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Impact of ACTH Signaling on Transcriptional Regulation of Steroidogenic Genes

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The trophic peptide hormone adrenocorticotropic (ACTH) stimulates steroid hormone biosynthesis evoking both a rapid, acute response and a long-term, chronic response, via the activation of cAMP/protein kinase A (PKA) signaling. The acute response is initiated by the mobilization of cholesterol from lipid stores and its delivery to the inner mitochondrial membrane, a process that is mediated by the steroidogenic acute regulatory protein. The chronic response results in the increased coordinated transcription of genes encoding steroidogenic enzymes. ACTH binding to its cognate receptor, melanocortin 2 receptor (MC2R), stimulates adenylyl cyclase, thus inducing cAMP production, PKA activation, and phosphorylation of specific nuclear factors, which bind to target promoters and facilitate coactivator protein recruitment to direct steroidogenic gene transcription. This review provides a general view of the transcriptional control exerted by the ACTH/cAMP system on the expression of genes encoding for steroidogenic enzymes in the adrenal cortex. Special emphasis will be given to the transcription factors required to mediate ACTH-dependent transcription of steroidogenic genes.

Keywords: adrenal cortex, steroidogenesis, cAMP, transcription factors, gene regulation

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INTRODUCTION

The adrenal gland is a key component of the hypothalamus–pituitary–adrenal (HPA) axis, thus playing a crucial role in the adaptation of organism to stressors. The adrenocorticotropic hormone (ACTH) belongs to this regulatory circuitry, being one of the most potent physiological modulators of adrenal cortex steroidogenesis and trophicity (1, 2). It exerts its role through the binding to the G protein-coupled receptor (GPCR) melanocortin 2 receptor (MC2R), which activates adenylyl cyclase cascade leading to cAMP production and subsequent activation of cAMP-dependent protein kinase A (PKA). PKA is the main kinase responsible for the phosphorylation of specific transcription factors, which in turn regulate free cholesterol availability and activate steroidogenic enzyme expression (3, 4). Among those transcription factors, steroidogenic factor 1 (SF-1), cAMP response element-binding protein (CREB), CRE modulator (CREM), CCAAT/enhancer-binding proteins (C/EBPs), and activator protein 1 (AP-1) have been extensively described for their implication in regulating the expression of the genes encoding for steroidogenic acute regulatory (StAR) protein and steroidogenic enzymes (5–8). Any perturbation of this ACTH/cAMP/PKA-dependent cascade may cause alteration in adrenocortical cell proliferation and dysregulation of steroidogenesis as occur in various human diseases. The aim of the present review is to provide an overview of the ACTH/cAMP-dependent transcriptional regulation of the steroidogenic process in the adrenal cortex.

ADRENAL STEROIDOGENESIS

Steroid hormones are implicated in the regulation of a plethora of developmental and physiological processes from fetal life to adulthood. Cholesterol is the precursor of all those hormones that hence share a closely related structure based on the cyclopentanophenanthrene 4-ring hydrocarbon nucleus. Cholesterol can be synthesized *de novo* from acetate in the adrenal (9). However, the main adrenal source of cholesterol is plasma low-density lipoproteins (LDLs) provided by dietary cholesterol (10). The sterol response element-binding proteins (SREBPs) are a family of transcription factors implicated in the regulation of genes participating in the biosynthesis of cholesterol and fatty acids (11). They are the main regulators of intracellular cholesterol metabolism. ACTH promotes the activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. It also stimulates the uptake of LDL cholesterol esters, which, once taken up by receptor-mediated endocytosis can be converted to free cholesterol for steroid hormone biosynthesis (12) or directly stored. Moreover, ACTH stimulates hormone-sensitive lipase (HSL) and inhibits acyl-coenzyme A (CoA):cholesterol acyltransferase (ACAT), thereby increasing the free cholesterol pool available for steroid hormone biosynthesis.

The initial step of steroidogenesis occurs in the mitochondria. Whereas the movement of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) is known to be promoted by StAR (see below), the molecular mechanism underlying cholesterol transport and loading into the OMM is still under investigation (13). The enzymes that participate in steroid biosynthesis are either cytochrome P450s (CYPs) or hydroxysteroid dehydrogenases (HSDs).

Cytochrome P450s are a group of oxidative enzymes. In the human genome, genes for 57 CYPs enzymes have been described. Seven of them are called "type 1" enzymes. They are targeted to mitochondria and receive electrons from NADPH via a flavoprotein (ferrodoxin reductase) and a small iron-sulfur protein (ferredoxin). "Type 2" enzymes are localized in the endoplasmic reticulum and received electrons form NADPH via a single 2-flavin protein called P450 oxidoreductase (14). Six P450 enzymes participate in steroidogenesis. The mitochondrial P450 side chain cleavage (SCC, encoded by the *CYP11A1* gene) catalyzes breakage of the bond between positions 20 and 22 in the cholesterol side chain (20, 22 desmolase). P450c11β (11β-hydroxylase, encoded by the *CYP11B1* gene) and P450c11AS (aldosterone synthase, encoded by the *CYP11B2* gene) catalyze 11β-hydroxylase, 18-hydroxylase, and 18-methyl oxidase activities. At the level of the endoplasmic reticulum, we distinguish: P450c17 (encoded by the *CYP17A1* gene) that catalyzes both 17α-hydroxylase and 17,20-lyase activities; P450c21 (encoded by the *CYP21A1* gene) that catalyzes 21-hydroxylation in the synthesis of both glucocorticoids and mineralocorticoids; and P450-Aro (encoded by the *CYP19A1* gene) that catalyzes the aromatization of androgens to estrogens.

The HSDs use nicotinamide adenine dinucleotides or their phosphate forms ($NADH/NAD^+$ or $NADPH/NADP^+$) as cofactors to either reduce or oxidize a steroid through a hydride transfer mechanism (15). Differently from steroidogenic reactions catalyzed by P450 enzymes, which are mediated by a single form of P450, each reaction catalyzed by HSDs involves at least two, often different isozymes. On the basis of their structures, those enzymes are divided into: short-chain dehydrogenase/reductase (SDR) family, which include 11β-HSDs 1, 2, and 17β-HSDs 1, 2, and 3 and aldo-keto reductase (AKR) family, which include 17β-HSD5 (15, 16). Under a physiological point of view, it is preferable to classify the HSDs as dehydrogenases or reductases. The former use NAD^+ as cofactors to oxidize hydroxysteroids to ketosteroids, whereas the latter mainly use NADPH to reduce ketosteroids to hydroxysteroids. Those enzymes act *in vitro* typically bidirectionally on the basis of the pH and cofactor concentration, while in intact cells they work mainly in one direction, with the direction established by the cofactors available (15, 16).

