J. Steroid Biochem. Molec. Biol. Vol. 43, No. 8, pp. 931–935, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

COMPARISON OF cAMP-RESPONSIVE DNA SEQUENCES AND THEIR BINDING PROTEINS ASSOCIATED WITH EXPRESSION OF THE BOVINE CYP17 AND CYP11A AND HUMAN CYP21B GENES

MICHAEL R. WATERMAN,* NORIO KAGAWA, ULRICH M. ZANGER, KYOKO MOMOI,† JOHAN LUND‡ and EVAN R. SIMPSON

Departments of Biochemistry and Obstetrics and Gynecology and The Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75235, U.S.A.

Summary—Maintenance of optimal steroidogenic capacity in the adrenal cortex requires the action of the peptide hormone ACTH. Upon binding to its cell surface receptor ACTH activates adenylate cyclase leading to elevated levels of intracellular cAMP which in turn enhances transcription of the genes encoding the enzymes involved in the conversion of cholesterol to the steroid hormones. By deletion analysis of their upstream regions, the genes encoding the steroid hydroxylases *P*450c17, *P*450c21 and *P*450scc (CYP17, CYP21B and CYP11A, respectively) were found to contain unique cAMP-responsive sequences (CRSs). These sequences are unique in the sense that they have not previously been described to be associated with other genes whose transcription is regulated by cAMP. Furthermore they appear to bind unique nuclear proteins or transcription factors not previously associated with cAMP-dependent transcription. This review summarizes the relatedness of these CRSs in the bovine CYP17 and CYP11A genes and the human CYP21B gene and provides an up-to-date summary of the properties of their nuclear DNA-binding proteins.

INTRODUCTION

Almost 20 years ago it was shown that following hypophysectomy, the levels of steroid hydroxylase activities and proteins decreased in the adrenal cortex [1]. Administration of ACTH to hypophysectomized animals restored the levels of the steroid hydroxylases. These experiments clearly established a role for ACTH in the maintenance of steroidogenic activity in the adrenal cortex. With the advent of the techniques of molecular biology and their application to investigation of steroidogenesis the details of the mechanisms by which ACTH regulates steroid hydroxylase gene expression are being elucidated. Binding of ACTH to its cell surface receptor activates adenylate cyclase leading to elevated levels of cAMP. ACTH through cAMP has both acute and chronic actions in the adrenal cortex. The acute action involves the mobilization of cholesterol into the steroidogenic pathway and is very rapid, occurring within minutes. The chronic action of ACTH is to coordinately enhance the transcription of the genes encoding enzymes involved in the steroidogenic pathway and this is a longer term process taking several hours to become manifest. Once the cDNAs encoding the steroid hydroxylases were cloned (work of several laboratories summarized in [2]), it was shown that ACTH enhances steroid hydroxylase gene expression at the transcriptional level [3]. Furthermore this event appears to be coordinated in the sense that transcription of the genes encoding *P*450c17, *P*450c21, *P*450scc and *P*450c11 (the four adrenocortical steroid hydroxylases in most species) is activated at the same time. Recently it has been shown that transcriptional activation via cAMP may not be the only mechanism by which ACTH enhances levels of the steroidogenic enzymes [4], but transcription appears to be a major mechanism.

Proceedings of the First International Symposium on A Molecular View of Steroid Biosynthesis and Metabolism, Jerusalem, Israel, 14–17 October 1991.

*To whom correspondence should be addressed at: Department of Biochemistry, Vanderbilt University School of Medicine Nashville, TN 37232-0146.

†Present address: Department of Biochemistry, National Cardiovascular Center, Research Institute, 5-7-1, Fujishirodai, Suita, Osaka 565, Japan.

‡Present address: Department of Medical Nutrition, Huddinge University Hospital F60, NOVUM, 5-141 86 Huddinge, Sweden.

