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PROMOTER ELEMENTS OF THE MOUSE 21-HYDROXYLASE (*Cyp*-21) GENE INVOLVED IN CELL-SELECTIVE AND cAMP-DEPENDENT GENE EXPRESSION

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Summary—*Cyp*-21 (the mouse steroid 21-hydroxylase gene) is expressed exclusively in cells of the adrenal cortex, is induced by ACTH and cAMP, and is required for corticosteroid synthesis. This review examines the molecular basis for the regulated expression of *Cyp*-21 in the ACTH-responsive, mouse adrenocortical tumor cell line, Y1. We demonstrate that 330 bp of 5′-flanking DNA from the *Cyp*-21 gene are sufficient for cell-selective and ACTH-induced expression of *Cyp*-21, and that this promoter region comprises multiple, closely spaced enhancer elements each of which is required for promoter function. Within this promoter, we define three related elements that contain variations of an **AGGTCA** motif and that contribute to the cell-selective expression of *Cyp*-21. Variations of these same **AGGTCA**-bearing elements are also involved in the expression of *Cyp*11a and *Cyp*11b in Y1 adrenocortical cells. These elements interact with the same or closely related nuclear proteins found only in steroidogenic cell lines. Taken together, these results suggest that shared elements contribute to the adrenal cell-selective expression of at least three steroidogenic cytochrome *P*450 genes.

The element at −170 and the related elements at −65, −140 and −210 in the *Cyp*-21 promoter are not active as enhancers in the mutant Y1 cell line, Kin-8. Kin-8 cells contain a mutation in the regulatory subunit of the type 1 cAMP-dependent protein kinase that renders the enzyme resistant to activation by cAMP. Therefore, these elements appear to be selectively dependent upon an intact cAMP-dependent protein kinase for enhancer function. Individually, none of these elements confer cAMP-dependence to a reporter gene driven by a heterologous promoter. On the basis of these observations, we suggest that ACTH- and cAMP-dependent expression of *Cyp*-21 requires the combined actions of the element at −170, and the related elements at −140, −210 and −65.

OVERVIEW

Glucocorticoids are adrenal steroid hormones that play critical roles in carbohydrate, protein and fat metabolism, regulation of endocrine and immune systems and response to stress. They are synthesized from cholesterol via an ordered series of enzymatic reactions involving several cytochrome *P*450 mixed-function oxidases. Certain of these cytochrome *P*450 enzymes, notably the steroid 21-hydroxylase and the steroid 11β-hydroxylase are expressed only in cells of the adrenal cortex and are uniquely required for the synthesis of the adrenal corticosteroids. Others, such as the cholesterol side-chain cleavage enzyme, are expressed more widely among steroidogenic tissues, but are not expressed elsewhere. The levels of the adrenal steroidogenic cytochrome *P*450 enzymes are coordinately increased by corticotropin (ACTH) via mechanisms involving increases in gene transcription, most likely mediated by cAMP [1, 2]. A number of other factors have also been implicated in the expression of the steroidogenic cytochrome *P*450 genes [e.g. 3, 4].

We have undertaken a series of investigations to determine the molecular bases for the cell-selective and ACTH-regulated expression of the steroidogenic cytochrome *P*450 enzymes in the mouse adrenal cortex. We have chosen to investigate steroidogenesis in the mouse adrenal cortex because an homologous, functionally-differentiated, mouse adrenocortical cell line (clone Y1) is available to study gene expression by transfection. This study reviews our work on the molecular bases for the cell-selective

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expression of genes encoding the mouse steroid 21-hydroxylase (*Cyp*-21),* steroid 11β-hydroxylase (*Cyp*11b) and cholesterol side-chain cleavage enzyme (*Cyp*11a) and their coordinate regulation by ACTH. Our results suggest that shared regulatory elements in the promoter regions of these genes contribute to their cell-selective expression in the adrenal cortex, that cAMP and cAMP-dependent protein kinase are required for both constitutive and hormone-induced expression of these genes and that, with the exception of *Cyp*11b, previously characterized cAMP-response elements are not involved in hormone-induced expression of these genes.