The pattern of steroid hormones secreted by each adrenal zone is determined by the enzymes expressed in each zone (17). Specifically, adrenal *zona glomerulosa* expresses angiotensin II receptors and P450c11AS, whereas it does not express P450c17. Indeed, *zona glomerulosa* produces aldosterone under the regulation of the renin–angiotensin system. In contrast, at the levels of *zona fasciculata*, angiotensin II receptors, and P450c11AS are not detected, whereas the ACTH receptor MC2R and P450c11β are expressed (18). The *zona fasciculata* also expresses P450c17, which catalyzes 17α-hydroxylation, exhibiting only little 17,20-lyase activity. Indeed, *zona fasciculata* secretes the two glucocorticoids, cortisol and corticosterone, under the influence of ACTH, but very little dehydroepiandrosterone (DHEA). Interestingly, patients displaying mutations in P450c17 are not able to produce cortisol, but show increased corticosterone production (19), like in rodents, which normally do not express P450c17 in their adrenals. Regarding the *zona reticularis*, it also expresses MC2R and large amounts of P450c17 and cytochrome b_5, thus displaying 17,20-lyase activity with subsequent DHEA production, the most part of which is sulfated to DHEAS by SULT2A1. In general, small amounts of DHEA are converted to androstenedione, and very little amounts of androstenedione are converted to testosterone, likely through AKR1C3/17βHSD5. In contrast, *zona reticularis* expresses very little P450c21 and P450c11β (thus producing only a minimal amount of cortisol) and relatively little 3βHSD2.

ACUTE RESPONSE TO ACTH: THE STEROIDOGENIC ACUTE REGULATORY PROTEIN

Steroidogenic cells are able to store very little amounts of steroids, which imply a rapid synthesis of new steroids in response to a sudden demand. ACTH exerts its role in promoting steroidogenic cell growth and stimulating steroidogenesis at three distinct levels. We first distinguish a long-term exposure to ACTH. It takes weeks or months to stimulate adrenal growth and it is mediated by ACTH-dependent production of cAMP, which in turn triggers IGF-II (20), fibroblast growth factor (FGF) (21), and epidermal growth factor (EGF) (22) synthesis. The concerted action of those growth factors is to promote adrenal cellular hypertrophy and hyperplasia. Second, ACTH can act over days through cAMP to stimulate the transcription of genes, which encode for different

steroidogenic enzymes (see below). Third, ACTH can mediate an acute response, which occurs within minutes and is inhibited by protein synthesis inhibitors (like puromycin or cycloheximide). This ACTH-mediated acute response is accompanied by a rapid stimulation of the StAR gene transcription in steroidogenic cells of the adrenal cortex, testis, and ovary (5, 23) and by the phosphorylation of Ser195 in the existent pool of StAR (24). Those events promote cholesterol flow from the OMM to the IMM, where cholesterol is converted to pregnenolone in the first and rate limiting step of steroid hormone biosynthesis. The first to show that ACTH acute steroidogenic response involved the rapid synthesis of a 37-kDa phosphoprotein were Orme-Johnson and coworkers (25, 26). Just a few years later, Stocco and colleagues cloned this protein and they gave it the name of "StAR" (27). StAR is an acutely regulated, cycloheximide-sensitive protein exhibiting a mitochondrial leader sequence by which it is directed to the mitochondria. Once inside the mitochondria, StAR is cleaved to a 30-kDa protein. It has been shown that the overexpression of a mouse StAR in the mouse Leydig MA-10 cells increased their basal steroidogenesis (27). Moreover, when expression vectors for both StAR and P450scc enzyme are cotransfected in non-steroidogenic COS-1 cells, the synthesis of pregnenolone is augmented respect to that obtained with P450scc alone (28). The pivotal role of StAR in the regulation of steroidogenesis was strengthened after the identification of mutations causing premature stop codons in the StAR gene of patients affected by the most common form of congenital lipoid adrenal hyperplasia (CAH) (28, 29), a rare disorder of steroid biosynthesis. In this disorder, glucocorticoid, mineralocorticoid, and sex steroids biosynthesis is impaired, which may lead to adrenal failure, severe salt wasting crisis and hyperpigmentation in phenotypical female infants irrespective of genetic sex (29). Moreover, it has been shown that in mice the targeted disruption of the *Star* gene causes defects in steroidogenesis, with consequent male pseudohermaphroditism and lethality within 1 week after birth (30, 31).

Several studies have been carried out to understand the mechanism of action of StAR (32), which still remains to be fully elucidated. It was hypothesized that the "mature" 30-kDa intramitochondrial form of StAR was the biologically active portion of the protein, due to its longer half-life respect to the short one of the 37 kDa precursor. However, when the two StAR forms are expressed in the cytoplasm or added to mitochondria *in vitro*, they are equally active (33). Moreover, while constitutively active when it is localized on the OMM, StAR results to be inactive at the level of the mitochondrial intramembranous space or the matrix (34). That evidence suggested that StAR exerts its action on the OMM, its steroidogenesis-promoting function tightly depending on the residency time on the OMM itself (33, 34). This implies that StAR activity is linked to its localization rather than to its cleavage to the "mature" form. When StAR interacts with the OMM, it undergoes to conformational changes (35, 36) that allow StAR to accept and discharge cholesterol molecules. Interestingly, steroidogenesis-promoting and cholesterol-transfer activities of StAR are distinct. Indeed, StAR-mediated transfer of cholesterol between synthetic membranes *in vitro* (37) is maintained also by the inactive R182L mutant, which impairs steroidogenesis, causing lipoid CAH (38). Finally, StAR activity on the OMM requires the translocator protein TSPO, also called peripheral benzodiazepine receptor (PBR) (39, 40), which has been identified as a key player in the flow of cholesterol into mitochondria to permit the initiation of steroid hormone synthesis. Moreover, it has been demonstrated that phosphorylated StAR interacts with voltage-dependent anion channel 1 (VDAC1) on the OMM, which in turn promotes processing of the 37-kDa phospho-StAR to the 32-kDa intermediate (41). In the absence of VDAC1, phospho-StAR undergoes degradation by cysteine proteases prior to mitochondrial import and subsequent cleavage to the 30-kDa protein. StAR phosphorylation by PKA requires phosphate carrier protein on the OMM, which seems to interact with StAR before it interacts with VDAC1 (41). Importantly, although StAR is necessary for the ACTH-mediated acute steroidogenic response, steroidogenesis still occurs in the absence of StAR (around 14% of StAR-induced rate) (29, 42). This may account for the steroidogenic capacity of tissues lacking StAR, like placenta and brain.