The coordinated transcriptional activation of the CYP17, CYP21, CYP11A and CYP11B genes suggested that a common mechanism possibly involving the same *cis*-regulatory sequence(s) which bind common DNA-binding protein(s) was involved in the cAMP-dependent increase of the transcription of all these genes. The details of this and other aspects of steroid hydroxylase gene expression (i.e. tissue-specific, developmental, cAMP-independent) are being investigated by several laboratories. In Dallas we have focused primarily on cAMP-dependent transcription of the CYP17, CYP21 and CYP11A genes and this review represents a progress report of studies addressing the mechanism of this chronic action of ACTH.

cAMP-RESPONSIVE DNA SEQUENCES (CRSs) IN STEROID HYDROXYLASE GENES

Using the now standard techniques of coupling 5′-flanking regions of the genes encoding steroid hydroxylases to a reporter gene (rabbit β-globin in this case [5]), the CRSs in the bovine CYP17, bovine CYP11A, and human CYP21B genes have been identified. These CRS elements are shown in Fig. 1. Within the 5′-flanking regions studied, the bovine CYP17 gene has two CRS elements while the bovine CYP11A and human CYP21B genes have single CRS sequences. The information in Fig. 1 provides the first surprise in this study. While sequence relatedness is apparent between the CRS elements from CYP21B and CYP11A, there is no relatedness between the two CRS elements in CYP17 and between them and CRS elements in CYP21B and CYP11A. This suggests that even though transcription in response to cAMP appears to be coordinated, it does not appear that the same system functions in each gene. This is particularly surprising based on the evolutionary profile developed for the CYP (*P*450) genes [6]. This profile suggests that CYP17 and CYP21 evolved from a common progenitor gene diverging from one another approx. 800 million years ago. The divergence of CYP11 from the path leading to the CYP17/CYP21 progenitor occurred much longer ago than that and, therefore, it is surprising that the CRS elements of CYP21B and CYP11A are more closely related than are the CYP21B CRS and CRSI from CYP17. However, the simple comparison of DNA sequences does not absolutely establish the lack of relatedness of the mechanism of cAMP responsiveness of transcription of these genes. Investigation of the nature of the proteins binding to these DNA sequences is crucial to a detailed understanding of the mechanism(s) by which cAMP enhances steroid hydroxylase gene expression.

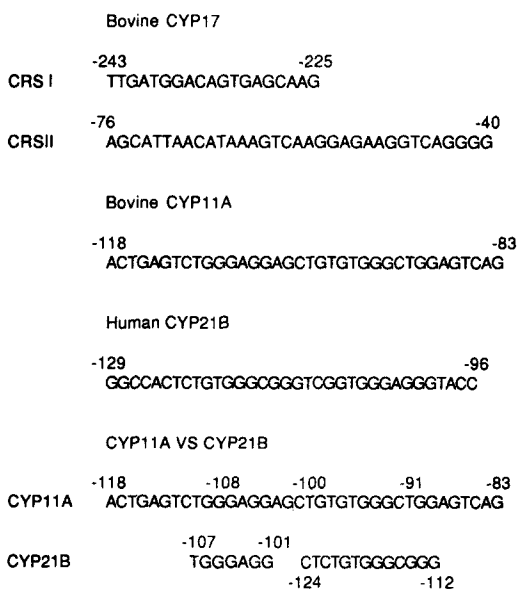


Fig. 1. CRSs found in the bovine CYP17 and CYP11A genes and the human CYP21B gene. This figure also shows the relatedness of the CRSs between CYP11A and CYP21B. Note that the sequence between -107 and -101 of CYP21B aligns with -109 and -104 of CYP11A while that between -124 and -112 of CYP21B aligns with -98 to -89 of CYP11A.

CHARACTERIZATION OF PROTEINS BINDING TO STEROID HYDROXYLASE CRS ELEMENTS

The nuclear proteins which bind to the CYP17 CRSI, the CYP21B CRS and the CYP11A CRS have been examined by DNA binding studies (gel shift and DNase I footprinting analysis) and by *in vitro* transcription.

CYP17 CRSI

*P*450c17 is expressed in several different steroidogenic tissues including adrenal cortex, placenta, ovary and testis. It is not present in nonsteroidogenic tissues including liver, kidney and lung. Gel shift analysis using the -243/-225 bp fragment (CRSI) from bovine CYP17 indicates that the protein(s) binding to this DNA element is found in all tissues examined and is perhaps ubiquitously distributed [7, 8]. Since CYP17 is expressed in only a limited subset of these tissues, we determined whether the ubiquitously expressed CYP17 CRSI binding protein was functional in all tissues. By *in vitro* transcription analysis we found that CYP17 CRSI activated the transcription of the