THE Y1 MOUSE ADRENOCORTICAL TUMOR CELL LINE

The Y1 mouse adrenocortical tumor cell line [5] behaves in many respects like cells from a normal adrenal cortex and thus provides a valuable model to study the regulation of adrenocortical function. The properties of the Y1 cell line have been reviewed in detail elsewhere [6, 7]. The cell line is nearly diploid and responds to ACTH with enhanced synthesis and secretion of C_{21} steroids. In addition, Y1 cells treated with ACTH undergo dramatic changes in cell shape, visible by phase contrast microscopy, and inhibition of cell growth at a point early in the G_1 phase of the cell cycle. The addition of ACTH to Y1 cells results in an acute steroidogenic response characterized by the increased conversion of cholesterol to pregnenolone [8]. ACTH also brings about a number of other characteristic intracellular changes that support steroidogenesis including increased glucose utilization [9], cholesterol mobilization [10], and induction of some of the enzymes of the steroidogenic pathway including adrenodoxin, *Cyp*11a and *Cyp*11b [11, 12]. A notable difference between Y1 cells and cells of the normal mouse adrenal cortex is that Y1 cells produce 20α-hydroxyprogesterone and 11β,20α-dihydroxyprogesterone as major steroid metabolites, instead of corticosterone [13, 14].

Another important feature of the Y1 cell line that has been particularly useful for studies of hormonal control of adrenal steroidogenesis is its susceptibility to genetic manipulation either through mutation [7] or through DNA-mediated transformation [15, 16]. Mutations in Y1 cells resulting from specific lesions in the ACTH-responsive adenylyl cyclase and the cAMP-dependent protein kinase have been used to evaluate the roles of cAMP and cAMP-dependent protein kinase in hormonal control of steroidogenesis [for review see 7]. DNA-mediated transformations have been used successfully to analyze the contributions of the promoter regions of various steroidogenic enzymes to regulated gene expression. Some of these latter studies are reviewed below.

EXPRESSION OF *Cyp*-21 IN Y1 ADRENAL CELLS

Regulated expression of the Cyp-21 gene transfected into Y1 cells

Cyp-21 is expressed at high levels in the normal mouse adrenal cortex; however, *Cyp*-21 is not expressed in Y1 mouse adrenal cells either in the presence or absence of ACTH (Fig. 1). Nevertheless, Y1 cells stably transfected with a cosmid clone containing the *Cyp*-21 gene from Balb/c mice were able to constitutively express the *Cyp*-21 gene at high levels, to increase the levels of *Cyp*-21 transcripts in response to ACTH (Fig. 1) and to synthesize corticosterone as a major steroid metabolite (Fig. 2). In contrast, mouse fibroblast L cells transfected with the same cosmid did not synthesize *Cyp*-21 transcripts (Fig. 1). Taken together, these results indicate that Y1 cells are unable to express their endogenous *Cyp*-21 gene, but can express a transfected wild-type *Cyp*-21 gene in a hormone-inducible and cell-selective manner. As a consequence of this defect, Y1 cells exhibit a truncated steroidogenic pathway [15, 17]. The inability of Y1 cells to express the endogenous *Cyp*-21 gene may have resulted from mutations or deletions in the coding or promoter regions of the gene or from non-mutagenic, reversible mechanisms.

Reversible cis modifications silence the endogenous Cyp-21 in Y1 adrenocortical tumor cells

In order to determine the basis for *Cyp*-21 deficiency in the Y1 cell line, we attempted to clone and characterize the endogenous *Cyp*-21 gene [18, 19]. A *Cyp*-21 gene was cloned from Y1 cells as a 9 kb *Bgl* II fragment characteristic of the mouse *Cyp*-21 gene [17]. This genomic fragment was transfected into Y1 cells, and stable transformants were isolated and analyzed

*In this review, we have attempted to follow the formal system of nomenclature for the *P*450 superfamily as proposed by Nebert *et al.* [45]. In some figures, however, *Cyp*-21 is referred to as 21-OHase, *Cyp*11b as 11β-OHase, and *Cyp*11a as SCC.

