Minireview

Biochemical Diversity of cAMP-dependent Transcription of Steroid Hydroxylase Genes in the Adrenal Cortex*

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One of the major advances in biology during the past decade has been the identification of the zinc finger-containing nuclear receptors of the steroid/thyroid hormone superfamily and the elucidation of the biochemical mechanisms defining their roles in regulating gene expression. Underlying the functional diversity of these nuclear receptors is the requirement for availability of specific ligands, which bind to and thereby activate these receptors. Many of the members of this superfamily are activated upon binding steroid hormones, which are produced from cholesterol by a variety of highly regulated pathways. These pathways are localized primarily in steroidogenic tissues such as adrenal cortex, gonads, and placenta and in the broadest sense are regulated by developmental, tissue-specific, constitutive (cAMP-independent), and cAMP-dependent processes. Once normal developmental and tissue-specific events have occurred, thereby programming the expression of the correct steroidogenic pathways in the proper cellular types, the primary regulation of steroid hormone biosynthesis throughout life is via peptide hormones derived from the anterior pituitary. Thus receptor-mediated steroid hormone action is itself dependent on the action of peptide hormones through their membrane-bound receptors on steroidogenic cells. The interaction between these two types of receptors is coupled by steroid hormone biosynthesis.

Steroidogenic Pathways

The most complex steroidogenic pathways are found in the adrenal cortex producing mineralocorticoids (aldosterone), glucocorticoids (cortisol or corticosterone depending on the species), and adrenal androgens (dehydroepiandrosterone and androstenedione) (Fig. 1). The ovary produces primarily estrogen and progesterone, the testis testosterone and dihydrotestosterone, and the placenta progesterone. There are considerable species variations among steroidogenic pathways, and those outlined in Fig. 1 are generic rather than representing a particular species. Peptide hormones regulate steroidogenesis via cAMP by two temporally distinct processes (1). An acute response involves the rapid mobilization of cholesterol from cellular stores into the mitochondrion where the conversion of cholesterol to pregnenolone, the initial step in all steroidogenic pathways, takes place. By a more chronic process, peptide hormones regulate the transcription of genes encoding enzymes of the steroidogenic pathways. The primary focus of this minireview is the biochemical details of the chronic regulation of transcription of steroid hydroxylase genes by peptide hormones, which leads to the maintenance of optimal steroidogenic capacity such that steroid hormones can be generated on demand via the acute response.

The steroid hydroxylases involved in steroid hormone biosynthesis are members of the cytochrome P450 (CYP) superfamily of hemoprotein, mixed-function oxidases (2), and transcription of each of the steroid hydroxylase genes in the bovine adrenal cortex is activated by cAMP (3). Before focusing attention on analysis of the biochemical features of cAMP-dependent transcription of these genes, a brief consideration of the other regulatory mechanisms associated with their transcription is warranted.

Developmental and Tissue-specific Regulation

The precise timing of the onset of expression of steroid hydroxylases during embryogenesis is not yet well established. In fact, while expression of these genes is known to occur in fetal organs it is not certain that fetal expression of steroidogenic pathways is important in development. One clear exception is the well known requirement for testosterone synthesis in development of male secondary sex characteristics (4). The pathway from cholesterol to testosterone includes two steroid hydroxylases, P450scc and P450c17 (see Fig. 1) and the timely onset of transcription of genes encoding these enzymes occurs in normal fetal testis. All steroid hydroxylase genes contain binding sites for the orphan receptor steroidogenic factor 1 (SF-1) (5) also known as Ad4 binding protein (Ad4BP) (6). One function of this transcription factor is probably in tissue-specific expression of these genes establishing steroidogenic pathways in the appropriate cell types; however, SF-1 has recently been found to have a much more profound role in development. The SF-1 knockout mouse is born without adrenal glands or gonads, indicating that the morphogenesis of these steroidogenic organs is dependent on this orphan receptor (7). These knockout mice are born and suckle but die within several days after birth. A homozygous P450scc-deficient rabbit is also born and survives for a few days without an obvious phenotype beyond the absence of maleness (8). A number of developmental processes including lung maturation have been thought to depend on steroid hormones, particularly glucocorticoids. Certainly the live birth of the SF-1 knockout mouse and the P450scc-deficient rabbit puts this in question, although maternal steroid hormones might participate in these developmental processes. Thus, even though it has been established in several species that steroid hydroxylase expression occurs shortly after the developmental appearance of steroidogenic organs, with the exception of development of maleness in the 46 XY individual, roles for fetal production of steroid hormones are not clear.