reporter gene in extracts from mouse adrenal Y1 tumor cells. Extracts from SVT2 cells (nonsteroidogenic mouse 3T3 fibroblast cells) by contrast were inactive, although as estimated by gel shift analysis the concentration of CRSI binding protein in SVT2 and Y1 nuclear extracts was approximately the same. Therefore, it is possible that Y1 extracts contain a functional CRSI binding protein while SVT2 cells do not. To test this hypothesis we partially purified this binding protein from Y1 nuclear extracts by affinity chromatography. Addition to SVT2 extracts and *in vitro* transcription analysis demonstrated that indeed the CRSI binding protein fraction from adrenal cells can complement the functional deficiency in nonsteroidogenic (SVT2) cells [7]. Thus, we believe that the CRSI-binding protein of SVT2 and probably other nonsteroidogenic cells cannot enhance CYP17 CRSI-dependent transcription, suggesting it to be in an inactive form. Thus tissue-specific as well as cAMP-dependent CYP17 expression appear to involve CRSI and its transcription factor. Perhaps activation of the CRSI-binding protein involves a tissue specific kinase, but further investigation of this point is required.

Figure 2 shows the results of a gel shift competition analysis in Y1 adrenal nuclear extracts using CRSI as the probe and double stranded oligonucleotide competitors for a variety of *cis*-elements associated with cAMP-responsive gene transcription. As none of these competitors had an effect, we conclude that CRSI is not related to either the CRS elements found in other steroid hydroxylase genes, nor to other cAMP response elements such as the somatostatin CRE or the AP2 binding site. As shown in Fig. 1, the bovine CYP17 gene contains a second CRS. However, at this point in time the nature of the DNA-binding proteins for CYP17 CRSII has not been examined.

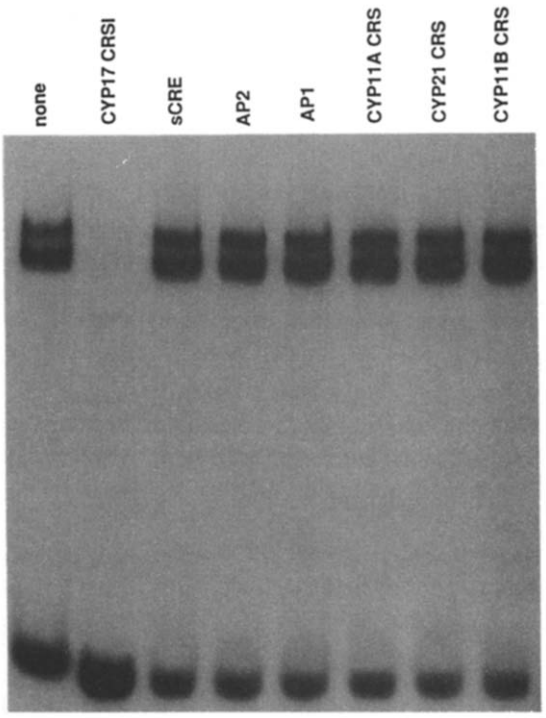
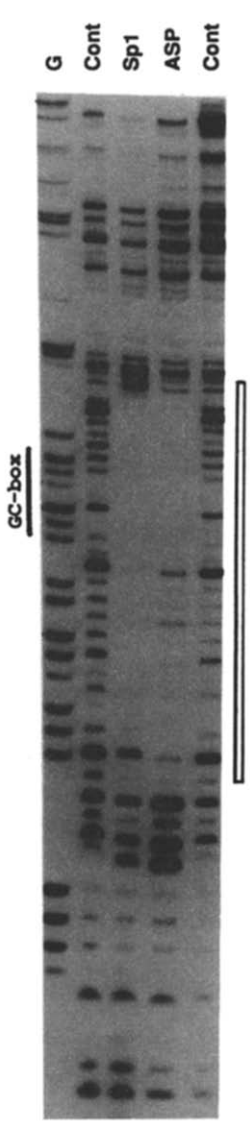


Fig. 2. Gelshift competition analysis of the bovine CYP17 CRSI in Y1 adrenocortical nuclear extracts. A double stranded oligonucleotide -243 to -225 of bovine CYP17 was used as probe and the indicated double stranded oligonucleotides were added as competitors in a 500× molar excess. sCRE, consensus CRE from the rat somatostatin gene; AP1 and AP2, consensus binding sequences for activator protein 1 purchased from Stratagene; CYP11A CRS, -118 to -83 bp from the bovine gene; CYP21 CRS, -129 to -96 bp from the human gene; CYP11B CRS, oligonucleotide for the cAMP response element Ad4 [19] of the bovine CYP11B gene; kindly provided by Dr Ken Morohashi.