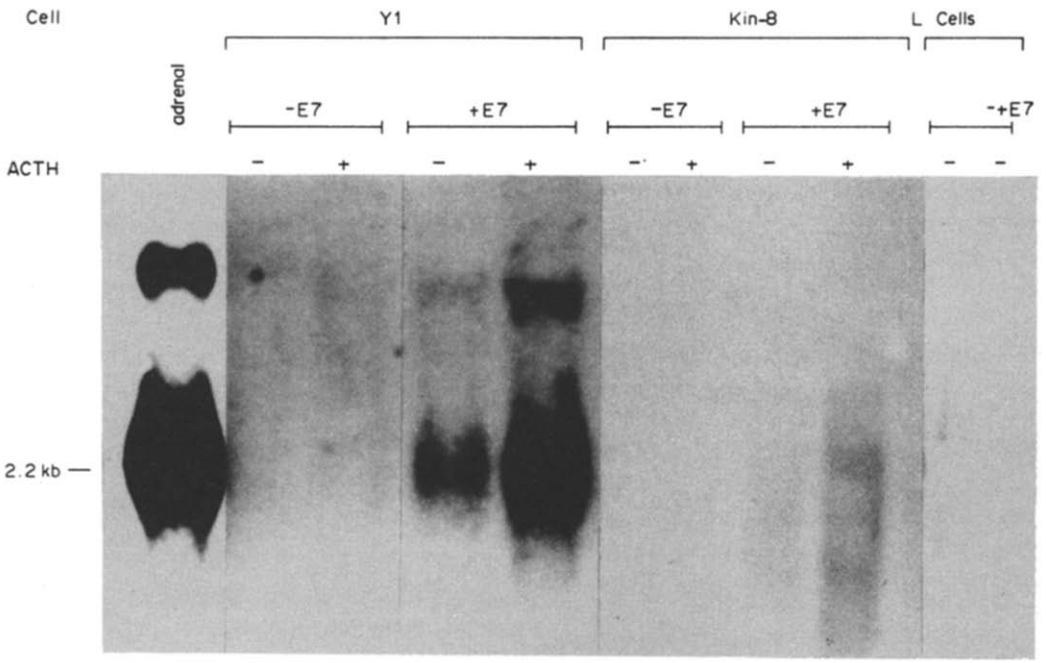


Fig. 1. Analysis of *Cyp*-21 RNA in transfected cells. Y1 mouse adrenocortical tumor cells, protein kinase-defective Kin-8 adrenal cells and mouse fibroblast L cells were co-transfected with a neomycin-resistance gene and a cosmid (E7) containing the mouse *Cyp*-21 gene and transformants were isolated. Total RNA from untransformed (−E7) and transformed (+E7) cells were analyzed for *Cyp*-21 transcripts by Northern blot hybridization with a *Cyp*-21 probe. As shown, the transfected cells expressed a 2.2 kb transcript that was indistinguishable from the transcript found in BALB/c mouse adrenal glands. Where indicated (+), cells were treated with ACTH for 24 h before RNA was isolated. Reprinted from p. 418 of Ref. [17] by courtesy of Marcel Dekker.

for expression of *Cyp*-21. Isolates containing the transfected gene from Y1 cells expressed *Cyp*-21 and acquired the ability to synthesize 21-hydroxylated steroids. These results demonstrate that Y1 cells contain a functional *Cyp*-21 gene that is silenced by reversible, *cis* modification. Cloning the *Cyp*-21 gene from Y1 cells and propagation of the genomic clones in bacteria removed the *cis* modification, most likely because bacterial cells do not contain the DNA