CHRONIC RESPONSE TO ACTH

The transcription of steroidogenic genes depends on the slower, chronic response to ACTH in the adrenal cortex. Indeed, ACTH interaction with specific membrane receptors leads to the activation of coupled G proteins, with subsequent stimulation of membrane-associated adenylyl cyclase catalyzing cAMP formation. cAMP-activated PKA hence phosphorylates multiple transcription factors, whose concerted action and interaction with different *cis*-regulatory elements direct StAR and steroidogenic gene expression. Furthermore, after transcription factor binding to gene promoters, posttranslational modifications, like phosphorylation/dephosphorylation and coactivator proteins binding, are required to activate gene expression. In the following section, the main transcription factors that direct the transcription of steroidogenic genes in response to ACTH will be described (**Table 1**; **Figure 1**).

Steroidogenic Factor 1

Steroidogenic factor-1 (SF-1; NR5A1) is an orphan member of the nuclear receptor superfamily, which acts as a key regulator of adrenogonadal development and tissue-specific gene expression in steroidogenic cells. Parker and Morohashi groups identified SF-1 by its capacity to bind to and activate transcription from multiple P450 steroidogenic enzyme promoters, which display one or more SF-1-binding sequences in close proximity to the TATA box (72, 73). Furthermore, it has been shown that SF-1 interacts with multiple coactivator and corepressor proteins that function as bridges between transcription factors and the basal transcription system (74–76).

In the human adrenocortical cancer cell line H295R *StAR* gene transcription is induced by both angiotensin II and cAMP via increased SF-1-binding to a cAMP-responsive region within the first 350 bp upstream of the transcription initiation site (45, 77–79). On the other hand, the mutation of the SF-1 response elements in the mouse promoter of the StAR gene does not impair cAMP-dependent StAR induction in MA-10 Leydig cells or Y1 adrenocortical cells (78).

TABLE 1 | Promoter elements implicated in basal and ACTH/cAMP-regulated expression of steroidogenic genes.

	Basal regulation	ACTH/cAMP-dependent regulation
StAR	Three SF-1-binding sites [−135; −95; −45; **mouse** promoter, Ref. (7)] Three SF-1-binding sites [−926/−918; −105/−96; −43/−36; **human** promoter, Ref. (43, 44)] Two Sp1-binding sites [−1159/−1153; −157/−151; **human** promoter, Ref. (44, 45)]	CRE2/AP-1 [−81/−75, **mouse** promoter; Ref. (6)] Two C/EBPs putative-binding sites [−119/−100; −50/−41; **human** promoter, Ref. (43)] Two SF-1-binding sites [−105/−65; −43/−36; **human** promoter, Ref. (43)] Highly conserved overlapping motif [−81/−72, **mouse** promoter, which recognizes the CRE/AP1 and C/EBPs family of proteins, Ref. (7, 46–48)]
CYP11A1	Proximal SF-1-binding site [P site, −46/−38; **human** promoter, reviewed in Ref. (49)] Imperfect Sp1-binding site [−111/−101; **human** promoter, reviewed in Ref. (49)] TReP-132 [−155/−131; **human** promoter, reviewed in Ref. (49)**]** Adrenal enhancer (AdE, −1850) composed of two binding regions (a) AdE1 (−1845) containing an imperfect Sp1 and an NF-1-binding site [**human** promoter, reviewed in Ref. (49)] (b) AdE2 (−1898) containing an imperfect Sp1-binding site [**human** promoter, reviewed in Ref. (49)] AP-1 motif [−319/−313; **mouse** promoter, Ref. (50)] TGAGTCA motif [termed SF-3-binding site, −120/−114; **mouse** promoter, Ref. (50, 51)] AGGTCA motif [termed SF-2-binding site, −73/−68; **mouse** promoter, Ref. (50, 51)] AGCCTTG motif [termed SF-1-binding site, −45/−39; **mouse** promoter, Ref. (50, 51)]	Proximal SF-1-binding site [P site, −46/−38; **human** promoter, reviewed in Ref. (49)] Upstream cAMP responsive sequence (U-CRS, −1600 bp), which includes (a) SF-1-binding site (−1617/−1609) (b) CREB/ATF-binding site (CRE; −1685/−1606) (c) Two AP-1-binding sites (−1559/−1553; −1633/−1626) (a)/(b)/(c) **human** promoter, reviewed in Ref. (49) AP-1 motif [−319/−313; **mouse** promoter, Ref. (50), although available data indicate that this motif is not a major contributor to the induction of CYP11A1 expression by ACTH/cAMP] TGAGTCA motif [termed SF-3-binding site, −120/−114; **mouse** promoter, Ref. (50, 51)] AGGTCA motif [termed SF-2-binding site, −73/−68; **mouse** promoter, Ref. (50, 51)] AGCCTTG motif [termed SF-1-binding site, −45/−39; **mouse** promoter, Ref. (50, 51)] (mutation of those elements reduced the expression levels of *Cyp11A1* gene following treatment with 8-Br-cAMP, although all mutated plasmids retained appreciable responsiveness to cAMP)
CYP11B1	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; **human** promoter, Ref. (52, 53) reviewed in Ref. (54)] Ad5 [−119/−111; **human** promoter, ERRalpha has been shown to be the nuclear protein interacting with this element under basal conditions, reviewed in Ref. (54)] SF-1-binding site [termed Ad4, −242/−234; **human** promoter, it seems to be less important for both CYP11B1 and CYP11B2 basal expression, reviewed in Ref. (54)] CRE-binding site [−74/−67; **mouse** promoter, reviewed in Ref. (54)] Two Ad5-binding sites [one at −122/−114 and the other one at −208/−200; **mouse** promoter, reviewed in Ref. (54)] SF-1-binding site [−247/−240; **mouse** promoter, reviewed in Ref. (54)]	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; **human** promoter, Ref. (52, 53), reviewed in Ref. (54)] Functional CRE consensus sequence [−56/−49, **mouse** promoter, Ref. (55)]
CYP11B2	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; **human** promoter; Ref. (53), reviewed in Ref. (54)] Ad5 [−119/−111; **human** promoter, ERRalpha has been shown to be the nuclear protein interacting with this element under the basal conditions, reviewed in Ref. (54)] Two SF-1-binding sites [one at −129/−114, **human** promoter, Ref. (56)]; The other one termed Ad4, −344/−337, **human** promoter. It seems to be less important for both CYP11B1 and CYP11B2 basal expression; reviewed in Ref. (54) Chicken ovalbumin upstream promoter transcription factor [COUP-TF, −129/−114; **human** promoter, Ref. (56)] CRE-binding site [−67/−60; **mouse** promoter, reviewed in Ref. (54)] Ad5 [−108/−100; **mouse** promoter, reviewed in Ref. (54)] SF-1 [−330/−323; **mouse** promoter, reviewed in Ref. (54)]	CRE-binding site [termed Ad1/CRE and resembling a consensus CRE, −71/−64; **human** promoter; Ref. (53, 56) reviewed in Ref. (54)] SF-1-binding site [−129/−114; **human** promoter, Ref. (56)] Chicken ovalbumin upstream promoter transcription factor [COUP-TF, −129/−114; **human** promoter, Ref. (56)] CRE-binding site [−67/−60; **mouse** promoter, Ref. (56), reviewed in Ref. (54)]
CYP17A1	ASP/Sp1-binding site [−8/−19; **human** promoter, Ref. (57)] SF-1-binding site [−58/−50; **human** promoter, Ref. (57)] Two nuclear factor 1 (NF-1)-binding sites [−107/−85; −178/−152, **human** promoter, Ref. (59)] −184/−206 region [The site within this sequence that confers basal activity is not known, although it does contain a sequence resembling an SF-1 site at −195/−200; **human** promoter, Ref. (57)] Sp1/Sp3-binding site [−227/−184, **human** promoter; Ref. (59)] SF-1 (−62/−40), Sp1 (−186/−177), and Pbx/Meis (−243/−225) binding sites [**bovine** promoter; Ref. (59–63)]	cAMP-regulatory sequence [CRS, −57/−38: SF-1, p54nrb/NonO, and poly-pyrimidine tract-binding protein-associated splicing factor (PSF) are the transcription factors shown to be associated to this region; **human** promoter, Ref. (58)] SF-1 (−62/−40)- and Pbx/Meis (−243/−225)-binding sites [**bovine** promoter; Ref. (60–63)]

