A cAMP-responsive Element Regulates Expression of the Mouse Steroid 11β-Hydroxylase Gene*

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Douglas A. Rice§, Leslie D. Aitken§, G. R. Vandenbark§, Andrea R. Mouw§, Amanda Franklin*, Bernard P. Schimmer* and Keith L. Parker§

From the §Howard Hughes Medical Institute and §Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710 and the §Banting and Best Department of Medical Research and Department of Pharmacology, University of Toronto, Toronto, Ontario M5G I6E, Canada

In Y1 mouse adrenocortical tumor cells, expression of steroid 11β-hydroxylase (11β-OHase) is stimulated by cAMP following a delay of 4–6 h. Our results demonstrate that a cAMP-responsive element (CRE) within the 11β-OHase promoter region is a major determinant of this induction. The 5′-flanking sequences from the mouse 11β-OHase gene were placed in front of a human growth hormone reporter gene and transfected into Y1 cells. Treatment of transfected cells with 8-bromo-cAMP increased expression directed by the 11β-OHase 5′-flanking region by 3.8-fold. In 5′-deletion analyses, 123 base pairs of 5′-flanking sequences were sufficient for cAMP induction, whereas cAMP treatment did not affect expression of a plasmid with only 40 base pairs of 5′-flanking sequence. Within these 123 base pairs, a region from -56 to -49 matched 7 of 8 bases comprising the consensus sequence for the CRE. 11β-OHase 5′-flanking sequences from -65 to -42, including the CRE-like sequence, conferred cAMP inducibility to promoters from the thymidine kinase and choriconic gonadotropin α-subunit genes. DNase I footprinting and Southwestern blotting analyses demonstrated that the protein which interacted with the CRE in the 11β-OHase promoter region was similar to the CRE-binding protein associated with other cAMP-regulated genes. Together, these results suggest that an interaction between the 11β-OHase CRE and CRE-binding protein mediates cAMP induction of the 11β-OHase gene.

Steroid 11β-hydroxylase (11β-OHase) is an adrenal-specific cytochrome P-450 enzyme that is required for the synthesis of both glucocorticoids and mineralocorticoids (1). Adrenocorticotrophic hormone (ACTH) increases transcription of 11β-OHase as part of its trophic action to maintain the steroidogenic capacity of the gland. cAMP also increases adrenocortical expression of 11β-OHase and has been proposed to mediate this action of ACTH (2).

In order to identify factors that are important in regulating expression of the 11β-OHase gene, we recently isolated the mouse 11β-OHase gene and began to study the cis-acting elements that determine its expression (3). These studies showed that the 5′-flanking region of the 11β-OHase gene directed adrenal-specific expression. Moreover, we demonstrated that expression of the transfected 11β-OHase/human growth hormone gene (hGH) in Y1 cells required a functional cAMP-dependent protein kinase (3), suggesting that the 5′-flanking sequences were a target of cAMP-mediated regulation. A sequence (TGACGTGA), which matched 7 of 8 bases of the consensus sequence for the cAMP-responsive elements (CREs) of a number of cAMP-induced genes (4–11), was shown by site-selected mutagenesis to be required for 11β-OHase promoter activity (3). These results implicated cAMP and cAMP-dependent protein kinase in expression of 11β-OHase and suggested that this regulatory element might function as a CRE to confer cAMP inducible gene expression. The presence, however, of a CRE-like sequence in the 11β-OHase 5′-flanking region did not prove that this element determined cAMP inducibility, as genes such as glucagon (12) and parathyroid hormone (13) contain the 8-bp CRE consensus sequence in their 5′-flanking regions but are not regulated by cAMP.

In this report, we directly assessed the role of the potential CRE in induction of 11β-OHase expression by cAMP. Our results show that this sequence functions as a CRE to mediate cAMP induction of this adrenal-specific steroidogenic enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and modification enzymes were obtained from Boehringer Mannheim. Radiouclides were purchased from DuPont-New England Nuclear. Reagents for cell culture were obtained from Gibco, and bacterial chloramphenicol acetyltransferase (CAT) and 8-Br-cAMP were purchased from Sigma. Poly(d1-dC/d1-dC) was obtained from Pharmacia LKB Biotechnology Inc. Nitroelulose and Nytan membranes were purchased from Schleicher and Schuell.

