not be done using this technology. An approach that would separate proteins to a greater extent and utilized a more sensitive detection method would probably be needed if the identity of this protein were to be uncovered. This approach had yielded positive results in studies by Nan Orme-Johnson and her colleagues who demonstrated the rapid synthesis of a series

of phosphoproteins in response to ACTH stimulation of adrenal cells in culture by labeling the cells with ^{35}S-methionine and separating them on 2-D polyacrylamide gel electrophoresis (PAGE) [38–45]. This turned out to be the first demonstration of the protein that was later cloned in our laboratory, but I must sadly admit to not being aware of her elegant work at the beginning of my own work.

At about the time when I was planning to work on this project, I wondered if it would it be possible to use 2-D PAGE followed by sensitive silver staining to demonstrate the presence of proteins in stimulated steroidogenic cells that were not present in unstimulated cells, and thus give us a clue to the identity of the putative regulatory protein. We began these studies in my laboratory using primary cultures of rat Leydig cells that we isolated using commonly accepted methods. A great number of protein "spots" were detected on the gels and we attempted to search for differences between unstimulated and stimulated cells that would appear rapidly and be consistently reproducible. This, we were unable to do, despite many attempts. While we thought we had candidate proteins, our results were not consistent, and obtaining sufficient numbers of Leydig cells was proving to be time-consuming and costly.

In 1981, Mario Ascoli published a manuscript in which he described the isolation of several clonal lines of Leydig cells from the M5480P tumor [46]. One of these lines, the MA-10 cell line had functional hormone/chorionic gonadotropin (hCG) receptors and produced copious amounts of steroid in response to hCG, cholera toxin, and 8-Br-cAMP. This cell line seemed to be an ideal tool with which to continue our studies on the search for the regulator. Mario was most generous in sharing this cell line with us and we began to shift our efforts from primary cells to Leydig tumor cells. We found that the cells grew well in our hands and produced high levels of progesterone (due to a lack of cytochrome P450 17A1, CYP17A1) in response to stimulation. We also saw that they synthesized very low basal amounts of steroid in the absence of stimulation, another perceived advantage for us since the acute regulator may be present in small amounts without stimulation and in high amounts following stimulation. We reasoned that this might allow us to see differences between unstimulated and stimulated cells more clearly. We began these studies still utilizing 2-D PAGE and silver staining but the results we obtained remained equivocal and unsatisfactory, not allowing us to make any definitive statements concerning a potential acute regulator. We decided to shift from the use of silver staining to autoradiography as a detection method with the rationale that the putative regulator was newly synthesized and therefore may be radiolabeled to a higher degree than other proteins following stimulation. We tried labeling the cells first using a ^{3}H-amino acid mixture and then ^{14}C-leucine. Again, these approaches were not satisfactory. It was around that time that I became aware of the Orme-Johnson approach and we decided to utilize incubation in ^{35}S-methionine as a labeling procedure. It did not take too long to observe in stimulated MA-10 cells the proteins that we referred to as the 30 kDa proteins and which were, in all probability, identical to the proteins that Orme-Johnson had seen earlier in cultured adrenal cells. In the middle of these studies, I took a sabbatical leave in Holland in 1986 and joined Henk van der Molen's group at Erasmus University in Rotterdam. I did not work on the acute

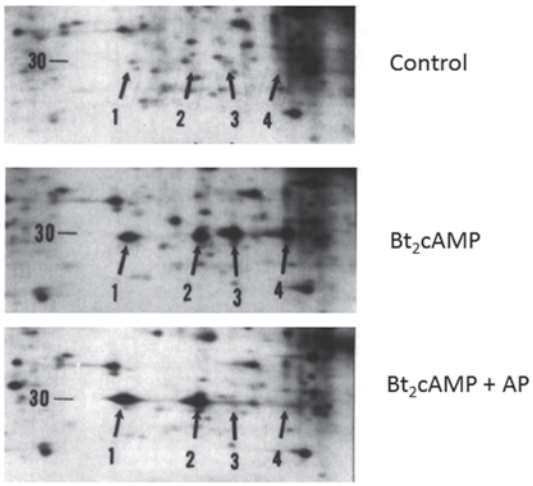

Fig. 2.1 Detection of the 30 kDa StAR protein by ^{35}S-methionine labeling and 2-D-PAGE**.** MA-10 mouse Leydig tumor cells were treated for 6 h with serum-free media containing ^{35}S-methionine in the absence (control) or presence of the cAMP analog dibutryl-cAMP (Bt_2cAMP, 1 mM). Mitochondria were isolated and the proteins separated by 2-D-SDS-PAGE. Shown are typical fluorograms of radiolabeled "spots on a gel" from this type of experiment. The arrows labeled 1–4 indicate the position of the StAR spots. The 30 kDa protein spots 1–4 appeared after cAMP treatment and correlated with steroid output (see text). Treatment of the isolated mitochondria with alkaline phosphatase (AP) resulted in loss of proteins 3 and 4 and recovery of radiolabel in protein spots 1 and 2. These data support that StAR is a phosphoprotein. Western blot analysis was used and confirmed that the protein spots 1–4 were StAR protein. *StAR* steroidogenic acute regulatory protein, *cAMP* cyclic adenosine-3′,5′-monophosphate

regulator while in Holland but did manage to learn a lot about male reproduction as the entire group in Rotterdam focused on this topic. I worked most closely with Focko Rommerts who had a great deal of experience in steroidogenesis and was quite familiar with the problem that I was trying to solve. We had many discussions concerning this topic and readily agreed that this was a difficult problem and that I would probably not be successful in solving it.

Upon returning to Texas, I began to work on the acute regulator once again. Our methodological approach was working well and we were readily able to reproduce the appearance of the 30 kDa proteins on 2-D gels and because of the purchase of a new computer-assisted imaging system, we were also able to accurately quantitate the proteins under many differing experimental conditions. We used this approach to do a series of experiments over the next several years to demonstrate that the 30 kDa proteins were induced by trophic hormone and cAMP analog, were rapidly synthesized in response to stimulation, were cycloheximide sensitive, were dose and time responsive to stimulation, and were localized to the mitochondria, the site of the regulated step [47–52] (Fig. 2.1). We also learned, at about the same time that the Orme-Johnson laboratory did, that the 30 kDa proteins were derived from a larger 37 kDa precursor much the same as many other mitochondrial proteins are

derived, having an N-terminal targeting sequence that was removed during import into the mitochondria [39, 52]. All of the experiments that we performed were supportive of the idea that these proteins might be involved in the acute regulation of steroidogenesis, but as I was so abruptly reminded by a colleague upon giving a talk at the European Testis Workshop one year, "yes Doug, but they are just spots." Of course, we understood that correlations do not provide proof of anything and that we needed a cause and effect relationship between the 30 kDa mitochondrial proteins and steroid synthesis in order to convince anyone that these proteins were involved in the acute regulation of steroid biosynthesis. It was clear that running a hundred or a thousand more 2-D gels that supported our hypothesis would not provide the proof we needed, so we began to think about cloning the complementary deoxyribonucleic acid (cDNA) for this protein. I say this protein because at this time, we knew that while there were 4, 30 kDa "spots" on the gels, these spots were in fact modifications of the same protein and thus were one gene product. This would ostensibly make our task a bit easier, though still difficult.