(Continued)

TABLE 1 | Continued

	Basal regulation	ACTH/cAMP-dependent regulation
CYP21	Two Sp1-binding sites [−118/−112 and −106/−100 within the recognition site −129/−96; **human** promoter, Ref. (64)] Two SF-1-binding sites [a putative one within the −300 bp proximal promoter and a second one within a distal region, lying approximately 4.8 kb upstream of the transcription start site; **human** promoter, Ref. (65)] Enhancer element [−330/−150; **mouse** promoter, Ref. (51)] Essential regulatory element [−210/−170; **mouse** promoter, highly conserved in the genes from human and bovine; Ref. (51, 66, 67)] A and B elements located 5.3 and 6 kb upstream of the transcriptional start site [**mouse** promoter, Ref. (69)]	Adrenal-specific protein (ASP)-binding site [−129/−113, within the recognition site −129/−96; **human** promoter, Ref. (64)] Enhancer element [−330/−150; **mouse** promoter, Ref. (51)] Essential regulatory element [−210/−170; **mouse** promoter, highly conserved in the genes from human and bovine; Ref. (51, 66, 67)] cAMP consensus sequence [−68/−62; **mouse** promoter, it matches part of the consensus sequence proposed for cAMP-regulated expression of other genes, Ref. (68)] Nuclear-binding response element (NBRE)/Nurr77 binding site [−65, **mouse** promoter, Ref. (66)] Regulatory elements containing variation of an AGGTCA motif at −170, −210, −140, −65 [**mouse** promoter; they show similarity to the CRE consensus, although they do not function as classical CREs, Ref. (68); variations of these same AGGTCA-bearing elements are also involved in the expression of *Cyp11a* and *Cyp11b* in Y1 adrenocortical cells, see above and Ref. (70)]
HSD3B2	Two SF-1/LRH-1-binding sites [−64/−56; −315/−307; **human** promoter, reviewed in Ref. (71)] Nuclear-binding response element (NBRE)/Nurr77 binding site [−131; **human** promoter, reviewed in Ref. (71)] GATA-binding site [−196/−190; **human** promoter, reviewed in Ref. (71)]	Nuclear-binding response element (NBRE)/Nurr77 binding site [−131, **human** promoter, reviewed in Ref. (71)]

Blue, red and green colours distinguish the different species (human, mouse and bovine, respectively).

Adrenocorticotropic hormone/cAMP-dependent transcription of the human *CYP11A1* gene, encoding for the mitochondrial enzyme P450scc responsible for cholesterol conversion to pregnenolone, requires the binding of SF-1 at two sites (−40 and −1600 bp) on the gene promoter (80). Remarkably, Omura and Chung laboratories have shown that the upstream promoter elements confer a large portion of CYP11A1 responsiveness to cAMP in Y1 or I-10 Leydig tumor cells and primary adrenal cells of transgenic mice (80, 81). SF-1-dependent activation of the *CYP11A1* promoter can be potentiated by cotransfection with c-Jun in steroidogenic JEG3 cells, but not in COS-1 cells (82). Thus, c-Jun and SF-1 act synergistically to activate the transcription of *CYP11A1*. It has also been shown that in the human adrenal cortex *CYP11A1* can be strongly activated by GATA-6 in a SF-1-dependent and DAX1-sensitive fashion (83).

Moreover, it has been shown that SF-1 interacts with the homeodomain-containing transcription factor pituitary homeobox 1 (Ptx/Pitx1) to synergistically promote *CYP11B1* gene transcription (84). Interestingly, in the bovine adrenal only one *CYP11B1* gene is expressed. Its promoter is characterized by the presence of both a SF-1- and a CRE (cAMP-response element)-like binding site, which are essential for cAMP-driven transcription (85, 86).

Adrenocorticotropic hormone/cAMP signaling also regulates the expression of the human *CYP11B2* gene in the *zona glomerulosa*, where it is responsible for mineralocorticoid production via an SF-1-binding site and a CRE (56).