**Cell Culture and DNA-mediated Gene Transfer**—The cell lines used in these studies were: Y1, an ACTH- and cAMP-responsive subclone of the mouse adrenocortical tumor cell line isolated by Yasumura et al. (14); Kin-8, a cAMP-resistant, protein kinase-defective mutant of Y1 (15); and JEG-3, a human choriocarcinoma cell line obtained from the American Type Culture Collection. Conditions for cell culture of Y1 (3), Kin-8 (16), and JEG-3 (6) cells were as described. The effects of 8-Br-cAMP on gene expression were determined by adding the inducer to the cells as described in the legends for Tables I and II. hGH levels in culture medium were measured by radioimmunoassay as described (16), using a kit from BioMega Diagnostics. Cell lysates were prepared by freeze-thawing, and levels of
CAT activity in cell lysates were determined using a described procedure (17). Measurements of CAT activity were in the linear range of the assay, as determined by a standard curve using bacterial CAT. Protein concentrations in the samples were determined by the Bradford method (18), using a kit obtained from Bio-Rad. Total cellular RNAs were prepared and analyzed by Northern blotting as described (3). Probes for hGH (6), mouse 118-OHase (3), and v-fos (American Type Tissue Culture Collection No. 41040).

Plasmids—The plasmids used in these studies were: p-1500GH, which contains 5'-flanking sequences from -1500 to +7 from the mouse 11β-OHase gene in front of the hGH gene (3); p-123GH, which contains 5'-flanking sequences from -123 to +7 from the mouse 11β-OHase gene in front of the hGH gene; p-40GH, which contains 5'-flanking sequences from -40 to +7 from the mouse 11β-OHase gene in front of the hGH gene; p-1500ΔCREGH, a plasmid derived from p-1500GH in which bases from positions -56 to -49 were mutated (3); pTKCAT, which contains thymidine kinase promoter sequences from -105 to +56 in front of the bacterial chloramphenicol acetyltransferase gene (19); pTKCAT/CRE(3x), which contains a trimer of 11β-OHase 5'-flanking sequences from -65 to -42 cloned into the BamHI site upstream of the thymidine kinase promoter in pTKCAT; pα100CAT, which contains the promoter region of the α-subunit of chorionic gonadotropin cloned in front of CAT (6); pα100CAT/CRE(1X), which contains a single copy of the CRE from the 11β-OHase gene cloned in front of pα100CAT.

DNase I Footprinting Analyses—Nuclear extracts were prepared from Y1 and Kin-8 cells using a modification of the procedure of Dignam et al. (20, 21). The DNA probe, containing sequences from -250 to +6 in the 11β-OHase 5'-flanking region, was end-labeled at the 3' end of the noncoding strand with the Klenow fragment of DNA polymerase I. DNase I footprinting experiments were performed as described (22), using 5 fmol of probe. Where indicated, specific duplex oligonucleotides at 100-fold molar excess over probe were used as competitors in the binding reactions.

Southern Blotting Analyses—Southern blot analyses were performed essentially as described (23). Samples containing 30 μg of nuclear extract from each tissue were resolved by electrophoresis on 12% SDS-polyacrylamide gels and transferred by electroblotting onto nitrocellulose membranes. After soaking for 30 min at 4 °C in binding buffer (25 mM Hepes, pH 7.9, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol), the filter was incubated for 30 min at 4 °C in binding buffer containing 5% (w/v) nonfat dry milk and then washed in several changes of binding buffer over 15 min. Hybridization with 32P-labeled oligonucleotides was performed overnight at 4 °C in binding buffer containing poly(dI·dC)/poly(dI·dC) at 10 μg/ml. The oligonucleotide probes were labeled with [γ-32P]ATP and polyadenylated kinase, concatenated using T4 DNA ligase, and added to the binding buffer at 10 000 cpm/ml. After hybridization, the filters were washed with several changes of binding buffer containing 0.25% nonfat dry milk and then exposed to Kodak XAR-5 film.