CYP21B CRS

As noted above, CYP17 and CYP21B are predicted to have arisen from a common CYP progenitor gene. Based on this hypothesis we would have imagined that there could be relatedness in the cAMP responsive systems associated with these two genes. Obviously as seen in Fig. 1 such relatedness was not apparent between CYP17 CRSI and CYP21B CRS. Using the CYP21B -129/-96 bp fragment in gel shift analysis, a more complex pattern than that observed for CYP17 CRSI is found to result from the binding of two proteins [9]. The CYP21B CRS contains a GC-box sequence (consensus = G/TGGGC/AGGG/AG/AC/T) which is known to bind the ubiquitous transcription factor Sp1 [10, 11]. Bands *a* and *c* of the 3-banded pattern observed with -129/-96 result from Sp1 binding and are observed in all tissues tested, both steroidogenic and nonsteroidogenic. Band *b*, however, was observed only in cells of adrenal origin and we have designated this protein to be ASP (adrenal specific protein). Gel shift analysis suggests that ASP binds more 5′-ward in -129/-96 than does Sp1, however, the binding sites are presumed to overlap significantly [9]. DNase I footprint analysis with purified samples of ASP and Sp1 show clearly how great the overlap between these two binding sites is (Fig. 3). The difference between proteins binding to CYP17 CRSI and CYP21B CRS was clearly established by gel shift competition analysis. Excess CYP17 CRSI oligonucleotides had no effect on the gel shift pattern observed with CYP21B CRS and excess



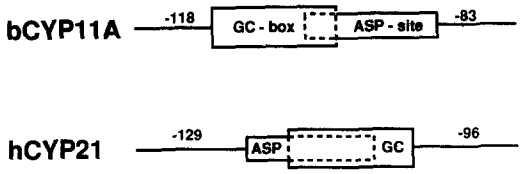


Fig. 4. Schematic comparison of Sp1 and ASP-binding sites within the CRSs from CYP11A and CYP21B.

CRS [14]. More recently we have localized the CRS to -118/-83 bp [15] as shown in Fig. 1. Gel shift analysis has shown that excess CYP21B CRS competitor DNA abolishes binding of proteins to CYP11A CRS and vice versa, clearly establishing that the same or very closely related proteins bind to both CRSs, namely ASP and a GC-box-binding protein which is presumably also Sp1. However, the two protein binding sites appear to be in the reverse orientation in

CYP11B and CYP21B are expressed solely in adrenal cortex while CYP17 and CYP11A are expressed in other steroidogenic tissues as well. Also CYP11A and CYP11B encode mitochondrial proteins while CYP17 and CYP21 encode microsomal proteins. Finally, based on evolutionary considerations, CYP17 and CYP21 arose from a common progenitor CYP gene while CYP11A and CYP11B arose from a different progenitor CYP gene. None of these obvious comparisons shed any light on why the mechanisms involved with cAMP-dependent expression of CYP21 and CYP11A seem to be related while those for CYP17 and CYP11B are different. Perhaps the detailed characterization of the relatedness, or lack thereof, of the transcription factors interacting with these different CRS elements will shed some light on this question. However, at this point in time the origin of the diversity of the mechanisms involved the cAMP-dependent transcription of the steroid hydroxylase genes and the physiological reasons for this diversity remain an enigma.

Acknowledgement—The support of USPHS Grant DK28350 is greatly appreciated.