I personally had no formal training in molecular biology and therefore the task of cloning this cDNA seemed daunting to me. I considered spending some time in Dallas where I was offered space in Evan Simpson's laboratory at the University of Texas Southwestern Medical Center, where I could work with my former student, Mike Kilgore, and learn the art of cloning and see if we could clone the cDNA for this protein. At that time, one of the most fortuitous and important things in my career happened. Barbara Clark had agreed to join my laboratory in 1992, just having finished her Ph.D. in Mike Waterman's laboratory at UT Southwestern in Dallas. Barbara was very well versed in all of the methodology that would be required to clone the cDNA for this protein. We talked about the project and she agreed to tackle it head on. It was an extremely difficult project as this work was performed prior to mass spectrometry and Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) and the fact that this protein represented approximately 0.2% of the total cell protein and 0.7% of the mitochondrial protein (as we later determined). Barbara diligently tried to purify the protein using fast protein liquid chromatography (FPLC). As we had no assay that we could utilize on the fractions collected, she attempted to "find" the protein by running mini 2-D gels on each of the eluted fractions, an unbelievable effort when you think about it. FPLC proved to be unsuccessful and Barbara then went through the arduous process of preparing mitochondrial mitoplasts, solubilizing the protein, running prep gels, cutting out the 28–32 kDa regions of the gels, extracting the proteins from these gels, concentrating the proteins, and running these concentrates on 2-D gels followed by silver staining to visualize the 30 kDa proteins. Then the proteins were carefully excised from the gels and retained until sufficient amounts of protein were obtained for microsequencing by the Harvard Microchemical Facility. The facility indicated to us that approximately 100 pM would be required for sequencing and approximately twice this amount was sent to them. Tryptic digestion of the 30 kDa protein, high-performance liquid chromatography (HPLC) separation of digested protein into separate peaks and sequencing of three of these peaks produced sequences of 19, 13, and 12 amino acids. With this information, degenerative nucleotides of 17–24 bases long were

synthesized and used to amplify the 30 kDa cDNA from a cDNA library using polymerase chain reaction (PCR). This resulted in a 400 base pair product that in turn was used to probe the cDNA library once again. This probe resulted in the isolation of a 1,456 base pair full-length cDNA clone that when sequenced was found to represent a novel cDNA. The open reading frame of this cDNA was found to be 852 base pairs in length and coded for a protein that was 284 amino acids in length that also proved to be a novel sequence. A number of steps were taken to assure that the correct cDNA had been cloned with perhaps the most definitive proof being that the open reading frame contained the exact same sequences that were provided to us by the Harvard Facility. While this all sounds very straight forward, I can assure you it was anything but that, as we encountered a number of starts and stops all along the way during these efforts, just as all laboratories do.

We were now at last in a position to determine if a cause and effect situation existed for this protein. Barbara cloned the cDNA for the 37 kDa precursor protein into a pCMV expression vector and transfected this vector into unstimulated MA-10 cells and compared steroid synthesis to empty pCMV-transfected cells. Expression of the 37 kDa/30 kDa protein resulted in a twofold to fourfold increase in progesterone production in the absence of hormone stimulation, and thus provided the cause and effect observation that was required to demonstrate the function of this protein. We strongly felt that this was the evidence that we needed to convince the scientific community that this protein functioned as the acute regulator of steroid hormone biosynthesis that had long been sought. We named the protein StAR, for Steroidogenic Acute Regulatory protein. During the course of these experiments, other individuals in my laboratory added significant observations to this story. Steven King performed in vitro transcription and translation of the 37 kDa cDNA and showed that this resulted in the synthesis of all 4 forms of the 30 kDa protein, a strong supporting piece of information [53]. Jeff Wells, a technician in our laboratory, worked tirelessly in growing and harvesting MA-10 cells that were needed for the isolation of the 30 kDa proteins. This was a team effort that culminated with the publication describing the purification, cloning, and expression of the StAR protein in the November 1994 issue of *The Journal of Biological Chemistry* [53].

Earlier in 1994, I met Jerry Strauss from the University of Pennsylvania, at a meeting in the UK. I was discussing some of our results with Jerry and eventually the discussion came around to the enigma of searching for the cause of the disease, lipoid congenital adrenal hyperplasia (lipoid CAH). Lipoid CAH results from an almost complete inability of the afflicted patients to synthesize steroid hormones. Lipoid CAH was originally thought to be a result of a mutation in the CYP11A1 gene, but this proved to be incorrect once the human gene was cloned and later sequenced from the tissue of patients having this disease [54]. Similarly, other protein candidates were discounted as the cause of lipoid CAH. We felt that malfunction of the 30 kDa protein that we were working on could be a candidate for causing lipoid CAH. We then sent the 400 bp cDNA fragment to Jerry and his laboratory was able to clone the human cDNA counterpart of the StAR gene. The information that resulted from cloning and sequencing the human StAR cDNA was sent to another colleague, Walter Miller at the University of California, San Francisco. Walter

had access to human tissue (testicular tissue) from patients who had been diagnosed with lipoid CAH. Using the sequence information he received from Jerry, Walter's laboratory was able to clone the StAR cDNA from lipoid CAH tissue. The first four clones that Walter's lab isolated and sequenced from lipoid CAH patients had mutations in the StAR cDNA. Cloning these mutated cDNAs into expression vectors and expressing them in COS-1 cells, transfected with the cholesterol side-chain cleavage enzyme system, indicated that these mutated StAR cDNAs could not support steroid synthesis while control StAR cDNAs could. In essence, nature had provided a StAR knockout and its phenotype proved that StAR was an essential element for acute steroid biosynthesis. These elegant and important results support the role of StAR and also provided unequivocal evidence for the cause of lipoid CAH. These results were published in *Science* in the spring of 1995 [55]. The human StAR mutation findings were corroborated in 1997 with the Proceedings of the National Academy of Sciences USA (PNAS) publication of the phenotype of the mouse StAR knockout by Keith Parker's laboratory [56]. The mouse phenotype essentially mirrored the human phenotype and thus, a rodent lipoid CAH model became available for further study. Since that time, less severe mutations in the StAR gene as well as mutations in the CYP11A1 gene have been identified in patients with lipoid CAH [55, 57–59].

These are the best recollections of the history of the discovery of StAR that I have as they pertain to my own laboratory. In a narrative such as this, it is impossible to chronicle each of the individual contributions made to this story and I apologize for leaving out the names of individuals who have so contributed. Individual contributions can, of course, be gleaned from the publications that have chronicled this series of investigations. As of this writing, more than 1730 manuscripts have been published that were identified using "steroidogenic acute regulatory" as a probe on PUBMED. Last, but certainly not the least, I would like to acknowledge the support of the National Institute of Child Health and Human Development (NICHD) of the NIH and the Robert A. Welch Foundation for making this work possible.

StAR (STARD1) Overview

Over a 20-year span (1994–2014), the biochemical and genetic data on StAR has confirmed its essential role in cholesterol transfer into mitochondria and in controlling steroidogenesis (reviewed in [60]). Of particular importance was the finding that mutations in the *hSTARD1* gene are the most common basis for lipoid CAH, as discussed above [55, 61]. The mechanism of cholesterol transport by StAR and lipoid CAH is the topic of Chap. 4 so we will only briefly summarize StAR protein function. StAR is a nuclear gene that encodes a protein that is synthesized in the cytoplasm as a precursor protein with an amino-terminal domain characteristic of a mitochondrial targeting sequence; e.g., a predicted amphipathic helix commonly found in matrix localized proteins [53]. Mitochondrial import and processing of the newly synthesized 37 kDa precursor StAR produces a 32 kDa intermediate product

and a mature 30 kDa matrix localized form (reviewed in [60]). The current model for StAR function is that StAR is active on the cytosolic side of the outer mitochondrial membrane and that import and processing "inactivates" StAR. The inactivation is a consequence of localization of StAR to the interior of the mitochondria rather than a loss of cholesterol binding and function. Indeed, key studies showed that in the absence of the N-terminal mitochondrial targeting sequence, StAR is composed solely of a START domain that does not get processed yet is capable of binding cholesterol and facilitating cholesterol transport across mitochondrial membranes [62]. Therefore, the import and processing of StAR by mitochondria is proposed to be an "off" switch for cholesterol transfer and thus steroidogenesis. However, studies from StAR knockout mice and StAR transgenic animals implicate that in vivo the targeting of StAR to mitochondria may be important for efficient cholesterol transfer and steroid production. StAR knockout mice accumulate significant amounts of cholesterol in the adrenals and gonads and the animals die shortly after birth due to the absence of adrenal hormones [63]. Lipid accumulation was attenuated and adrenal and gonadal steroidogenesis were restored in StAR knockout mice that expressed a StAR transgene. On the other hand, when an amino terminal truncated StAR transgene was expressed, the mice retained lipid accumulation in the adrenal and gonads and had partially restored steroidogenesis in a tissue- and gender-specific manner [64]. The StAR transgenic animal studies support that StAR is capable of functioning without being targeted to the mitochondria, but highlight the importance of correct and efficient subcellular localization of the protein for full function in vivo.