Furthermore, SF-1 is implicated in the transcriptional regulation of *CYP17A1*, the gene encoding the P450c17 enzyme, which catalyzes both the 17α-hydroxylation of pregnenolone and progesterone (required for cortisol biosynthesis) and the 17,20-lyase reaction of 17α-hydroxylated steroids (leading to androgen production). Studies carried out on H295R cells revealed that CREs are located within the first 63 bp upstream of the transcriptional initiation site and that a second basal transcription element lies between −184 and −206 bp (87). SF-1 forms a complex with $p54^{nrb}$/NonO and polypyrimidine tract-binding protein-associated splicing factor (PSF), which binds within those first 60-bp upstream of the transcriptional start site, stimulating CYP17 expression in response to ACTH/cAMP signaling (58). As for *CYP11A1*, GATA-6 promotes the SF-1-dependent transcription of *CYP17* in H295R cells (83).

Many studies have revealed that the ACTH-regulated expression of the *CYP21A1* gene, coding for the P450c21 enzyme, which has a key role in the production of cortisol and aldosterone, requires the binding of the nuclear receptors SF-1 and Nur77 to its promoter (88–90). Interestingly, SF-1 binds to a distal region that lies approximately 4.8 kb upstream of the *CYP21A1* transcription start site driving adrenal-specific expression of the human gene (65).

cAMP Response Element/Binding Protein/CRE Modulator/Activating Transcription Factor

A family of cAMP-responsive nuclear factors mediates transcriptional regulation by ACTH/cAMP signaling pathway. This family is composed by a large number of proteins, which are encoded by the CREB, CREM, and ATF genes. Those proteins recognize and bind the 8-bp 5′-TGACGTCA-3′ palindromic sequence or a minor variation, called the CRE, which lie within 100 nucleotides of the TATA box in the promoters of eukaryotic cAMP responsive genes (91–93). The members of the CREB family are characterized by their DNA-binding leucine zipper (bZIP) domains and generally they interact with each other to mediate cAMP-dependent transcriptional response (91). Interestingly, the sequences of the mouse (27), human (94), and rat *StAR* promoters, which exhibit an extensive homology, lack a consensus CRE, similarly

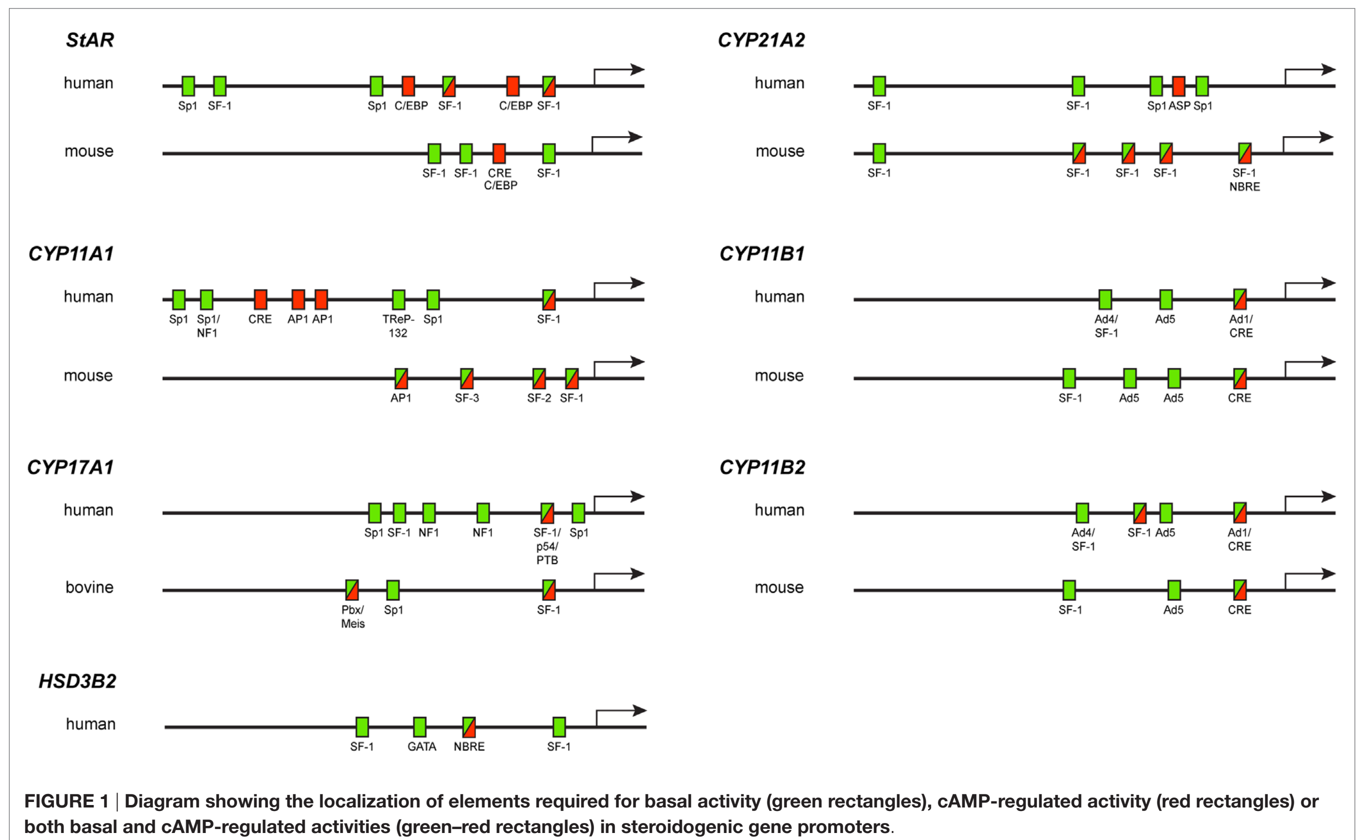

FIGURE 1 | Diagram showing the localization of elements required for basal activity (green rectangles), cAMP-regulated activity (red rectangles) or both basal and cAMP-regulated activities (green–red rectangles) in steroidogenic gene promoters.

to the promoters of different steroid hydroxylase genes whose transcription is regulated by ACTH/cAMP signaling (57). It has been shown that the cAMP-responsive region of the StAR gene promoter exhibits a highly conserved motif (50-TGACTGATGA-30 corresponding to 281/272 bp in the mouse promoter) to which different bZIP families of transcription factors, like not only CREB, CREM, and ATF1, but also AP-1 and C/EBPs (see below) bind to drive StAR transcription (7, 46, 47, 95, 96).