RESULTS

CAMP Induces the Expression of 11β-OHase and c-fos in Y1 Adrenocortical Cells with Different Kinetics—Previous work had suggested that the kinetics of induction of the adrenal steroidogenic enzymes by cAMP differed from those of other genes in which CREs had been defined (24, 25). To determine whether this difference resulted from differences in the genes or from differences between adrenal cells and other cell lines studied, we analyzed the effect of CAMP on expression of 11β-OHase and c-fos in Y1 adrenocortical cells. As shown in Fig. 1, induction of c-fos was maximal 1 h after addition of 8-Br-CAMP and steady-state mRNA levels declined by 2 h. In contrast, 11β-OHase expression showed no induction until 4 h and did not peak until 6 h. These differences in the kinetics of CAMP induction are consistent with previous reports that directly measured transcription (24, 25), and strongly suggest that the different kinetics reflect intrinsic differences in the regulatory elements of these genes rather than differences in the cell lines studied.

The 5'-Flanking Sequence of 11β-OHase Determines CAMP Inducible Expression—In order to study the contribution of the 5'-flanking sequences to CAMP induction, Y1 adrenocortical cells were transfected with p-1500GH, a plasmid containing 1500 bp of 5'-flanking sequence from the mouse 11β-OHase gene fused to the hGH reporter gene. Following transfection, cells were cultured in 4 ml of fresh growth medium for 24 h and then incubated in the presence or absence of 3 mM 8-Br-CAMP for an additional 72 h. At the end of the incubation, the culture medium was assayed for hGH content. Values for hGH production were presented as means ± standard errors. The number of transfections for each plasmid are indicated in parentheses.

| Plasmid       | hGH       |
|---------------|-----------|
|               | ng/plate  |
| p-1500GH (25) | 24.1 ± 1.8| 94 ± 14.5 | 3.8 ± 0.4 |
| p-123GH (9)   | 17.5 ± 2.5| 43.6 ± 8.0| 2.5 ± 0.5 |
| p-40GH (15)   | 4.5 ± 0.5 | 5.0 ± 0.4 | 1.1 ± 0.06|
| p-1500ΔCREGH (3)| 4.5 ± 0.2 | 3.5 ± 0.4 | 0.9 ± 0.07 |

*The fold stimulation of hGH production by 8-Br-cAMP was determined by paired analysis of basal and stimulated samples in the separate transfection experiments.
as a major determinant of cAMP induction of the mouse 11β-
OHase gene.

The Potential CRE at −56 Confers cAMP Inducibility to Heterologous Promoters—In order to determine if the sequence at −56 in the 11β-OHase promoter functioned as a CRE, we analyzed its effect on expression of the CAT reporter gene driven by promoters from the thymidine kinase and chorionic gonadotropin α-subunit genes. The 11β-OHase sequence included the potential CRE (from −56 to −49), as well as 9 and 7 bp of flanking sequences at the 5′ and 3′ ends, respectively. Y1 cells were transfected with the parent plasmid, pTKCAT, or with pTKCAT/CRE(3x), which contains three copies of the putative CRE placed 5′ of the thymidine kinase promoter. As shown in Table II, addition of the CRE trimer did not affect basal expression of CAT driven by the thymidine kinase promoter, but led to a 4-fold increase in expression after treatment with 8-Br-cAMP. Furthermore, in a preliminary experiment performed in triplicate, the addition of 2 copies of the 11β-OHase CRE to palOOCAT led to 5-fold increases in expression of CAT after treatment of transfected Y1 cells with 8-Br-cAMP (500 ± 200 cpm control versus 2400 ± 400 cpm after treatment with 8-Br-cAMP).

More marked cAMP induction occurred in experiments using plasmids in which the 11β-OHase CRE was placed in front of palOOCAT and transfected into JEG-3 choriocarcinoma cells; this combination of the human choric gonadotropin α-subunit promoter and JEG-3 cells has been shown to be particularly sensitive to the addition of CREs (6). As shown in Table II, the addition of a single copy of the 11β-
OHase CRE to palOOCAT had minimal effects on basal expression but led to a 23-fold increase in CAT expression upon induction with 8-Br-cAMP. These results showed that the potential CRE of the 11β-OHase promoter region conferred cAMP inducible expression to two heterologous promoters.