Since continual synthesis of 37 kDa precursor StAR is required to maintain the active cytosolic form of StAR associated with mitochondria, the mechanisms that control StAR protein expression control steroidogenesis. Both transcriptional and posttranscriptional mechanisms regulate StAR gene expression, and this chapter provides an overview of the mechanisms controlling StAR expression in the gonads and adrenal. First, a general overview for transcription initiation and hormonal regulation of steroidogenesis is provided to set the context for StAR regulation.

Basic Mechanisms for Transcription Initiation

A gene that is transcribed by RNA Polymerase II (RNAPII) has distinctive functional elements that are required to initiate and enhance transcription. The promoter region of a gene contains sequence-specific cis-acting DNA elements, or regulatory elements, that are recognized and bound by trans-acting DNA-binding proteins (transcription factors) required to initiate transcription. Studies that characterize gene promoters provide the location for these cis-acting regulatory elements and, by convention, the position of these elements are designated relative to the transcription start site (TSS). The TSS is designated as the first nucleotide transcribed into pre-mRNA and is denoted numerically by +1. The core promoter is

defined as the minimal region required for RNAPII to initiate transcription. There are two major classes of core promoter sequences for RNAPII binding; an AT-rich DNA sequence containing a consensus TATAAAA element called the TATA box is found in approximately 25% of mammalian genes, and CG-rich regions referred to as a TATA-less promoter found in the remaining 75% of the genes. In general, TATA-box elements are found in highly regulated, cell-specific expressed genes while CG-rich regions serve as promoters for many housekeeping or commonly expressed genes. Recruitment of RNAPII to the core promoter and proper transcription initiation is controlled by the assembly of the general transcription complex that together with RNAPII forms the transcription preinitiation complex (PIC). The assembly of the PIC is enhanced by activator and coactivator proteins that bind to regulatory elements within the promoter.

Activator proteins are transcription factors that bind their respective sequence-specific DNA regulatory elements and function in either a constitutive manner (always bound to DNA) to maintain steady-state levels of gene expression or in a developmental or regulated manner to activate gene transcription in response to external stimuli such as hormonal signaling. The sequence-specific DNA regulatory elements typically contain a twofold symmetry and the activator proteins bind as homo- or heterodimers. Common DNA and protein interacting motifs identified within activator proteins include the helix-turn-helix (HTH), basic helix-loop-helix (bHLH), zinc finger (Zn finger), and basic leucine zipper (bZIP) motifs. bZIP proteins have a conserved region aligned by the spacing of leucine resides every 7th amino acid that folds into an α-helix containing a hydrophobic face with evenly spaced leucines. The interdigitation of the leucines between these α-helices forms the leucine zipper and promotes protein dimerization. A basic region is adjacent to each helix and binds the half-site of the response element; therefore, dimerization is necessary for function (DNA binding). bZIP motifs are commonly found in activators that are regulated in response to acute cellular signaling. For example, the c-FOS and c-JUN proteins of the AP-1 transcription family are phosphorylated and activated by protein kinase C (PKC) signaling and the cAMP responsive element-binding protein, CREB, is phosphorylated and activated by the protein kinase A (PKA) signaling pathway. Another common DNA-binding motif is the zinc finger; a structure formed by the coordination of Zn ions by specifically spaced cysteine (Cys4) or cysteine and histidine (Cys2His2) residues that generates adjacent finger-like structures that bind the major groove of DNA and promote protein dimerization. The Zn finger Cys_2Cys_2 motif is shared by members of the steroid hormone/nuclear receptor superfamily where ligand binding enhances homo- or heterodimer formation and DNA binding.

Coactivator and corepressor proteins do not bind DNA directly but are recruited to promoters via protein interactions with the DNA-bound activator proteins (reviewed in [65–67]). Conserved protein interacting motifs found on activating proteins include the LXXLL motif for nuclear receptor-coactivator interactions, and kinase interacting domain (KID) for CREB/activated transcription factor (ATF) family members and coactivators [67, 68]. Protein-protein interactions can be dependent upon posttranslational modification(s), such as phosphorylation of CREB

on Ser133 by PKA or Ca^{2+} signaling, which results in increased association with the coactivator CREB-binding protein (CBP/p300 [68–71]). Coactivators classically have intrinsic histone acetyltransferase activity (HAT) and acetylate core histone proteins (H2A, H2B, H3, and H4) leading to open chromatin structure for active transcription. Members of the HAT family include CBP/p300, P/CAF (p300/CBP Associated Factor), and the p160 family that includes SRC-1(NCoA-1), SRC-2 (NCoA-2), and SRC-3. Additional histone modifying enzymes have been shown to contribute to coactivator function [72].

Conversely, a gene may be actively silenced by the constitutive presence of a corepressor complex on the promoter. Corepressor proteins, like coactivators, are associated with DNA bound activator proteins via protein-protein interactions thereby localizing the corepressor to specific promoter regions of the gene [67]. The repressor protein then recruits HDAC proteins, enzymes with intrinsic histone deacetylase activity that help maintain histones in the deacetylated state and promote gene silencing. mSin3 and nucleosome remodeling and histone deacetylation (NuRD) are two common repressors utilized by multiple activator proteins to recruit HDAC1 to the promoter. Nuclear receptors also can utilize the corepressors silencing mediator of retinoid and thyroid receptors (SMRT) and nuclear receptor corepressor (NCoR) to either recruit HDACs directly or indirectly via Sin3A complex formation [73, 74].

Hormonal Regulation of Steroidogenesis and StAR Function

The hypothalamic-pituitary-gonadal axis is the major hormonal signaling cascade that controls sex hormone output by the testis and the ovary while the hypothalamic-pituitary-adrenal axis is the major hormonal signaling cascade that controls glucocorticoid synthesis by the adrenal gland (Fig. 2.2a). In brief, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) or corticotropin-releasing hormone (CRH) that then stimulate the gonadotropes or corticotrophes of the anterior pituitary, respectively, to synthesize and secrete the gonadotropins leutinizing hormone [75] and follicle stimulating hormone (FSH) or adrenocorticotropin hormone (ACTH). LH stimulates testosterone synthesis by testicular Leydig cells and androgen production by ovarian theca cells while FSH stimulates estrogen production by ovarian granulosa cells. ACTH stimulates glucocorticoid synthesis by the adrenal gland. Mechanistically, ACTH, LH, and FSH bind to their respective 7-transmembrane G-protein-coupled receptors in their target tissues and activate signal transduction pathways leading to increased expression and/or activation of transcriptional activator proteins and subsequent increase in StAR and other genes within the steroid hormone biosynthetic pathway (see Fig. 2.2b and legend for more detailed description). The cross-talk between these pathways leads to maximal increase in steroid output. The cAMP-PKA signal transduction pathway is central to