cAMP response element-binding protein has been demonstrated to be the principal player in mediating stimulus-transcription coupling in the ACTH/cAMP pathway. However, knockout mouse CREB studies showed that this action can be compensated by other CRE-binding proteins like CREM and ATF-1 (97). This mechanism also seems to work in the regulation of steroiodogenesis, as CREB family members directly induce *StAR* gene transcription (6, 7, 47). Interestingly, whereas CREB gene products usually function as positive transactivators, CREM can either activate or repress CRE-mediated transcription (98, 99). Alternative splicing of the CREM gene originates multiple isoforms that can act as either activators (τ, τ1, and τ2) or repressors (α, β, and γ) of transcription (100). Identical functional regions have been identified in CREB and CREMτ proteins (99). When overexpressed, either CREB or CREMτ display qualitatively comparable effects toward cAMP-dependent StAR gene transcription in murine adrenal and gonadal cells (6, 7, 101), whereas CREMα and CREMβ have been shown to repress StAR transcription (7). Further, CREM proteins can bind to CREs as homodimers or as heterodimers with CREB/ATF displaying similar functional outcomes to those of CREB (91). Remarkably, it has been shown that CREB and CREM associate with the proximal rather than the distal StAR promoter upon cAMP analog treatment (47, 48). Sugawara and coworkers compared the implication of CREB and CREM in cAMP-mediated StAR gene expression and identified CREM as the main mediator in H295R cells (101). In contrast, another group showed that CREB and ATF-1, but not CREM, mainly bound to the StAR promoter upon ACTH/cAMP stimulation (47). Besides CREB and CREM, also the CRE-binding protein, ATF-1 is a key regulator of StAR gene expression. ATF-1 differs from CREB and CREM as it lacks the glutamine-rich Q1 domain, although this does not affect its ability to work as a transcriptional activator (91, 102). Interestingly, two paralogs of ATF-1, called CRE-binding protein 1(CRE-BP1 or ATF-2) and ATF-a, display alternative exon splicing and bind to CREs, but they are not able to mediate cAMP-responsive transactivations (91).

cAMP response element-binding protein/CREM/ATF are activated by PKA, PKC, and other kinases that phosphorylate them at specific residues within their N-terminus. Indeed, phosphorylation of CREB at Ser133/119 or CREM at Ser117 leads to CREB–CREM interaction with coactivators like CREB-binding protein/p300 (CBP/p300) (see below) with subsequent stimulation of their transcriptional activity (47, 103–105). It has been reported that cAMP analogs increase CREB phosphorylation in a time-dependent manner in steroidogenic cells. This

phosphorylation event correlates with the association of both phosphorylated CREB and CBP to the proximal promoter of the StAR gene (47, 48, 106). The phosphorylation of Ser133 is required for CREB activation; however, it is not sufficient for full activation of the protein. Indeed, it has been shown that a short region C-terminal to the PKA phosphorylation site within CREB protein is required for CREB transcriptional activation (91, 107, 108). The crucial role of CREB phosphorylation by PKA has been also observed *in vivo* where transgenic mice, which express a non-phosphorylatable mutant of CREB (called CREB-M1, Ser133Ala), exhibit somatotroph hypoplasia and dwarfism (109). Moreover, expression of CREB-M1 in adrenal and gonadal cells strongly decreases cAMP-induced StAR gene expression (6, 7, 101).

cAMP response element-binding protein participates together with GATA-6 and AP-1 to the SF-1- and AP-2-dependent *CYP11A1* gene transcription in rodent placenta and ovary (110). Interestingly, the ACTH-stimulated transcription of the human *CYP11B1* gene depends on the CREB family member ATF-2 (52). Moreover, as reported above, ACTH/cAMP signaling regulates *CYP11B2* gene transcription via a CRE and a SF-1-binding site (56). Similarly, CREB binds to a CRE at the level of the mouse *Cyp11b1* gene promoter to drive ACTH-dependent transcription (55).

CCAT/Enhancer-Binding Proteins

CCAT/enhancer-binding proteins are a family of transcription factors containing a highly conserved bZIP at the C-terminus that is involved in dimerization and DNA binding. C/EBPs bind with different affinities to a consensus site consisting of a dyad symmetrical repeat (A/GTTGCGC/TAAC/T) (111). At least six members of the family have been cloned and characterized, named from C/EBPα to C/EBPζ. C/EBPα and C/EBPβ are expressed in steroidogenic cells, the expression levels of C/EBPβ being increased in the nucleus by the action of LH and analogs of cAMP (112, 113). The cAMP-inducible domains of C/EBPs, with the exception for C/EBPβ, lack a PKA phosphorylation site, which implies that C/EBPs are able to mediate cAMP-dependent responses by indirect mechanisms. In contrast, C/EBPβ phosphorylation by PKA within its bZIP domain affects its DNA-binding activity (114).

C/EBPβ targets a binding region in the promoter of the mouse *Star* gene (281/272 bp), which is also bound by CREB/ATF (see above) and Fos/Jun (see below). Further, two putative C/EBP-binding sites have been identified within the human StAR promoter (43). Consequently, an implication for both C/EBPα and C/EBPβ in StAR gene transcription has been reported (43, 115, 116). C/EBPβ phosphorylation on Thr325 increases its association to the proximal StAR promoter, thus inducing StAR transcription (117, 118). Interestingly, it has been shown that GATA-4 and C/EBPβ directly interact *in vitro* and *in vivo* and synergistically activate the StAR promoter only in the presence of PKA (95, 117). This suggests that GATA-C/EBP transcriptional cooperation might promote ACTH/cAMP-dependent StAR transcription in all steroidogenic tissues, as this kind of PKA-dependent synergy has been shown for other members of GATA and C/EBP families. Finally, the disruption of either C/EBPα or C/EBPβ impairs normal reproductive development in female mice, with consequent reduced or altered ovulation and corpus luteum formation inability (112, 119).