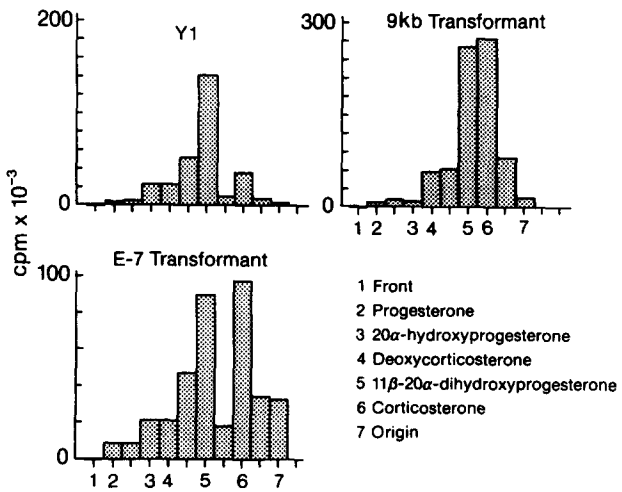


Fig. 2. *Cyp*-21 enzymatic activity in transfected Y1 cells. Y1 cells and a *Cyp*-21 transfectant (E-7 transformant) were incubated with [1,2-^{3}H]progesterone for 24 h. The distribution of radioactive steroid products in the culture medium was analyzed following chromatography of the samples on silica gel. The relative positions of steroid standards on the gel are indicated. As shown, the *Cyp*-21 transformant produced appreciable amounts of corticosterone whereas the Y1 parent lacked *Cyp*-21 activity. This figure also demonstrates that a 9 kb *Bgl* II fragment from E7 cosmid is sufficient for expression of *Cyp*-21. Reprinted from p. 417 of Ref. [17] by courtesy of Marcel Dekker.

modifying enzymes usually found in mammalian cells [20].

A possible role for DNA methylation in regulation of Cyp-21 expression

DNA methylation may be a contributing factor in the reversible *cis* modification of the *Cyp*-21 gene from Y1 cells. This gene is highly methylated in Y1 cells when compared with the gene in normal mouse adrenal glands. The *Cyp*-21 gene cloned from Y1 cells is hypomethylated, but becomes progressively hypermethylated when transfected back into Y1 cells. These changes in the methylation status of the Y1 *Cyp*-21 gene are accompanied by an extinction of *Cyp*-21 expression and seem to result from a *Cyp*-21-specific *de novo* DNA methylase activity expressed in Y1 cells. These changes in methylation state, however, cannot completely explain the silencing of the *Cyp*-21 gene. The demethylating drug 5′-azacytidine was unable to induce the expression of the endogenous *Cyp*-21, raising the possibility that other reversible *cis* modifications contribute to the silencing of the gene in Y1 cells [18, 19].

PROMOTER ELEMENTS OF *Cyp*-21

Minimum promoter requirements for cell-selective and hormone-induced expression in the Y1 adrenal cell line

The availability of genomic clones encoding *Cyp*-21 together with the Y1 adrenal cell expression system prompted an analysis of the contributions of the promoter region of *Cyp*-21 to adrenal cell-selective and ACTH-induced gene expression. As determined by 5′-deletion analysis, 230 bp of 5′-flanking DNA from the mouse *Cyp*-21 promoter ahead of the *Cyp*-21 structural gene was sufficient for constitutive *Cyp*-21 expression in the Y1 cell line [21]. Furthermore, a plasmid containing 330 bp of 5′-flanking DNA from *Cyp*-21 placed ahead of a human growth hormone reporter gene (p-330GH) generated high levels of growth hormone when transfected into Y1 adrenal cells, but was poorly expressed following transfection into steroid secreting, mouse Leydig tumor cells [Fig. 3(A)]. These 330 bp of flanking DNA also encoded sufficient information for ACTH-induced expression of growth hormone in the Y1 cell line [Fig. 3(B)]. Truncation of the 5′-flanking DNA to 156 bp resulted in a loss of both constitutive and ACTH inducible gene