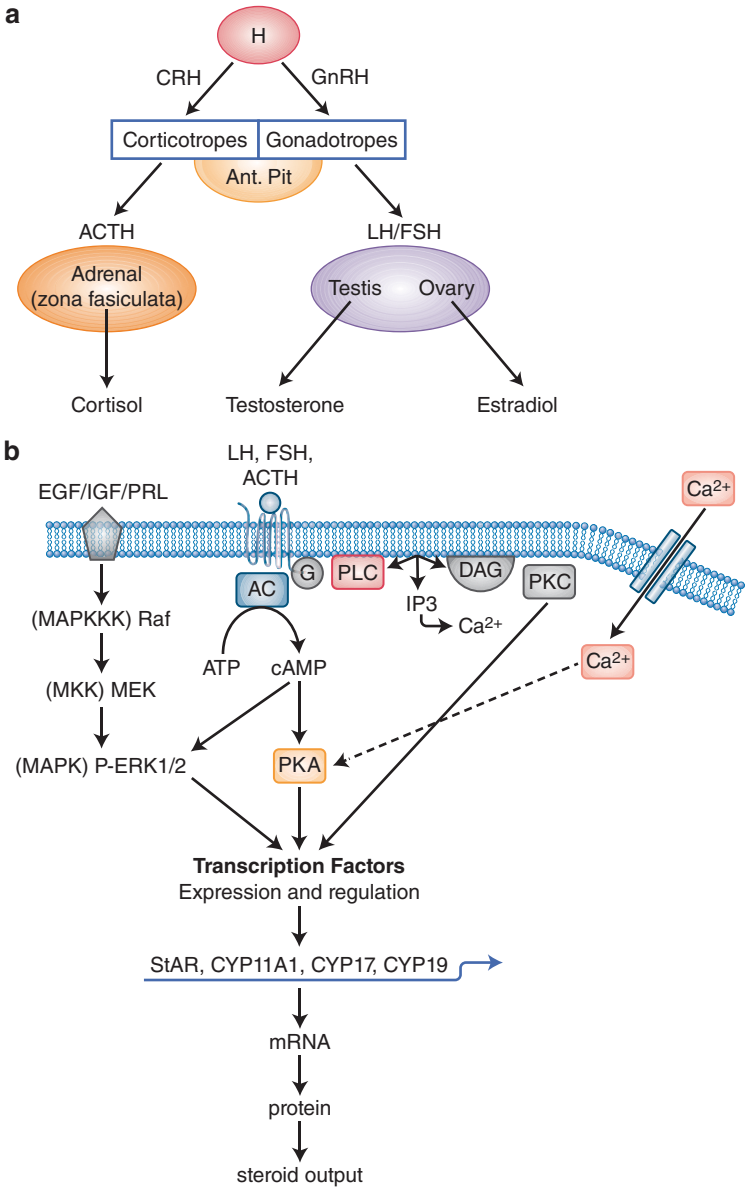

Fig. 2.2 Mechanisms for tropic hormone regulation of steroidogenesis. **a** The hypothalamic-pituitary-adrenal/gonadal axis controlling steroid hormone production. The pathway is described in the text. **b** Mechanisms for increased StAR and steroidogenic gene expression following tropic hormone stimulation. Shown is a model of the major signal transduction pathways activated by tropic hormone (LH, FSH, ACTH) stimulation of their respective G-protein-coupled receptors. Activated second messenger pathways include adenylyl cyclase (AC)-mediated increase in cAMP levels and the subsequent activation of protein kinase A (PKA), phospholipase C (PLC)-mediated increase in diacylglycerol (DAG) and inositol 1, 4, 5 trisphosphate (IP3). DAG then activates protein kinase C (PKC) while IP3 increases intracellular calcium levels that also promote PKC activation. Epidermal growth factor (EGF), insulin-like growth factor (IGF), and prolactin (PRL),

hormone-dependent activation of gonadal sex hormone and adrenal glucocorticoid and androgen synthesis. In addition, aldosterone, a critical steroid hormone that regulates salt balance and blood pressure, is produced by the adrenal gland. Major regulators of aldosterone synthesis are angiotensin II (Ang II) and K^+. Ang II is a peptide hormone that binds to its respective G protein-coupled cell surface receptor and activates the protein kinase C (PKC) and calcium signaling pathways while K^+-mediated membrane depolarization activates voltage-gated calcium channels and subsequent calcium-PKC- signaling pathways (reviewed in [76]). Similar to cAMP-PKA signaling, Ang II and K^+ lead to transcription factor phosphorylation and increased target gene expression, including StAR, resulting in increased aldosterone synthesis [77–80]. This chapter focuses on the cAMP-PKA-dependent mechanisms that activate StAR gene expression. The unifying theme for StAR gene regulation is that hormone-dependent activation of the signaling pathways that regulate transcription factor activation and recruitment to the StAR promoter functions in a cell-type-dependent manner resulting in cell-specific mechanisms that control StAR expression.

PKA-dependent phosphorylation of StAR is a key mechanism controlling StAR function. StAR was originally observed to be a phosphoprotein, a modification that was established by the early observations of four protein spots after 2-D gel electrophoresis; two of which were sensitive to alkaline phosphatase treatment (Fig. 2.1). Two consensus PKA phosphorylation sites at Ser56/57 and Ser194/195, in murine and human StAR, respectively, are *bona fide* PKA targets [81, 82]. StAR protein harboring a Ser194/95Ala mutation fails to mediate cholesterol transport into mitochondria, although the cholesterol-binding property is not changed due to phosphorylation [81–83]. In addition, PKC-dependent synthesis of wild-type StAR without subsequent PKA-dependent phosphorylation does not promote steroid production, supporting a role for posttranslational modification of StAR for function [83]. While the importance for StAR phosphorylation is established, the mechanistic link between StAR phosphorylation and function remains to be determined.

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activate the Ras/Raf and mitogen-activated protein kinases (MAPK/ERK) pathways, which have been shown to modulate the cAMP-PKA-dependent control of StAR expression. The activation of these protein kinases results in the increased expression and/or phosphorylation of transcription factors that control StAR and steroidogenic enzymes within the steroidogenesis pathway, leading to an increase in steroid output. The potential cross-talk between these pathways through cAMP-mediated activation MAPK or calcium signaling modulating PKA illustrates the potential for multi signals converging on activation of key transcription factors that contribute to increased gene expression. The cAMP-PKA pathway is the key pathway regulating StAR expression and steroidogenesis in steroidogenic tissues. This may be attributed, in part, to the PKA-dependent phosphorylation of StAR that is required for function. The model pathway in **b** is adapted from [114]. CYP11A1, cytochrome P450 side chain cleavage enzyme; CYP17, cytochrome P450 17a-hydroxylase, 17, 20 lyase; CYP19, cytochrome P450 aromatase are used as examples of regulated genes within the steroid hormone biosynthetic pathway *H* hypothalamus, *CRH* corticotrophin-releasing hormone, *GnRH* gonadotropin-releasing hormone, *Ant. Pit.* anterior pituitary, *ACTH* adrenocorticotropin hormone, *LH* luteinizing hormone, *FSH* follicle stimulating hormone, *StAR* steroidogenic acute regulatory protein, *cAMP* cyclic adenosine-3′,5′-monophosphate, *DAG* diacylglycerol