Constitutive (cAMP-independent) Regulation

Primary cultures of bovine adrenocortical cells maintained for several days in the absence of ACTH lose approximately 50% of their normal levels of steroid hydroxylases, except for

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1The abbreviations used are: SF-1, steroidogenic factor 1; Ad4BP, Ad4 binding protein; CR, cAMP-responsive sequence(s); PKA, protein kinase A; CRE, consensus cyclic AMP response element; ORBE, CRE binding protein.
P450c17, which essentially disappears (9). This demonstrates that in bovine adrenal cortex, steroid hydroxylase levels, with the exception of P450c17, can be regulated in part by cAMP-independent processes. These regulators presumably include growth factors and cytokines, and while the biochemical mechanisms are not at all clear, considerable species variation exists at this level of regulation. It can be expected that cAMP-independent regulation provides a basal level of transcription of steroid hydroxylase genes in steroidogenic tissues upon which cAMP-responsive transcriptional regulation is superimposed. The unique physiology associated with steroidogenesis in different species and different steroidogenic tissues may be an important contributor to the variation in cAMP-independent regulation of steroid hydroxylase levels.

**cAMP-dependent Regulation**

In experiments carried out as early as 1969 it was shown that hypophysectomy of rats leads to a substantial decline in the activities of steroid hydroxylases and that treatment of hypophysectomized animals with pharmacological doses of peptide hormones derived from the anterior pituitary (ACTH in the bovine adrenal cortex) can restore these enzymatic activities (10,11). We now know that these peptide hormones activate adenylate cyclase leading to elevated levels of intracellular cAMP. The action of ACTH leading to elevated levels of steroid hydroxylase activities (via cAMP) is at the transcriptional level, and it takes several hours for enhanced transcription to be observed (3). This suggested that the CRE/CREB system might not be involved since it responds more rapidly to cAMP. Also, enhanced transcription of the different steroid hydroxylase genes in the bovine adrenal cortex was observed at approximately the same time indicating that these genes were turned on in a coordinate fashion. Thus as schematized below, the hypothesis at the outset of "promoter bashing" of the steroid hydroxylase and adrenodoxin genes coupled to reporter genes has been examined primarily in three species, human, bovine, and mouse.

In addition to studies on the expression of the genes encoding P450scc, P45011B, P450aldo, P450c21, and P450c17, regulation of the gene encoding the iron-sulfur protein adrenodoxin required for function of all mitochondrial P450s including P450scc, P45011B, and P450aldo has also been examined. ACTH-dependent transcription of the adrenodoxin gene was coordinate with that of the steroid hydroxylase genes (3) suggesting the same CRS.

Analysis of the 5'-flanking regions of the bovine steroid hydroxylase and adrenodoxin genes coupled to reporter genes has led to the surprising conclusion that each gene contains its own different cAMP-responsive elements (Table 1). This result is particularly surprising since SF-1 (Ad4BP) binding sites that presumably are important in developmental and/or tissue-specific expression are found in each of the steroid hydroxylase genes. As depicted in Fig. 2, the steroid hydroxylase genes have distinct evolutionary patterns that might provide an explanation for the origin of different CRS elements in these genes. However, the presence of SF-1 binding sequences in each of these genes makes the evolutionary distance between them a less likely explanation for the diversity among CRS elements.