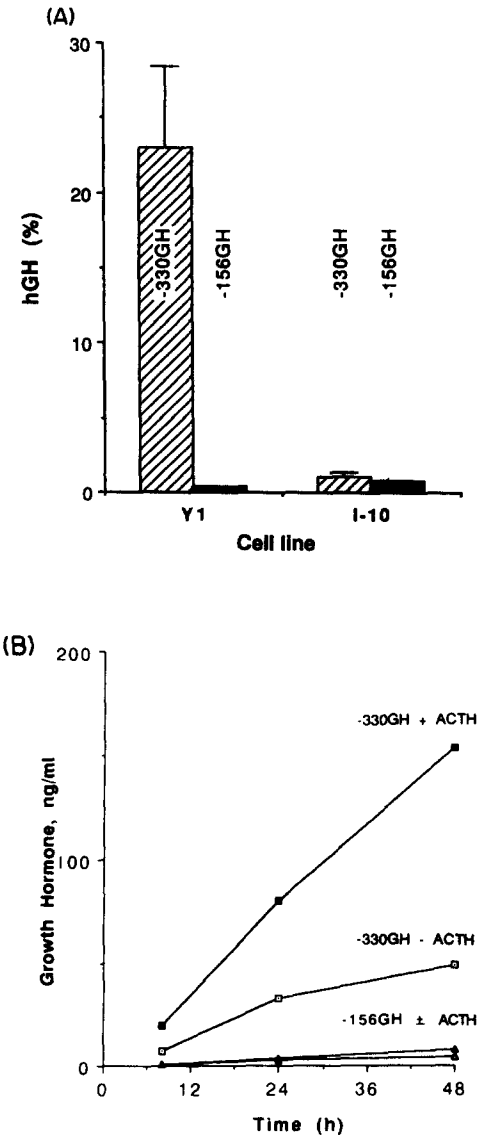


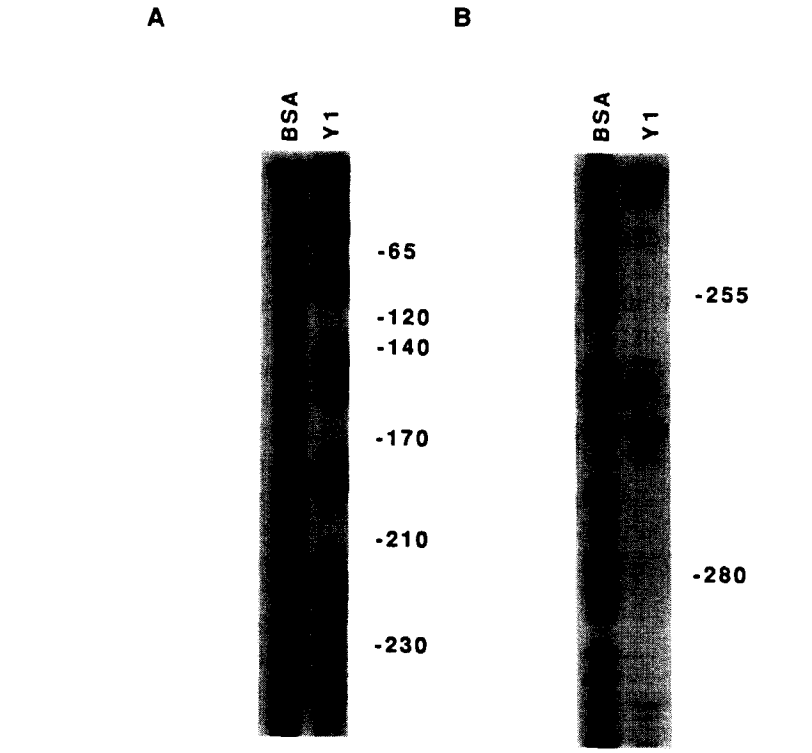
Fig. 3. Contributions of *Cyp*-21 5′-flanking DNA to cell-selective (A) and ACTH-induced (B) gene expression. (A) Y1 adrenal and I-10 Leydig tumor cells were assayed for human growth hormone (hGH) production following transfection with plasmids containing 330 bp (p-330GH) or 156 bp (p-156GH) of *Cyp*-21 5′-flanking DNA ahead of the hGH reporter gene. Results were expressed as a percentage of the hGH level obtained from cells transfected with a reference plasmid to adjust for differences in transfection efficiencies between the two cell lines. (B) Y1 cells were transfected with p-330GH or p-156GH, incubated at 37°C for 40 h to allow for hGH expression and then incubated with or without ACTH (5 mU/ml). Aliquots were taken at the times indicated and assayed for hGH content. Figure 3(A) is reprinted from [22] by courtesy of the American Society for Biochemistry and Molecular Biology.