We previously showed that 11β-OHase expression in Y1 cells absolutely required cAMP-dependent protein kinase activity (3). Accordingly, we analyzed the effect of 8-Br-cAMP treatment on expression of pTKCAT(3x) in Kin-8, a Y1 derivative defective in cAMP-dependent protein kinase activity. When Kin-8 cells were transfected with pTKCAT/CRE(3x), treatment with 8-Br-cAMP did not alter gene expression, indicating that the CRE required functional cAMP-dependent protein kinase activity (data not shown).

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OHase CRE to palOOCAT led to 5-fold increases in expression of CAT after treatment of transfected Y1 cells with 8-Br-cAMP (500 ± 200 cpm control versus 2400 ± 400 cpm after treatment with 8-Br-cAMP).

These results are consistent with previous reports that cAMP-dependent protein kinase activity was required for CRE-mediated increases in gene expression (4, 26) and suggest that the CRE determines the dependence of 11β-OHase expression on cAMP-dependent protein kinase activity.

DNase I Footprinting Experiments Define a CRE-binding Protein in Y1 Cells—To determine further the relationship between the CRE in the 11β-OHase gene and those in other cAMP-regulated genes, we performed DNase I footprinting experiments with nuclear extracts from Y1 cells and a probe containing 11β-OHase 5′-flanking sequences from −250 to +6. As shown in Fig. 2, a footprint that protected bases from −63 to −47 closely corresponded to the location of the CRE (−56 to −49). Encoding of the coding strand also showed a footprint over the CRE. Addition of duplex oligonucleotides containing the homologous sequence from the 11β-OHase promoter region (TGACGCTGA) or the CRE sequences from the somatostatin promoter (TGACGCTA (4)) or from the adenoviral E4 gene (TGACGCTG (27)) inhibited the footprint. In contrast, no inhibition was observed with oligonucleotide competitors containing regulatory elements at −210 (AGAGGTCA (28)) and −65 (AAAGGTCA (29)) in the steroid 21-hydroxylase (21-OHase) promoter. The latter sequences share 6 and 5 bases, respectively, with the CRE consensus and have been implicated as important regulators of 21-OHase expression (28). These results strongly suggest that the CREs from the 11β-OHase promoter and from other cAMP-regulated promoters are recognized by the same protein in Y1 nuclear extracts; in contrast, the 21-OHase regulatory elements at −210 and −65 do not interact with this protein.

In view of the demonstration that 11β-OHase expression requires cAMP-dependent protein kinase activity, we analyzed DNA-protein interactions using nuclear extracts prepared from Kin-8 cells. Despite the virtual absence of cAMP-dependent protein kinase activity in Kin-8, there was no

| Table II |
|---|
| The potential CRE at −56 confers cAMP inducibility to heterologous promoters |

Y1 and JEG-3 cells were seeded on 100-mm tissue culture dishes at 1 × 10^6 cells/plate and transfected with 2.5 μg of the indicated plasmids as described (3). Following incubation in the absence (+) or presence (+) of 8-Br-cAMP (2 mM), cells were harvested, and CAT assays performed as described under “Experimental Procedures.” All values were corrected for protein concentration, and all values are the means of triplicate transfections ± standard deviation. The increases in expression of 8-Br-cAMP-treated samples relative to control are shown in parentheses.

| Plasmid            | 8-Br-cAMP | cpm/mg protein |
|--------------------|-----------|----------------|
| Y1 cells           |           |                |
| pTKCAT             | −         | 1,700 ± 150    |
| pTKCAT/CRE(3x)     | +         | 1,500 ± 700    |
|                   | +         | 2,000 ± 600    |
|                   | +         | 6,550 ± 1,100 (3.6x) |
| JEG-3 cells        |           |                |
| palOOCAT           | −         | 1,100 ± 50     |
| palOOCAT/CRE(1x)   | +         | 2,300 ± 400    |
|                   | +         | 1,550 ± 400    |
|                   | +         | 35,700 ± 6,100 (23X) |