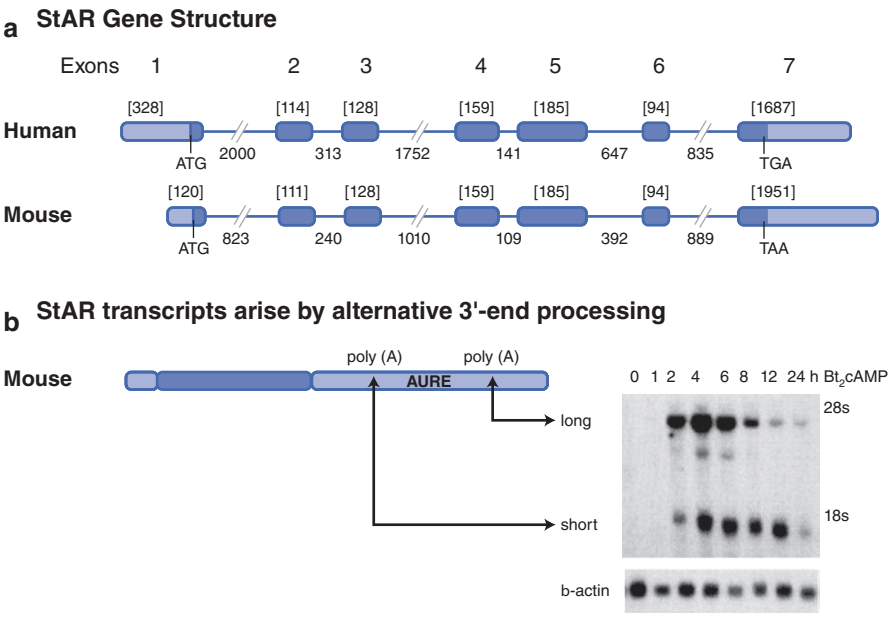

Fig. 2.3 StAR gene and primary transcript structure. **a** Schematic representation of the human and mouse StAR gene structure. The sequence information obtained from human gene ID 6770, NG_011827 and mouse gene ID 20845, NM_011485 were used to cartoon the gene structures for human and mouse StAR, respectively. Exons are depicted by dark boxes with the 5′ and 3′ untranslated regions shown in lighter color and the size of the exons are given above each box. Introns are depicted by a line with the size shown below the line. The start (ATG) and stop (TGA/TAA) codons are indicated in the schematic. Exon 2 of human StAR contains an additional 3 base pairs relative to the mouse sequence. **b** Alternative 3′-end processing results in two major StAR transcripts. Shown is a schematic of the mouse StAR primary transcript. The coding region is depicted by the dark box with the 5′ and 3′ UTR in lighter shade. Two major polyadenylation signals (poly(A)) within the 3′-UTR of mouse StAR that promote differential processing leading to a long (~3.5 kb) and short (~1.6 kb) transcript in rodent cell lines. The two major transcripts are shown by Northern blot analysis of StAR mRNA in MA-10 mouse Leydig tumor cells after treatment with the cAMP analog Bt_2 cAMP for the indicated time. The presence of destabilizing AUREs (AU-rich elements) in the longer transcript are proposed to contribute to the shorter half-life of the longer transcript [173]. Figure for Northern blot data is adapted from data presented in [84] *StAR* steroidogenic acute regulatory protein, *mRNA* messenger ribonucleic acid, *cAMP* cyclic adenosine-3′,5′-monophosphate, *AU* adenosine-uridine

StAR Gene Structure

Shortly after we cloned the mouse StAR cDNA, we characterized the mouse gene [84]. The gene is comprised of 7 exons and 6 introns that span a relatively short distance, 6.25 kb, located on chromosome 8 [84]. The cDNA contains an open reading frame of 852 bases encoding a protein of 284 amino acids with predicted molecular weight of 31.6 kDa [53]. The human StAR gene shares the same general structure with 7 exons-6 introns spanning ~8 kb that maps to chromosome 8p11.23 [85] (Fig. 2.3). The human cDNA encodes a protein of 285 amino acids due to an

additional 3 nucleotides in exon 2 that results in an insertion of a serine at position 47. Many of the mammalian StAR proteins are 285 amino acids in length, with the mouse and rat being the apparent exceptions. The additional amino acid has no apparent functional advantage since deletion of the amino terminal 65 amino acids from StAR does not decrease activity for cholesterol transfer.

The core promoter of StAR contains a TATA-box, common for highly regulated genes. To localize the cAMP-responsive region of StAR promoters, early studies used reporter gene assays and tested the activity of a series of 5′-deletion constructs beginning with >3 kb of the 5′-flanking sequence upstream of the transcription start site (TSS). A region within 250 bp of the TSS was shown to be sufficient for maximal promoter activity of mouse, human, rat, and bovine StAR genes in the adrenal and gonads. Alignment of StAR promoter regions from multiple species revealed highly conserved sequence-specific DNA regulatory elements within the first 150 bp region immediately upstream of the TSS (Fig. 2.4a). This proximal promoter region of StAR has been most extensively studied utilizing mouse and human cell lines. These studies demonstrated that the conserved regulatory elements bind their cognate transcription factors in a cell-type- and species-specific manner. The major transcription factors found to activate StAR transcription are steroidogenic factor-1 (SF-1 also referred to as adrenal 4-binding protein and encoded by NR5A1), NUR77 (NR4A1), CCAAT/enhancer-binding protein beta (C/EBPβ), GATA-4, activator protein-1 (AP-1) family members, and CREB/CREB-modulator (CREM) family members (Fig. 2.4a).

Zinc-Finger Transcription Factors Involved in StAR Gene Expression

SF-1 is an orphan member of the nuclear receptor superfamily that binds as a monomer to the consensus DNA sequence, 5′- AGGTCA-3′; a sequence that was originally found to be common in promoters of several steroid hydroxylase genes [86–89]. SF-1 is highly expressed in steroidogenic cell types where it functions to help control the tissue-specific expression of genes involved in the steroid hormone biosynthesis pathway [90, 91]. Additionally, gene knockout studies in mice showed that SF-1 is critical for development and differentiation of the endocrine and reproductive systems, demonstrating it has multiple functions [92]. SF-1 function has been linked to both basal and cAMP-PKA-dependent gene expression, with promoter- and cell-specific mechanisms contributing to SF-1-dependent target gene expression. Mutational analysis of a non-consensus SF-1 element located directly upstream of the CRE/AP-1 element in StAR proximal promoter verified SF-1 plays a major functional role in StAR expression (Fig. 2.4). SF-1 binds the RNA coactivator SRA (steroid receptor activator RNA) and SRA is important for SF-1-dependent gene activation in the adrenal and gonads [93]. While the cAMP-PKA-dependent mechanisms controlling SF-1 function are not fully elucidated, current working

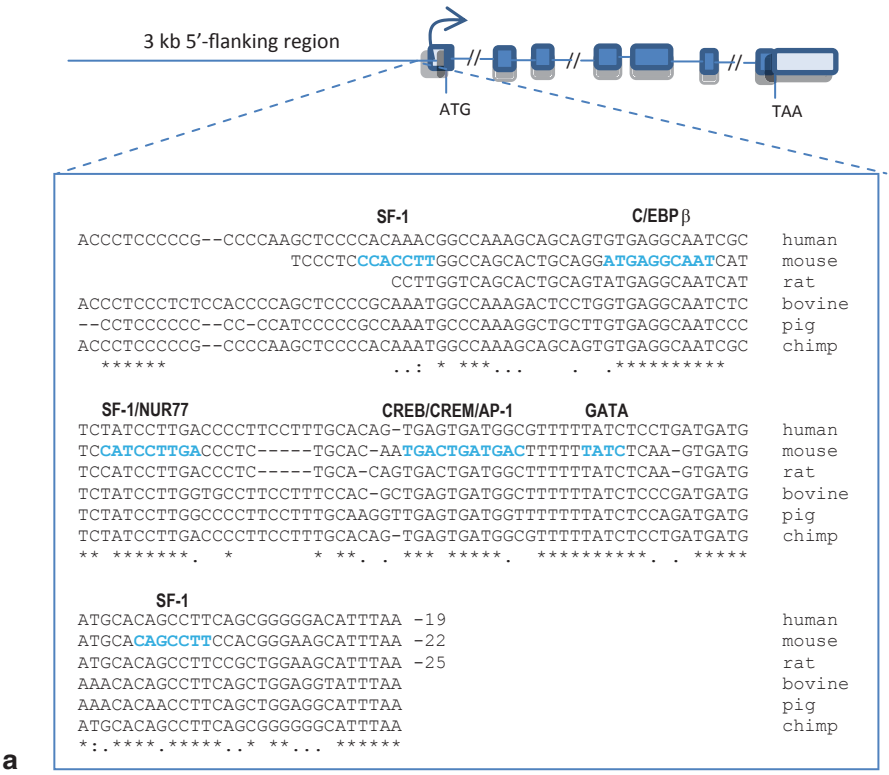

Fig. 2.4 Multiple Sequence Alignment of the StAR Proximal Promoter. **a** Shown is a ClustalW alignment of human, mouse, rat, bovine, pig, and chimpanzee StAR promoter sequences between the TATA box (TTTAA) and the distal SF-1 element located at -145 bp in the mouse promoter sequence. The binding sites for SF-1, C/EBPβ, CREB/CREM/AP-1 are shown in *light blue* and labeled with the names of the respective transcription factor(s). **b** Comparison of the cAMP-responsive region of the human and mouse StAR proximal promoter. Shown is a short 53 bp region that encompasses non-consensus SF-1 and CRE/AP-1 elements and a consensus GATA element. Each of these DNA elements has been shown to bind their respective transcription factors and increase StAR transcription. The divergence between the mouse and human sequence within the critical elements and in the flanking DNA sequences may contribute, in part, to promoter (species)-specific differences in transcription factor binding. *StAR* steroidogenic acute regulatory protein, *C/EBP* CCAAT-enhancer-binding protein—beta, *CREB* cyclic-AMP responsive element-binding protein, *CREM* CREB modulator protein, *AP-1* activator protein 1, *SF-1* steroidogenic factor-1, *cAMP* cyclic adenosine-3′,5′-monophosphate, *DNA* complementary deoxyribonucleic acid

models include indirect mechanisms by which cAMP-PKA signaling promotes production of SF-1 ligands and SF-1 cofactors to enhance SF-1 function [94].