expression [Fig. 3(A and B)]. These results indicated that the sequences required for cell-selective and ACTH-induced expression of *Cyp*-21 resided within 330 bp of DNA flanking the structural gene and localized an important

regulatory element to the sequence from −330 to −156 relative to the transcriptional start site of *Cyp*-21 [22, 23].

Multiple regulatory elements are required for Cyp-21 expression

In order to identify those regulatory elements within the 330 bp of 5′-flanking DNA that were important for the regulated expression of the *Cyp*-21 gene, we first used DNase I footprint experiments to examine interactions between the DNA sequence and nuclear proteins from Y1 cells [24]. As shown in Fig. 4, nuclear extracts from Y1 adrenal cells generated multiple, closely spaced footprints within the 330 bp of 5′-flanking DNA. The footprint over the region from −110 to −150 resulted from two distinct DNA–protein interactions—one centered at −120 and one centered at −140. The DNA sequence at −120 contained a CCAAT motif and was competitively displaced by an oligonucleotide competitor containing a nuclear factor I consensus sequence. Therefore, it is likely that the sequence at −120 is a nuclear factor I



Footprint	Position	Sequence
-65	-74/-62	GCAAAGGTCAGAG
-120	-128/-113	CTGTGCCAATGTGAAA
-140	-150/-137	GATTCTCCAAGGCT
-170	-177/-162	GATGGTCCCATC
-210	-214/-203	AGAGAGGTCAGG
-230	-237/-226	AGTTAGGAAACT
-255	-259/-250	TCCGCCTACT
-280	-288/-277	TGGTGGGAGGGG

Fig. 4. Interactions between Y1 nuclear proteins and the *Cyp*-21 promoter region. DNase I footprinting experiments (A and B) were performed using a probe containing *Cyp*-21 sequences from −330 to +5 and 50 μg of Y1 nuclear extract (Y1) or bovine serum albumin (BSA). C, provides a summary of the protein–DNA interactions on the *Cyp*-21 promoter as well as the specific DNA sequences involved. Reprinted from [24] by courtesy of the American Society for Biochemistry and Molecular Biology.

binding site. Footprints were also observed over sites centered at −65 and −210, −230, −255 and −280 (Fig. 4). Interestingly, the sequences protected in the footprints at −210 and −65 each included the hexamer **5′-AGGTCA-3′** and resembled motifs associated with second messenger- and steroid hormone-induced gene expression [Fig. 5(A)]. A similar motif was noted within the sequence at −140. As will be discussed later in this paper, the sequences at −210, −65 and −140 interact with the same or related nuclear proteins and likely reflect similar transcription regulatory elements. The footprint at −280 spanned a sequence that matched in 8 out of 12 bp a putative cAMP-response element within the human *Cyp*-21 gene [Fig. 5(B)]; [25, 26]. Further inspection of the 330 bp of 5′-flanking DNA revealed a TATA sequence at −26 relative to the start of transcription [27], and a sequence (**5′-TTCTTCTTGA-3′**) from −185 to −176 that is conserved in the 21-hydroxylase genes of several species [21]. Figure 6 lists the complete nucleotide sequence of the *Cyp*-21 promoter from positions −330 to +1 relative to the transcriptional start site.