FIG. 2. Interaction between the potential CRE in the 11β-
OHase promoter region and proteins in nuclear extracts from Y1 cells. Preparation of nuclear extracts and DNase I cleavage of probe containing 11β-OHase sequences from −250 to +45 were performed as described under “Experimental Procedures.” Competitors were added as indicated at 100-fold molar excess over probe. These competitors included: −65, which contains sequences from −88 to −45 in the 21-OHase promoter (29); −210, which contains sequences from −215 to −195 in the 21-OHase promoter (30); somatostatin, which contains the CRE from the somatostatin promoter (4); 11β-
OHase, which contains sequences from −65 to −42 in the 11β-OHase promoter; ATF which contains 18 bp that include the CRE/ATF binding site from the adenoviral E4 promoter (27). The bases included within the protected region are shown at the left for the noncoding strand, and the bases comprising the CRE are boxed. BSA, bovine serum albumin.
cAMP Regulation of Steroid 11β-Hydroxylase

FIG. 3. The CRE from the 11β-OHase promoter region interacts with a protein with an apparent Mr of 43,000. Nuclear extracts from the indicated tissues (30 μg) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes by electroblotting, and hybridized with 32P-labeled oligonucleotide probes as described under “Experimental Procedures.” The probe used contained bases from −65 to −42 in the 11β-OHase promoter. The numbers at the left show the approximate molecular masses in kilo Daltons of the major bands, as determined by comparison with the migration of molecular mass standards on the same gel.

difference in the CRE footprint in the 11β-OHase promoter region (data not shown). This result indicates that protein kinase activity is not required for the interaction of the CRE and its binding protein; the same conclusion was reached from DNase I footprinting analyses of the CREs in the 5′-flanking regions of the somatostatin (30) and vasoactive intestinal polypeptide genes (10).

The CRE from the 11β-OHase Gene Interacts with a 43-kDa Protein in Y1, HeLa, and PC-12 Cells—To characterize the protein in Y1 nuclear extracts that interacted with the CRE, we performed Southwestern blotting analyses. Nuclear extracts from Y1, HeLa, and PC-12 cells were subjected to SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membranes, and hybridized with 32P-labeled oligonucleotides containing either the CRE from the somatostatin promoter region (30). Both CAMP-dependent protein kinase and protein kinase C phosphorylated CREB in vitro; although phosphorylation of CREB by CAMP-dependent protein kinase did not affect its binding to the CRE, it markedly enhanced the stimulatory effect of purified CREB in an in vitro transcription assay (32). These results thus provide an attractive model to explain how cAMP increases transcription of the somatostatin gene. Studies using CREs from a variety of cellular and viral promoters, including the ATF binding site from the somatostatin gene, have suggested that the same, or very similar protein(s) interact with multiple CREs (10, 27, 30). It remains unclear, however, whether CREB mediates all effects of cAMP on gene expression, or if other proteins play a role in the cAMP induction of some genes. In addition, the demonstration of CRE-like sequences within the 5′-flanking regions of genes that are not cAMP responsive suggests that the presence of the 8-bp palindromic sequence does not necessarily identify an authentic CRE (13).

Less progress has been made in elucidating the mechanisms determining cAMP inducibility of the steroidogenic enzymes. Clearly, cAMP increases transcription of the adrenal steroidogenic cytochrome P-450 enzymes (25); the time courses of these transcriptional increases are similar, suggesting coordinate regulation by a common pathway. The kinetics of cAMP induction of the steroidogenic enzymes, however, are significantly delayed relative to those of genes such as phoshoenolpyruvate carboxykinase, c-fos, and the α-subunit of glycoprotein a-subunit promoters (31). Finally, there is an additional band at approximately 31 kDa in the Y1 lane that is not seen in the other samples. A similar band is variably present in different nuclear extracts and possibly derives from proteolysis of one of the larger proteins.

DISCUSSION

Considerable attention has been directed at defining the mechanisms that determine hormonally inducible gene expression. Several studies have defined an 8-bp palindromic sequence in the 5′-flanking region of many cAMP-regulated genes, termed the cAMP-responsive element, that confers cAMP inducibility to heterologous promoters (4–11). Furthermore, a protein, designated the CRE-binding protein (CREB), was shown to bind to this element in the somatostatin promoter region (30). Both CAMP-dependent protein kinase and protein kinase C phosphorylated CREB in vitro; although phosphorylation of CREB by CAMP-dependent protein kinase did not affect its binding to the CRE, it markedly enhanced the stimulatory effect of purified CREB in an in vitro transcription assay (32). These results thus provide an attractive model to explain how cAMP increases transcription of the somatostatin gene. Studies using CREs from a variety of cellular and viral promoters, including the ATF binding site from the somatostatin gene, have suggested that the same, or very similar protein(s) interact with multiple CREs (10, 27, 30). It remains unclear, however, whether CREB mediates all effects of cAMP on gene expression, or if other proteins play a role in the cAMP induction of some genes. In addition, the demonstration of CRE-like sequences within the 5′-flanking regions of genes that are not cAMP responsive suggests that the presence of the 8-bp palindromic sequence does not necessarily identify an authentic CRE (13).