NUR77, encoded by the NR4A1 gene and also referred to as nerve growth factor induced-B (NGFI-B) is an orphan nuclear receptor that binds as a monomer to the cis-acting consensus sequence 5′-AAAGGTCA-3′ called the NGFI-B response element (NBRE). As the name implies, NUR77, and related family members NOR1 and NURR1, were characterized for their function in the nervous system as part of the immediate early response to stimuli such as growth factors and membrane depolarization [95]. In steroid producing tissues NUR77 expression is increased by hormonal stimulation and NUR77 has been shown to regulate steroidogenic gene expression [96–102]. The NBRE shares sequence identity with the SF-1 element and a NBRE overlaps the SF-1 element in the mouse STAR promoter (Fig. 2.4a). Calcium signaling contributes to the cAMP-dependent increase in steroidogenesis and a calcium-dependent increase in NUR77 expression leads to increased binding to the SF-1/NBRE element in the mouse StAR promoter [102–104] (Fig. 2.4a). However, NUR77 does not bind this region in the human StAR promoter [105, 106], likely due to flanking sequence variation at the SF-1/NBRE element between the mouse and human promoters (Fig. 2.4a). The inability for NUR77 to bind to the human StAR promoter, and potentially compensate for SF-1 function, may help explain the dependence on SF-1 for StAR promoter activity in human cell lines [78, 107–110].

The GATA family of zinc-finger DNA-binding proteins is named for the cis-acting DNA core consensus sequence, 5′- GATA-3′, that they bind. This family is composed of six members, GATA-1–GATA-6, with GATA-4 associated with gene transcription and development in the heart. However, GATA-4 is expressed in steroidogenic tissues as well as in the heart, lung, liver, and small intestine and has been shown to play a broader role in gonadal development and tissue-specific gene expression [111–113]. GATA proteins function with FOG (friend of GATA) and p300/CBP to activate target gene expression. StAR and several steroid hydroxylase gene promoters contain GATA elements and GATA-4 has been shown to regulate these genes in the testis and ovary.

bZIP Transcription Factors Involved in cAMP-Dependent StAR Gene Expression

Comprehensive reviews of the role of bZIP proteins in StAR gene regulation summarize the studies that identified the roles for members of this family of transcription factors that bind to a non-consensus CRE/AP-1 element in the StAR promoter and control transcriptional activation or repression [114, 115]. Therefore, only a broad overview highlighting the current models for StAR gene regulation is provided herein.

The *CREB, CREM, and ATF-1* genes encode a family of transcription factors that bind to cAMP-responsive elements [116] that have the consensus sequence

5′-TGACGTCA-3′. CREB/CREM/ATF-1 family members can form homo- or heterodimers through the bZIP domain and can functionally compensate for each other in activating target gene expression in response to cAMP-PKA signaling [117–120]. CREB is known generally as a transcriptional activator while multiple CREM isoforms, generated via alternative splicing or use of alternative promoters of the CREM gene, both transcriptional activators (CREMτ, τ1, and τ2) or repressors (CREMα, β and γ) and ICER (inducible cAMP early repressor). CREMτ is the major transcriptional activator isoform in the testis, with strong expression in germ cells where it controls expression of genes critical for spermatogenesis [121]. In mouse Leydig cell cultures, both CREB and CREM are phosphorylated in response to PKA activation and the phosphoproteins bind to the StAR CRE/AP-1 element and contribute to the cAMP-dependent increase in StAR transcription [122, 123]. CREB is not expressed in the adrenal and in this tissue, CREM isoforms bind to the CRE/AP-1 element and activate StAR transcription [78, 110].

AP-1 is a transcriptional complex composed of Fos and Jun proteins. Members of the Fos family (c-Fos, FosB, Fra-1, and Fra-2) are found only as a heterodimer partner with members of the Jun (c-Jun, JunB, and JunD) proteins and with some members of the CREB/ATF family, while Jun members form either homodimers or heterodimers [124–126]. AP-1 family members are phosphorylated in response to PKA or PKC activation and also bind to the CRE/AP-1 element [122, 127]. c-Jun functions as a potent trans-activator of *StAR* transcription in Leydig cells, possibly through the formation of heterodimers with CREB family members [127], while c-Jun/c-Fos heterodimers can both activate and repress StAR [128–131].

The C/EBP family members bind to CCAAT box DNA elements with the consensus sequence A/GTTGCGC/TAAC/T [132]. The C/EBP family is best known for regulating genes that control cell differentiation and are differentially expressed in a tissue-specific manner [133–135]. In steroidogenic cells, C/EBPα and C/EBPβ isoforms are detected and C/EBPβ expression is increased by cAMP-mediated mechanisms in mouse testicular Leydig and ovarian granulosa cells [136–138]. The functional C/EBP element in the StAR promoter is highly conserved and C/EBPβ in MA-10 Leydig cell, rodent granulosa-luteal cell, and human granulosa-luteal cell nuclear extracts was shown to bind to the mouse and human StAR proximal promoter [139–141]. Protein-protein interactions between C/EBPβ and SF-1 and C/EBPβ and GATA-4 have been proposed to contribute to Leydig and granulosa cell StAR gene expression, respectively.

Model for cAMP-PKA-Dependent Activation of STAR Transcription

A cAMP responsive region, referred to as the CAN or CRE/AP-1 site, is a non-consensus CRE with overlapping specificity for members of the CREB/CREM and AP-1 protein families (Fig. 2.4b). This region is the most complex of the StAR proximal promoter due to the complex pattern of transcription factor binding that

occurs in a species- and tissue-specific manner. Potential heterodimer formation between CREB, AP-1, and C/EBP bZIP proteins provide a level of control to integrate gene expression through one common element yet distinct trans-activating proteins.

One unifying theme identified by studies on StAR gene regulation from multiple laboratories is full promoter activity and a robust cAMP-dependent response involves SF-1 and GATA-4 binding to their respective elements in the StAR promoter (Figs. 2.4b and 2.5). Mutation of either the SF-1 or GATA-4 site does not diminish cAMP induction of StAR promoter activity, yet the loss of both of these factors attenuates overall promoter activity. The SF-1 and GATA-4 elements flank the critical CRE/AP-1 element, and protein-protein interactions between SF-1-GATA-4, SF-1-CREB, and C/EBPβ-GATA-4 have been reported and these interactions may help stabilize CREB/CREM/AP-1 factor binding at the CRE/AP-1 element. However, the fine-tuning of the hormone-dependent response is controlled by factor binding to the CRE/AP-1 element. Given that several combinations of bZIP proteins bind to this element, it is likely that the relative cellular levels of CREB/CREM/AP-1 proteins and preferential activation (phosphorylation) of these factors by hormone-dependent activation of the PKA and/or PKC signaling pathways regulate bZIP protein heterodimer partner formation controlling StAR gene expression in a cell type dependent manner.

The temporal pattern for transcription factor recruitment is nicely demonstrated by chromatin immunoprecipitation (ChIP) assays. The data support that upon 8-Br-cAMP treatment of MA-10 mouse Leydig tumor cells, the immediate response is recruitment of SF-1, GATA-4, CREB/CREM/AP-1, and CBP to the mouse StAR promoter followed by histone H3 acetylation and increased StAR mRNA levels. CREB, AP-1, and GATA-4 phosphorylation increases immediately following cAMP treatment of MA-10 cells with no change in total protein expression [142], indicating that phosphorylation of these factors promotes their recruitment to the StAR promoter and facilitates increased coactivator interactions [122, 140] (Fig. 2.5).