The most closely related steroid hydroxylase genes are those encoding mitochondrial steroidogenic P450s, the CYP11 gene family. Of the three members of this family, P450scc, P45011B, and P450aldo, bovine adrenal glands contain only P450scc and P45011B. Even though these genes are closely related, they seem to have quite distinct CRS elements. Bovine P450scc contains two CRSs near the promoter region, which play an important role in cAMP responsiveness of this gene (12,13). Each of these is a binding site for Sp1, a ubiquitous transcription factor that is not generally thought to be involved in cAMP-dependent transcription. Nevertheless, these Sp1-binding elements have been located through deletion analysis of the 5'-flanking region of bovine CYP11A by virtue of their ability to enhance transcription of reporter genes in response to elevation of cAMP levels in steroidogenic cells. Also, overexpression of the catalytic subunit of cAMP-dependent protein kinase (PKA) enhances transcription of reporter genes driven by these
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The bovine CYP17 gene contains two distinct CRS elements, which may be involved in a manner similar to that observed for CYP11A. However, this CRS has also been found to bind another protein that has yet to be characterized. Perhaps unlike the bovine P450scx gene, additional DNA-binding proteins as well as Sp1 are required for cAMP-dependent transcription of the adrenodoxin gene.

Characterization of the cAMP-responsive mechanisms for adrenocortical steroid hydroxylases in other species shows both similarities and differences to results obtained with the bovine genes. Humans and mice contain two members of the CYP11B subfamily: CYP11B1 encoding P45011B1 and CYP11B2 encoding P450aldo. The mouse gene encoding P45011B1 does not contain a CRE-like sequence and responds slowly to cAMP (22), while that encoding P450aldo does contain such a sequence and responds more rapidly to cAMP (23). The human adrenodoxin gene contains an Sp1 binding site (24) although comparison of binding proteins to this CRS with those binding to the bovine adrenodoxin CRS has not yet been made. The two Sp1-binding regions near the TATA box in the bovine CYP11A gene are also present in the human, mouse, and rat genes and are located within regions that are reported to participate in cAMP responsiveness (25–27). The human CYP11A gene also contains a CRS far upstream from the Sp1-binding sites that participates in cAMP responsiveness (25, 28, 29). This contains a CRE, and CREB binding may function in combination with Sp1 binding in achieving the full cAMP responsiveness of this gene. Dele-

**Table I**

| cAMP-responsive sequences in bovine steroid hydroxylase genes |
|---------------------------------------------------------------|
| Bovine CYP11A, CRS | -118 | -100 |
| Bovine CYP21, CRS | -129 | -115 |
| Bovine CYP17, CRS | -243 | -225 |
| Bovine adrenodoxin, CRS | +698 | +745 |

**Fig. 2. Evolutionary relationship of genes encoding adrenocortical steroid hydroxylases.** This figure is adapted from an evolutionary scheme of the CYP superfamily by Nebert et al. (36).

The bovine mitochondrial steroid hydroxylase, P450scx, is regulated by cAMP through a near consensus CRE sequence that presumably binds CREB (14). Transcription mediated by this near perfect CRE is strongly enhanced by cooperation of an upstream AddBP (SF-1) binding site (15). Thus the two bovine mitochondrial steroid hydroxylases that are members of the same gene family (CYP11) utilize quite different cAMP-responsive systems for their coordinate expression in response to ACTH.

The bovine adrenocortical microsomal steroid hydroxylases are the sole known members of two different gene families, CYP21 encoding P450c21 and CYP17 encoding P450c17. The CRS in bovine CYP21 most closely resembles one of the CRS elements in the bovine CYP11A gene; it contains overlapping binding sites for two nuclear proteins, one being Sp1 and the other being a 78-kDa protein (16). Binding of this latter protein, designated ASP, is required for CAMP responsiveness of bovine CYP21, not binding of Sp1. An ASP binding site is located next to but not overlapping with the upstream CYP11A CRS. However, bovine CYP21 requires ASP binding for cAMP-dependent transcription while bovine CYP11A requires Sp1 binding for this purpose.

The bovine CYP17 gene contains two distinct CRS elements, each binding its own group of nuclear proteins. This gene is novel among the bovine steroid hydroxylase genes in that it seems to be strictly regulated by cAMP. In primary cultures of bovine adrenocortical cells maintained in serum but in the absence of ACTH, the levels of the steroid hydroxylases decline. P450c17 disappears (18), while P450scx, P45011B, P450c21, and adrenodoxin decline by 50% or more to levels that are maintained throughout the lifetime of the cultures. The CYP17 CRS closest to the promoter (CRS II) binds SF-1 and other proteins (perhaps two of them), including Coup-TF. The upstream bovine CYP17 CRS (CRS I) is found to bind four nuclear proteins (19). Two of these contain peptide sequences not found in the protein data base. The other two are homeodomain proteins (helix-turn-helix DNA-binding motif) of the PBX family. These PBX proteins contain a putative PKA phosphorylation site that may be phosphorylated in response to ACTH. CRS I of bovine CYP17 is the first cellular target for PBX to be discovered, although this DNA-binding protein is expressed in most tissues. It is interesting that partially purified CRS I binding proteins from nonsteroidogenic cells are unable to activate CRS I-dependent transcription, whereas the proteins purified from steroidogenic cells can (20). Gel shift analysis indicates that the same or similar proteins are present in both samples. Perhaps CRS I-binding proteins from steroidogenic cells are activated by a tissue-specific event, which renders them competent to activate CRS1-dependent transcription.