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Southwestern blotting analyses (Fig. 3) showed that an oligonucleotide containing the 11β-OHase CRE interacted with a 43-kDa protein present in nuclear extracts from Y1, HeLa, and PC-12 cells. These results all indicate that cAMP induction of the 11β-OHase gene involves an interaction between a CRE and a protein very similar to that previously described as CREB and ATF.

In contrast, the mouse 21-OHase promoter region from -330 to +8 also contains sufficient information for cAMP induction but lacks an identifiable CRE. Regulatory elements at -210 and -65 share 6 and 5 bases, respectively, with the CRE but did not compete for binding of CREB to the 11β-OHase CRE (Fig. 2). Moreover, although proteins in Y1 cells produced multiple footprints on the 21-OHase 5' flanking region, none of these interactions were competed by oligonucleotides containing authentic CRE sequences. These observations strongly suggest that the 5' flanking region of the mouse 21-OHase gene does not contain a typical CRE. Similarly, no sequences within 800 bp of 5' flanking sequences in the mouse or human cholesterol side chain cleavage enzyme genes closely match the CRE consensus (34). The presence of a CRE in the promoter region of the 11β-OHase gene may thus be unique among the adrenal steroidogenic cytochrome P-450 enzymes of the mouse.

Bokar et al. (30) recently proposed that certain genes which show delayed induction by cAMP and sensitivity of this induction to cycloheximide may utilize a distinct pathway of transcriptional regulation. The absence of identifiable CRE-like sequences within regions that determine hormonal induction of 21-OHase and cholesterol side chain cleavage enzyme is consistent with this model. The demonstration of a CRE in the promoter region of the 11β-OHase gene, however, suggests that this model does not apply to all genes with delayed kinetics of cAMP induction. The basis for the delayed induction of 11β-OHase in Y1 cells, despite the presence of a functional CRE, remains an unresolved issue that will require further study. In addition, it will be very interesting to compare and contrast the mechanisms that provide cAMP induction to the 21-OHase and cholesterol side chain cleavage enzyme genes with that defined here for 11β-OHase regulation.

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In contrast, the mouse 21-OHase promoter region from -330 to +8 also contains sufficient information for cAMP induction but lacks an identifiable CRE. Regulatory elements at -210 and -65 share 6 and 5 bases, respectively, with the CRE but did not compete for binding of CREB to the 11β-OHase CRE (Fig. 2). Moreover, although proteins in Y1 cells produced multiple footprints on the 21-OHase 5' flanking region, none of these interactions were competed by oligonucleotides containing authentic CRE sequences. These observations strongly suggest that the 5' flanking region of the mouse 21-OHase gene does not contain a typical CRE. Similarly, no sequences within 800 bp of 5' flanking sequences in the mouse or human cholesterol side chain cleavage enzyme genes closely match the CRE consensus (34). The presence of a CRE in the promoter region of the 11β-OHase gene may thus be unique among the adrenal steroidogenic cytochrome P-450 enzymes of the mouse.

Bokar et al. (30) recently proposed that certain genes which show delayed induction by cAMP and sensitivity of this induction to cycloheximide may utilize a distinct pathway of transcriptional regulation. The absence of identifiable CRE-like sequences within regions that determine hormonal induction of 21-OHase and cholesterol side chain cleavage enzyme is consistent with this model. The demonstration of a CRE in the promoter region of the 11β-OHase gene, however, suggests that this model does not apply to all genes with delayed kinetics of cAMP induction. The basis for the delayed induction of 11β-OHase in Y1 cells, despite the presence of a functional CRE, remains an unresolved issue that will require further study. In addition, it will be very interesting to compare and contrast the mechanisms that provide cAMP induction to the 21-OHase and cholesterol side chain cleavage enzyme genes with that defined here for 11β-OHase regulation.

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