REFERENCES

1. Purvis J. L., Canick J. A., Mason J. I., Estabrook R. W. and McCarthy J. L.: Lifetime of adrenal cytochrome *P*450 as influenced by ACTH. *Ann. N.Y. Acad. Sci.* **212** (1973) 319–343.
2. Waterman M. R. and Simpson E. R.: Regulation of steroid hydroxylase gene expression is multifactorial in nature. In *Recent Progress in Hormone Research Vol. 45* (Edited by J. H. Clark). Academic Press, New York (1989) pp. 533–566.
3. John M. E., John M. C., Boggaram V., Simpson E. R. and Waterman M. R.: Transcriptional regulation of steroid hydroxylase genes by ACTH. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 4715–4719.
4. Hanukoglu I., Feuchtwanger R. and Hanukoglu A.: Mechanism of corticotropin and cAMP induction of mitochondrial cytochrome *P*450 system enzymes in adrenal cortex cells. *J. Biol. Chem.* **265** (1990) 20602–20608.
5. Westin G., Gerster T., Muller M., Schaffner G. and Schaffner W.: OVEC a versatile system to study transcription in mammalian genes and cell-free extracts. *Nucleic Acids Res.* **15** (1987) 6787–6797.
6. Nebert D. W., Nelson D. R., Coon M. J., Estabrook R. W., Feyereisen R., Fujii-Kuriyama Y., Gonzalez F. J., Guengerich F. P., Gunsalus I. C., Johnson E. F., Loper J. C., Sato R., Waterman M. R. and Waxman D. J.: The *P*450 superfamily: update on new sequences, gene mapping and recommended nomenclature. *DNA Cell Biol.* **10** (1991) 1–14.
7. Zanger U. M., Lund J., Simpson E. R. and Waterman M. R.: Activation of transcription in cell-free extracts by a novel cAMP responsive sequence from the bovine CYP17 gene. *J. Biol. Chem.* **266** (1991) 11417–11420.
8. Lund J., Ahlgren R., Wu D., Kagimoto M., Simpson E. R. and Waterman M. R.: Transcriptional regulation of the bovine CYP17 (*P*45017α) gene. Identification of two cAMP regulatory regions lacking the consensus CRE. *J. Biol. Chem.* **265** (1990) 3304–3312.
9. Kagawa N. and Waterman M. R.: Evidence that an adrenal-specific nuclear protein regulates cAMP responsiveness of the human CYP21B (P450C21) gene. *J. Biol. Chem.* **266** (1991) 11199–11204.
10. Kadonaga J. T., Jones K. A. and Tjian R.: Promoter-specific activation of RNA polymerase transcription by Sp1. *Trends Biochem.* **11** (1986) 20–23.
11. Letovsky J. and Dynan W. S.: Measurement of the binding of the transcription factor Sp1 to a single GC box recognition sequence. *Nucleic Acids Res.* **17** (1989) 2639–2653.
12. Zanger U. M., Kagawa N., Lund J. and Waterman M. R.: Distinct biochemical mechanisms for cAMP-dependent transcription of CYP17 and CYP21. *FASEB J.* **6** (1992) 719–723.
13. Picado-Leonard J. and Miller W. L.: Cloning and sequence of the human gene for P450C17 (steroid 17α-hydroxylase/17,20-lyase: similarity with the gene for P450C21. *DNA* **6** (1987) 439–448.
14. Ahlgren R., Simpson E. R., Waterman M. R. and Lund J.: Characterization of the promoter-regulatory region of the bovine CYP11A (*P*450scc) gene: basal and cAMP dependent regulation. *J. Biol. Chem.* **265** (1990) 3313–3319.
15. Momoi K., Waterman M. R., Simpson E. R. and Zanger U. M.: Cyclic AMP dependent transcription of the CYP11A (cholesterol side chain cleavage cytochrome *P*450) gene involves a DNA response element containing a putative binding site for transcription factor Sp1. *Molec. Endocrl.* (1992) In press.
16. Jackson S. P., MacDonald J. J., Lees-Miller S. and Tjian R.: GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. *Cell* **63** (1990) 155–165.
17. Rice D. A., Aitken L. D., Vandenbark G. R., Mouw A. R., Franklin A., Schimmer B. P. and Parker K. L.: A cAMP-responsive element regulates expression of the mouse 11β-hydroxylase gene. *J. Biol. Chem.* **264** (1989) 14011–14015.
18. Morohashi K. and Omura T.: Tissue-specific transcription of *P*450(11β) gene *in vitro*. *J. Biochem.* **108** (1990) 1050–1056.
19. Honda S., Morohashi K. and Omura T.: Novel cAMP regulatory elements in the promoter region of bovine P450(11β) gene. *J. Biochem.* **108** (1990) 1042–1049.