AP-1 Family of Transcription Factors

The AP-1 family of transcription factors participates in the regulation of cellular responses to multiple stimuli regulating proliferation, transformation, and cell death (120). It recognizes and binds to a DNA sequence known as the AP-1/phorbol 12-*O*-tetradecanoate 13-acetate responsive element [AP-1/TRE; TGA(C/G)TCA]. AP-1 is composed by a mixture of homo- and hetero-dimers formed between Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) family members (121–123). Fos members heterodimerize with Jun proteins and with specific members of the CREB/ATF family, but they are not able to form homodimers, whereas Jun members function as homodimers or heterodimers among themselves or with members of the Fos and CREB/ATF families (124). Both Jun and Fos family proteins belong to the bZIP group of DNA-binding transcription factors and their dimerization is necessary for specific and high affinity binding to the palindromic DNA sequence TGAC/GTCA (see above) (125). Studies carried out on the mouse *Star* promoter identified a highly conserved element (TGACTGA, −81/−75 bp), which shows homology with the AP-1/TRE sequence and overlaps also with the CRE2 sequence (6). Interestingly, it has been shown that Fos and Jun bind to this element, called CRE2/AP-1, thus regulating *Star* gene transcription (46, 96, 126). Moreover, two additional putative AP-1-binding sites have been identified within the rat *Star* promoter. c-Fos reduces basal, cAMP-, and c-Jun-mediated rat *Star* gene transcription in Y-1 adrenocortical cells (127). Indeed, a functional comparison between Fos and Jun revealed that c-Jun is the most powerful AP-1 family member for *Star* gene transactivation. Accordingly to this, it has been shown that only c-Jun, but not other AP-1 members, plays a pivotal role in the regulation of PKC-mediated *Star* transcription and steroidogenesis in Leydig and adrenal cells (96, 128).

Not only Protein kinase A but also PKC phosphorylate several Ser and Thr residues on c-Jun and c-Fos. In particular, the treatment with a cAMP analog or a growth factor increases the phosphorylation of c-Jun Ser63 and c-Fos Thr325. Those phosphorylation events are associated to StAR gene transcription and steroidogenesis in mouse Leydig cells (47, 96, 126, 129). Interestingly, ACTH/cAMP-dependent c-Jun and c-Fos phosphorylation increases the association between p-c-Jun/p-c-Fos and the CBP/p300 cofactors (see below), with consequent recruitment of CBP/p300 to the StAR promoter (47, 96, 126). The phosphorylation of c-Fos and c-Jun can alter their capacity to interact with other transcription factors, affecting their dimerization and DNA-binding specificity (124). This explains why the crosstalk between CREB and c-Fos/c-Jun can be associated with both gain and loss of function on the same *cis*-element in the context of a fine regulation of the transregulatory elements which participate in StAR gene transcription (126).

Sp Family of Transcription Factors

The Sp family of transcription factors is characterized by the presence of three conserved Cys2His2-type zinc fingers at their

C-terminus that form the sequence-specific DNA-binding domain (130). They can bind and exert their action through GC/GT-rich promoter elements to regulate the expression of multiple target genes (130, 131). Sp1 is the most well characterized member of the family and it exhibits similar structure and a high homology with Sp3 at the level of its DNA-binding domains, both being able to enhance ore repress promoter activity. However, although Sp1 and Sp3 recognize the same consensus-binding sites, it has been reported that both their DNA-binding properties and regulatory functions differ and depend on the promoter context and cellular background (130, 132, 133).

Regulatory elements for Sp1 and Sp3 have been identified within the human TSPO proximal promoter (134). They have been demonstrated to be strong positive elements for the promoter activity, although differences have been detected in the mechanism by which TSPO is regulated in non-steroidogenic versus steroidogenic cells (134). Sp1 is also involved in the cAMP-dependent transcription of the *CYP11A1* gene in human (135), bovine (136, 137), and porcine (138) adrenals. Furthermore, a cooperation between SF-1, Sp1, and CBP has been reported to drive cAMP-dependent *CYP11A1* transcription in bovine adrenal (136). Sp1 and Sp3, together with the nuclear fator-1C (NF-1C), bind to the second basal element of the *CYP17* gene promoter. This event is crucial for optimal basal transcription (59). Sp1 can also form a complex with GATA-4 or GATA-6 to regulate the expression of *CYP17* in the adrenal gland (139). Finally, Sp1 and adrenal-specific protein (ASP) bind to the *CYP21* gene promoter to regulate its cAMP-dependent transcription (140). This synergistic action seems to be required for maximal *CYP21* induction (140).

DAX-1

DAX-1/NR0B1 encodes an unusual member of the nuclear hormone receptor family of transcription factors. Its mutations cause adrenal hypoplasia congenita (AHC) associated with hypogonadotropic hypogonadism (HHG) (141, 142). *DAX-1* expression pattern, mostly restricted to steroidogenic tissues, suggested that it may have a role in the regulation of steroidogenesis. Indeed, in adrenocortical cells, DAX-1 works as a global negative regulator of basal and cAMP-regulated transcription of steroidogenic genes, both through direct binding to gene promoters and interaction with SF-1 and other transcription factors [(143–145); reviewed in Ref. (146)]. In addition, consistently with DAX-1 negative action on steroidogenesis, activation of the PKA pathway by ACTH in adrenocortical cells (147) and FSH in Sertoli cells (148) down-regulates Dax-1 expression.

ROLE OF COREGULATORS

Transcriptional coregulators are crucially implicated in nuclear receptor-mediated transcriptional activation (149–151) and transactivation by other factors, exerting their roles in multiples processes, like histone modification (152–154), chromatin remodeling (155), post-translational modification of transactivation complex members (156, 157), and ordered recruitment of basal transcriptional machinery (158, 159).

The role of coactivators and corepressors in the transcriptional regulation of steroid hydroxylase genes and *StAR* has been shown by several studies (74, 76). CBP and its functional homolog p300 are transcriptional coactivators that contain multiple functional domains and display intrinsic histone acetyltransferase activity (151, 160), by which they increase transcription factor accessibility to nucleosomal DNA. Classically, ACTH/cAMP signaling triggers the phosphorylation of specific transcription factors, which in turn can bind and recruit CBP/p300 (105, 160–162). As already reported (see above), when phosphorylated at Ser133 CREB is able to interact with CBP (104, 105). Interestingly, CBP/p300 contain PKA consensus sites, the phosphorylation of which is involved in regulating their functions. CBP/p300 play a key role in the transcriptional regulation of the *StAR* gene. Different studies have shown that cAMP-dependent phosphorylation of CREB at Ser133, c-Jun at Ser63, and c-Fos at Thr325 promotes the association and recruitment of CBP/p300 to the proximal *StAR* promoter (47, 48, 95, 126). cAMP-stimulated phosphorylation of C/EBPβ at Thr325 also increases C/EBPβ association to the proximal *StAR* promoter (117). Other factors like SF-1 and GATA-4 (see above), that are bound to the proximal *StAR* promoter, once phosphorylated in response to ACTH might enhance CBP/p300 recruitment to the promoter. This correlates with the CBP/p300-dependent increased effects of C/EBPβ and GATA-4 on *StAR* expression (95). Further, when overexpressed, CBP/p300 potentiated CREB, Fos/Jun, C/EBPβ, and GATA-4 transcriptional activity on the *StAR* gene (95, 126). This event is attenuated by the adenovirus E1A oncoprotein, which acts impairing CBP/p300 histone acetyltransferase activity and/or their interaction with other transcription factors or with the basal transcription machinery.