One model for cAMP-PKA stimulated StAR transcription that integrates the above studies is tropic hormone stimulation results in PKA-dependent phosphorylation of CREB/CREM and/or c-Fos/c-Jun (AP-1) and increases recruitment of these transcription factors to the CRE/AP-1 site in the StAR proximal promoter. SF-1 and GATA-4 bind to their respective elements that flank the CRE/AP-1 site. PKA-dependent phosphorylation of GATA-4 enhances both recruitment of this factor to the StAR promoter and protein-protein interactions. Protein-protein interactions between GATA-4 and SF-1, AP-1 and/or CREB and possible CREB- SF-1 interactions help stabilize the transcription complex and enhance recruitment of the coactivator CBP and RNAPII to the StAR promoter and increase transcription. SF-1-GATA-4 binding appear to be a common mechanism for mouse and human StAR gene activation in multiple cell types while the homo- or heterodimer partners (CREB/CREM or AP-1 or CREB-cJun) binding to the CRE/AP-1 element is dependent upon the cell type and stimulus.

An example of integrating StAR gene expression through one common element, yet distinct trans-activating proteins can be found in the follicular to luteal phase transition in the rodent ovary. During this transition, regulation of steroid

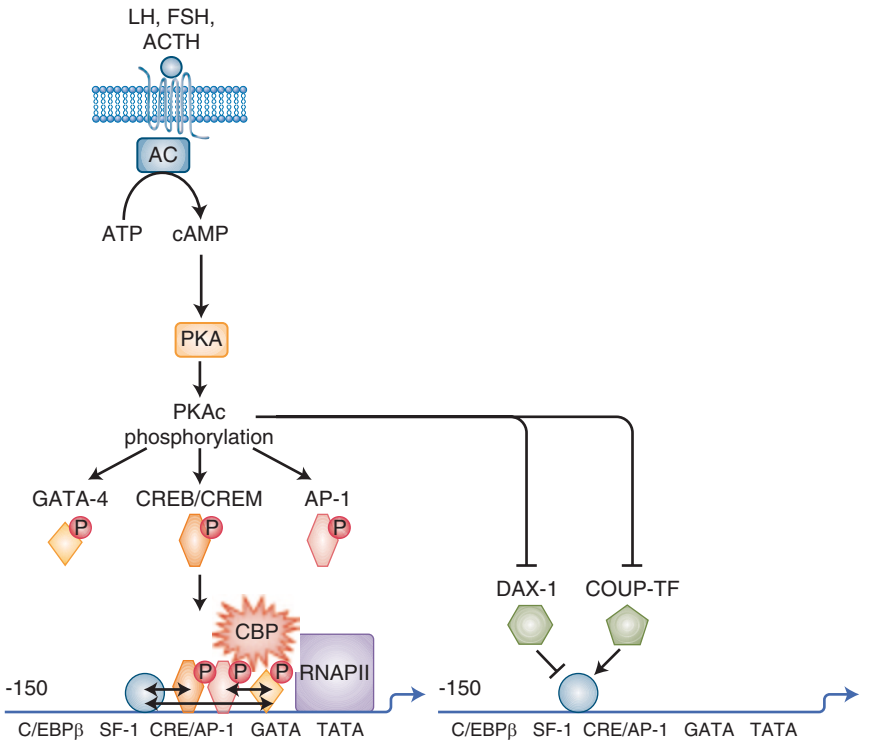

Fig. 2.5 Model for the cAMP-PKA-dependent activation of the mouse StAR gene. Tropic hormone (LH, FSH, ACTH) stimulation of their respective G-protein-coupled receptors (R) activates adenylyl cyclase (AC) and increases cAMP levels resulting in subsequent activation of protein kinase A (PKAc). PKAc-dependent phosphorylation of CREB/CREM, c-Fos/c-Jun (AP-1), and GATA-4 results in increased recruitment of these transcription factors to the StAR proximal promoter. The SF-1-GATA-4 binding appear to be a common mechanism for mouse and human StAR gene activation in multiple cell types while the bZIP homo- or heterodimer partners (CREB/CREM or AP-1 or CREB-cJun) binding to the CRE/AP-1 element is dependent upon the cell type and stimulus. Protein-protein interactions between GATA-4 and SF-1, AP-1 and/or CREB and possible CREB- SF-1 interactions help stabilize the transcription complex and enhance recruitment of the coactivator CBP and RNAPII to the StAR promoter and increase transcription. SF-1 may be constitutively bound to the promoter with increased association after cAMP-PKA activation. A switch from cAMP-dependent to cAMP-independent regulation of StAR transcription involves C/EBPb binding to its respective element and loss of CREB binding to the CRE/AP-1 element during luteolysis. NUR77 binds to a sequence overlapping the SF-1 element and modulates calcium-dependent regulation of StAR. Protein kinase A (PKAc)-dependent loss of DAX-1 and COUP-TF repressor functions: Both DAX-1 and COUP-TF function to interfere with SF-1 activation of the StAR promoter and actively repress StAR transcription (see text for details). Hormone stimulation and activation of PKAc results in decreased DAX-1 or COUP-TF expression in steroidogenic cells, thereby alleviating repression and promoting activation by allowing SF-1 and other factors to activate StAR gene expression *StAR* steroidogenic acute regulatory protein, *C/EBPb* CCAAT-enhancer-binding protein-beta, *CREB* cyclic-AMP responsive element-binding protein, *CREM* CREB modulator protein, *AP-1* activator protein 1, *cAMP* cyclic adenosine-3′,5′-monophosphate, *LH* luteinizing hormone, *FSH* follicule stimulating hormone, *ACTH* adrenocorticotropic hormone, *DAX-1* dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome gene 1, *COUP-TF* chicken ovalbumin upstream promoter transcription factor I and II, *CBP* CREB-binding protein, *RNAPII* ribonucleic acid polymerase II, bZIP basic leucine zipper, *AMP* adenosine-3′,5′-monophosphate

production switches from a cAMP-dependent (follicular phase) to cAMP-independent (luteal phase). StAR expression and steroid production are important during both phases and StAR gene regulation switches from a cAMP-dependent to a cAMP-independent mechanism. The switch is mediated by an AP-1 family member replacing CREB at the CRE/AP-1 element and recruitment of C/EBPβ to its functional element upstream of the CRE/AP-1 site [141]. ChIP analysis using mouse granulosa-luteal cells showed that C/EBPβ was recruited to the StAR promoter with no apparent increase in CREB/CREM association, indicating a tissue-specific difference in StAR regulation. These data are consistent with a switch in StAR regulation from a CREB/CREM-mediated (cAMP-dependent) to AP-1-C/EBPβ-mediated (cAMP-independent) mechanism in the ovary.

While much less is known about regulation of human StAR promoter activity, SF-1, GATA-4, and CREM remain important factors [78, 106–110]. The cAMP-dependent response in H295R human adrenocortical cells is CREM-mediated, and a CREM isoform has been shown to bind to the CRE/AP-1 element in the human StAR promoter [78, 110]. Overexpression of AP-1 family members JunB-Fos/FosB or JunB-ATF3 can transactivate StAR promoter-reporter gene expression in H295R cells, most likely via binding to the CRE/AP-1 element [105]. However, sequence differences in the CRE/AP-1 element between mouse and human StAR promoters may influence transcription factor binding, and this may broaden the transcription factor diversity for activating human StAR in response to different signaling pathways (Fig. 2.4b) [78].