The bovine adrenodoxin gene contains two CRS elements, the most important of which is located within the first intron (21). This CRS contains two putative CREs, the putative CRE-binding proteins to this CRS with those binding to the bovine adrenodoxin CRS suggesting that it may be regulated in a manner similar to that observed for CYP11A. However, this CRS has also been found to bind another protein that has yet to be characterized. Perhaps unlike the bovine P450scx gene, additional DNA-binding proteins as well as Sp1 are required for cAMP-dependent transcription of the adrenodoxin gene.

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3 P. Venepally, personal communication.

4 P-Y. Cheng, personal communication.
tion analysis of the promoter region of human CYP17 suggests that a functional CRS lies in a region that has no sequence homology to either bovine CRS I or CRS II (30). The mouse P450c17 gene contains a CRS that contains no sequence homology to CRS elements in either the human or bovine CYP17 genes (31). The mouse P450c21 gene like the mouse P450sc17 gene contains multiple elements involved in cAMP responsiveness (32). One such element binds SF-1 and the transcription factor NGFI-B (33), although the biochemical roles of these factors in cAMP responsiveness have yet to be elucidated. Human CYP21 contains an ASP-binding site that is nearly identical to that in bovine CYP21 and participates in cAMP-dependent transcription (16), in addition to a CRE-like sequence far upstream of the promoter (34).

**Conclusion and Perspective**

The original working hypothesis that ACTH coordinately regulates transcription of genes encoding steroid hydroxylases in the adrenal cortex via a common cAMP-responsive system is not correct. On the contrary, each gene utilizes its own unique cAMP-responsive system(s), even though the transcription of these genes appears temporally coordinated. The basis of this diversity might have been attributed to the different evolutionary patterns of these CYP genes (Fig. 2), but each gene contains one or more binding site for the developmentaUtissue-specific transcription factors (CREB) and non-traditional CAMP-dependent transcription factors (CRE). Perhaps the notion of coordinate transcription (16), in addition to a CRE-like sequence far upstream of the promoter, remains an enigma.

Results from studies of CYF17 may provide a hint, however, as to why this gene has a novel cAMP-responsive system relative to other bovine steroid hydroxylase genes. As noted, bovine CYP17 is strictly dependent on cAMP. During development, expression of this gene in the fetal adrenal gland is turned off during the middle third of gestation due to transient absence of ACTH in the fetal circulation (35). Thus a developmental need for the absence of cortisol seems coupled with ACTH-dependent maintenance of expression of CYP17. Perhaps, then, it is a necessity to couple cAMP-dependent transcription with other components of the multifactorial regulation of steroidogeneses. The coupling requirements may be different for each gene, thus leading to different cAMP-dependent regulatory systems for each.

Investigation of the biochemical basis of ACTH-dependent transcription of steroid hydroxylase genes in the adrenal gland has uncovered roles for traditional cAMP-dependent transcription factors (CREB) and non-traditional cAMP-dependent transcription factors (Sp1, SF-1, NGFI-B) as well as the first cellular target for the homeodomain protein PBX1. A common participant in cAMP-dependent transcription of each of these genes is PKA. Elucidation of the coupling of PKA activity to the variety of transcription factors required for maintenance of optimal steroidogenic capacity in the adrenal cortex and the subsequent coupling of these transcription factors to the basal transcription machinery are now the goal. These biochemical events occurring between PKA and transcription of steroid hydroxylase genes are an integral part of the connection between the action of peptide hormones from the anterior pituitary and the function of the steroid hormone receptor superfamily.

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