As already reported, coactivators and corepressors also play a key role in the transcriptional regulation of steroid hydroxylase genes. We report here some examples. Coexpression of CBP/p300 with the zinc finger protein TReP-132 has an additive effect on human *CYP11A1* gene promoter activity (163). Similarly, it has been shown that the coactivators CBP/p300, steroid receptor coactivator (SRC)-1, and transcriptional intermediary factor-2 (TIF-2) enhance SF-1-mediated bovine *CYP17* transcription, whereas the corepressors nuclear receptor corepressor (N-CoR) and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) increase the repressive activity of chicken ovalbumin upstream-transcription factor 1(COUP-TF-1) (164).

Finally, Sewer and coworkers have shown that a corepressor protein called mSin3A inhibits human *CYP17* gene transcription by the recruitment of a histone deacetylase to the SF-1/NonO/PSF complex that binds to the *CYP17* promoter (58). They have also described that coregulator exchange and sphingosine-sensitive cooperativity of SF-1, general control non-derepressed 5 (GCN5), p54, and p160 coactivators regulate cAMP-dependent *CYP17* transcription rate in H295R cells (165). The same group has shown that ACTH/cAMP signaling pathway promotes acid ceramidase (*ASAH1*) gene transcription via the binding of CREB to multiple region of the *ASAH1* promoter. This event triggers the recruitment of CBP/p300 with a related increase in the trimethylation of Lys4 on histone H3 (H3K4) on the *ASAH1* promoter in H295R cells (166).

ROLE OF PHOSPHATASE ACTIVITY ON ACTH/cAMP-DEPENDENT STEROIDOGENIC GENE TRANSCRIPTION

As already discussed, the ACTH-dependent increase in intracellular cAMP levels leads to the activation of PKA, which, in turn, phosphorylates specific nuclear factors to drive steroidogenic gene transcription. Remarkably, in the adrenal cortex ACTH regulation of steroidogenesis depends not only on PKA-mediated Ser/Thr phosphorylation, but also on the activity of protein tyrosine phosphatases (PTPs), which have been implicated in *StAR* expression and steroidogenesis (167–172). Indeed, the phospho-dephosphorylation of intermediate proteins is considered as a key event in the regulation of steroid biosynthesis. In 1999, Paz and coworkers showed that *in vivo* treatment with ACTH leads to an increase in total PTPs activity in adrenal *zona fasciculata*. The stimulation was characterized by a rapid onset (5 min), reached a peak after 15 min of ACTH administration (around twofold) and returned to basal levels after 30 min (168). They showed that the increase in PTPs correlated with a decrease in phosphotyrosine proteins (168). Moreover, the PTPs inhibitors pervanadate (PV) and phenylarsine oxide (PAO) inhibited ACTH- and 8Br-cAMP (a permeant analog of adenosine 3′,5′-phosphate)-dependent steroidogenesis in a dose-dependent fashion, whereas they did not affect steroid production supported by a cell-permeant analog of cholesterol (168). Those studies clearly indicated that PTPs activity has a key role in ACTH/cAMP signaling pathway, acting downstream of PKA activation and upstream of cholesterol transport across the mitochondrial membrane. The same group evaluated steroid production and StAR protein levels in Y1 cells upon PTP inhibition. They reported that PAO reduced ACTH-dependent stimulated steroidogenesis in those cells in a concentration-dependent manner and abrogated StAR protein induction (169). Those effects have been reproduced by a second PTPs inhibitor, benzyl phosphonic acid, which has a different mechanism of action (169). Altogether, those results show that PKA-mediated PTP activation in the steroidogenic system exerts the functional role of mediating StAR protein induction (169). The ACTH/cAMP/PKA signaling pathway stimulates also the release of arachidonic acid (AA) in adrenal and Leydig cells by the concerted action of two enzymes: an acylCoA-thioesterase (Acot2) and an acyl-CoA-synthetase (ACS4) (170, 173). Several reports have shown that AA and its metabolites play a key role in the hormonal control of steroidogenesis by regulating both the expression and function of StAR protein (174, 175). The ACTH/PKA system has been shown to control this pathway upregulating the ACS4 protein levels in adrenal and Leydig cells (175). Two PTP inhibitors both abrogate the ACTH/PKA-dependent ACS4 induction and reduced the effects of cAMP on steroidogenesis and StAR protein levels (175). Interestingly, exogenous AA is able to overcome this PTP-dependent inhibitory effect on StAR protein expression and steroidogenesis (176, 177). Furthermore, Sewer and Waterman have shown that PTP activity is essential for cAMP-dependent transcription of the human *CYP17* gene in H295R cells (178). They also investigated whether the inhibition of PTP activity can impair cAMP-dependent mRNA expression of other steroidogenic genes in the adrenal cortex. They have reported that *CYP11A1*, *CYP11B1/2*, and *CYP21A1* also require PTPs for cAMP-dependent mRNA expression, as the inhibition of both serine/threonine and tyrosine phosphatase activities negatively affected this event (178). Those evidences led those authors to propose a model whereby PKA phosphorylates and activates a dual-specificity phosphatase (DSP), which is able to mediate ACTH/cAMP/PKA-dependent transcription of steroidogenic genes (178, 179). The specific DSP has been identified as mitogen-activated protein phosphatase 1 (MKP-1), whose levels are increased by ACTH and cAMP in H295R cells (180). Moreover, the evidence showing that MKP1 overexpression promotes the transcriptional activity of a human *CYP17* promoter-reporter construct and its silencing decreases cAMP-stimulated *CYP17* gene expression, suggest a role for MKP-1 in cAMP-dependent *CYP17* transcriptional activation (180).

SUMMARY AND CONCLUSION

The studies outlined here have given an important contribution to the understanding of the impact of ACTH on the regulation of steroidogenic gene expression in the adrenal cortex. Given the pivotal role played by ACTH/cAMP signaling in the acute and chronic regulation of steroid secretion and the implication of those hormones in diverse arrays of fundamental physiological processes, an in-depth investigation is needed to completely elucidate the ACTH-dependent transcriptional regulatory mechanisms that control steroid secretion. Indeed, some aspects addressed in this review still represent a challenge for future studies, which might provide the scientific community with a broader picture of the complex signaling pathways and the intricate transcriptional circuitries that coordinately ensure optimal hormonal output.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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