Repression of StAR Transcription

In MA-10 mouse Leydig cells, a repressor complex composed of Sp3-mSin3A-HDAC proteins assembles on the mouse StAR promoter ~150 bp upstream of the CRE/AP-1 element [143]. Since the binding site for the repressor complex is not conserved, this may represent a species-(promoter)-specific repression of mouse StAR. In contrast, several repressor proteins have been shown to suppress StAR transcription by both direct and indirect mechanisms in several model systems and promoters. Repressor proteins can bind DNA and recruit corepressor complexes (direct), or block a trans-activator protein from binding DNA (indirect), thereby blocking assembly of a transcription initiation complex. The most extensively studied repressor for StAR is dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome gene 1 (Dax-1), a member of the nuclear receptor superfamily. DAX-1 is atypical in that it lacks a DNA-binding domain and contains a strong C-terminal repressor domain [144]. Expression patterns for DAX-1 and StAR in steroidogenic cell types are inversely related, providing correlative support for DAX-1 working as a repressor of StAR expression [145–153]. Despite the absence of a classical DNA-binding domain, DAX-1 has been reported to bind to a hairpin loop formed within promoters, including the StAR promoter [147, 154], which prevents SF-1 and/or NUR77 binding. In addition, direct SF-1-DAX-1 interactions that tether DAX-1 to the promoter via DNA-bound SF-1 results in recruitment of corepressor proteins and transcriptional repression of StAR [144, 154–156].

Chicken ovalbumin upstream promoter transcription factor I and II (COUP-TF), an orphan nuclear receptor, can function as either an activator or repressor protein [157]. As a repressor, COUP-TF can compete with SF-1 for binding to the AGGTCA element and recruit a corepressor complex, thus blocking SF-1-mediated transactivation [157–159]. COUP-TF can bind to SF-1 elements in the STAR promoter and overexpression of COUP-TF has been shown to block the hormone-stimulated increase in bovine StAR mRNA and human StAR promoter activity in bovine adrenal glomerulosa cells [160]. Thus, both DAX-1 and COUP-TF can function to interfere with SF-1 activation of the StAR promoter and represses StAR transcription. One possible mechanism for switching from repression to activation is hormone stimulation and activation of PKA resulting in decreased DAX-1 or COUP-TF expression, thereby allowing SF-1 and other factors to activate StAR gene expression (Fig. 2.5) [146, 153, 160, 161].

FOXL2, a member of the forkhead/hepatocyte nuclear factor 3 (FKH/HNF3) gene family, binds to the STAR proximal promoter (−42 bp) and represses StAR activity in the mouse ovary [162]. As with the other repressors, StAR and FOXL2 expression levels are inversely related; StAR mRNA levels are low in granulosa cells with high FOXL2. Differentiation of the follicle is associated with decreased FOXL2 and increased StAR, thus, hormone-dependent signaling appears to result in loss of a repressor and concomitant activation of transcription factors and coactivators to control StAR expression [141].

c-Fos and Yin yang 1 (YY1) have both activator and repressor functions on StAR promoter activity [78, 130, 163]. The rat StAR promoter has been well characterized for c-Fos, YY1, and DAX-1-mediated repression of StAR as a mechanism(s) contributing to suppression of steroid hormone biosynthesis and regression of the corpus luteum [130, 153, 163, 164]. Prostaglandin F2α (PGF2α) is a major signaling molecule for luteal regression in the rat ovary and PGF2α treatment was shown to increase c-Fos, YY1, and DAX-1 protein expression with subsequent repression of StAR promoter activity by both direct and indirect mechanisms.

Post-Transcriptional Regulation of StAR Expression

Two major StAR transcripts are detected for most mammalian species due to use of alternative polyadenylation sites in the 3′-UTR of the terminal exon [165] (Fig. 2.3). The differential processing of the 3′-end produces a short and long form of StAR mRNA with the long form retaining ~1800 nucleotides. The temporal pattern of expression of the two major transcripts after cAMP-PKA activation shows that both transcripts increase in parallel followed by a more rapid decrease in the long form relative to the short form [84, 85, 107, 166–168]. However, the steady-state mRNA levels, as determined by Northern blot analysis, indicates the short transcript is relatively minor compared to the long transcript, suggesting a preference for processing at the downstream polyadenylation site (84, 85, 107, 166–168]. The significance of the longer 3′UTR length is the presence of AU-rich destabilizing elements [169]

that are commonly found in short-lived transcripts, consistent with the temporal patterns of expression observed with StAR long v short transcripts. The AURE-binding protein, TIS11b (zinc finger protein, Znf36L1, also known as BRF1), binds to the StAR AURE and facilitates mRNA degradation [170–172]. Classically, destabilizing element-binding proteins, such as TIS11b, function by recruiting de-capping and deadenylation enzymes to initiate mRNA degradation although the mechanism for StAR mRNA degradation remains to be determined. TIS11b expression in the adrenal and gonads is increased by cAMP-PKA [170], providing a mechanism for decreasing StAR steady-state mRNA levels as another control point to regulate StAR protein levels in response to hormonal stimulation [173].

In addition to mRNA stability, the 3′UTR of StAR mRNA may serve to localize the transcript to the mitochondria by binding to the A kinase anchor protein, 121 (AKAP 121; 174,175). AKAP 121 is a scaffold protein associated with mitochondria that binds PKA regulatory subunits thereby localizing PKA to this organelle [176]. PKA was shown to be part of a protein complex at the mitochondria outer membrane that also includes outer mitochondrial membrane translocator protein (TSPO), TSPO-associated protein (PAP7, an AKAP family member), and StAR[177]. The cAMP-dependent formation of this complex facilitates cholesterol transfer into the mitochondria and the presence of StAR is an absolute requirement for the function of this complex [177]. In the absence of AKAP121, StAR protein expression is diminished in MA-10 mouse Leydig cells, suggesting StAR mRNA localization to the outer mitochondrial membrane is important for efficient translation [97, 178]. Given that StAR mRNA may be recruited to the mitochondria by AKAP-121 and that AKAP-PKA associate at the mitochondria, this would allow for localized translation and posttranslational modification of StAR.

Summary

The journey from detecting four new protein spots by 2-D-PAGE after treatment of rat adrenal cells or MA-10 mouse Leydig tumor cells with cAMP analogs to activate PKA signaling to cloning the cDNA encoding the protein spans over a decade from the early 1980s to 1994. However, as with all research, the work over this time was building on seminal studies from the 1960s–1980s that provided the evidence for an acute regulator of steroidogenesis which should fit the following criteria: trophic hormone stimulation of steroidogenic cells should induce the rapid synthesis of a labile protein(s), and that this newly synthesized protein(s) should function at the site of mitochondria and facilitate the translocation of cholesterol across the mitochondrial membranes for delivery to the cytochrome P450 side chain cleavage enzyme which catalyzes the conversion of cholesterol to pregnenolone for the first enzymatic step in steroidogenesis. StAR fits this description and an impressive amount of work in the mid to late 1990s demonstrated that mutations in the StAR gene are the genetic basis for lipoid CAH, StAR knockout mice are unable to produce steroid hormones, and that StAR binds cholesterol and functions at the outer membrane of

mitochondria for cholesterol transport into the organelle. Thus, relatively quickly, key data confirmed StAR's role as the acute regulator of steroidogenesis. Given the importance of the criteria for new protein synthesis for the acute regulation of steroidogenesis, understanding the molecular mechanisms that control the increase in StAR expression following trophic hormone stimulation would be important for understanding the mechanisms that control steroidogenesis. The cAMP responsive region of mouse and human StAR promoter is a non-consensus CRE/AP-1 element with overlapping specificity for members of the CREB/CREM and AP-1 protein families. Thus, the cAMP-PKA-dependent control of StAR was found to be rather complex and shown to occur in a species- and tissue-specific manner, driven mainly by the potential species- and tissue-specific heterodimer formations between bZIP CREB and AP-1 family members. Binding of other transcription factors, e.g., SF-1 and GATA-4, to their respective elements that are highly conserved between species may influence binding of the bZIP heterodimer partners at the non-consensus CRE/AP-1. Loss of transcriptional repression via loss of transcriptional repressor proteins DAX-1 and/or COUP-TF is another mechanism that works in concert with the direct activation mechanisms to promote the cAMP-PKA-dependent increase in StAR expression. Lastly, StAR mRNA stability is controlled by the AURE-binding protein, TIS11b, which binds to the StAR 3′ UTR AURE and facilitates mRNA degradation in a cAMP-PKA-dependent manner. Together these studies highlight the importance for fine control of StAR expression and that altering the expression or function of any of these transcription factors could influence StAR expression and lead to cell-specific alterations in steroid hormone biosynthesis, which would have implications for development and reproduction.

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