Two approaches were undertaken to test the functional importance of the various sequences within the *Cyp*-21 promoter identified by DNase I protection. First, each of these sequences was mutated within the context of the structural *Cyp*-21 gene plus 1.7 kb of 5′-flanking DNA and analyzed for expression of *Cyp*-21 transcripts following transfection into Y1 adrenal cells [24]. Mutation of each of the elements diminished expression of the *Cyp*-21 gene. Mutation of the sequence around position −255 had the least effect and diminished expression by approx. 50%. The other mutations each reduced expression from 80 to 100%. None of the mutations increased *Cyp*-21 gene expression, suggesting the absence of negative regulatory elements in this region of the promoter. In the second approach, selected elements were evaluated for their abilities to enhance the expression of p-$40_{11\beta\text{-OH}}$GH—a plasmid containing the structural gene encoding human growth hormone and the core promoter region from a mouse *Cyp*11b gene [28]. As shown in Fig. 7, single copies of the sequences centered at −65, −140, −170, −210 and −280 significantly enhanced the expression of the human growth hormone construct. When assayed individually, the apparent order of potency of the elements was $-170 \gg -280 > -140 > -65 \geqslant -210$.

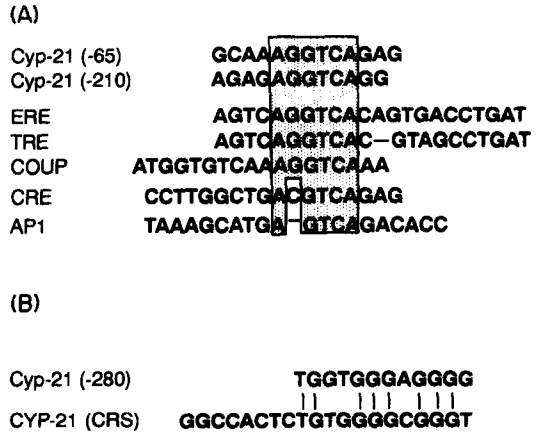


Fig. 5. Sequence comparisons of *Cyp*-21 promoter elements with elements involved in steroid hormone- and second messenger-induced gene expression. (A) The *Cyp*-21 elements at −65 and −210 are compared with an estrogen response element (ERE; [47]), a thyroid hormone response element (TRE; [48]), the chicken ovalbumin upstream promoter element (COUP; [35]), a cAMP-response element (CRE; [49]), and a phorbol ester-response AP1 binding site (AP1; [50]). (B) The *Cyp*-21 element at −280 is compared with a cAMP-response sequence (CRS) identified in the human *CYP*-21 promoter [25, 26].

Taken together, these results indicate that the *Cyp*-21 promoter comprises several closely spaced elements, each capable of enhancing transcription in Y1 adrenocortical tumor cells. Although the elements can function individually, their concerted actions seem to be required when they are aligned along the *Cyp*-21 promoter and further removed from the TATA box [24]. Similar interactions among modular regulatory elements have been observed previously in viral promoters [29] and may reflect a requirement for cooperative interactions.

ELEMENTS INVOLVED IN CELL-SELECTIVE EXPRESSION OF *Cyp*-21

Identification of potential elements based on DNase I footprinting and gel mobility shift assays

In order to identify promoter elements responsible for the adrenal cell-selective expression of *Cyp*-21, we first attempted to identify sequences in the *Cyp*-21 promoter that interacted specifically with proteins in nuclear extracts from adrenocortical cells. Towards this end, we examined the interactions of nuclear extracts from different cell lines with the *Cyp*-21 promoter. Nuclear extracts from steroidogenic and non-steroidogenic cell lines produced approximately equivalent interactions in DNase I protection assays with the elements at −120,

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-330  TCTAGAACAA  ATGCACTCCA CTCAGCTGGT CCAAGGGGAG GTATGGTGGG

-280  AGGGGCCGGC TCCAGTGGAC TCTCCGCCTA CTTGGCTGAA ACAGAGTTAG

-230  GAAACTGACT  GCGATACAGA GAGGTCAGGG TCTTGCATCC CTTCCTTTCT

-180  TCTTGATGGA  TGGTCCCATC TTTGATCCAC AGATTCTCCA AGGCTGATGG

-130  GGACTGTGCC  AATGTGAAAA CATACTGTTC TGTGTTGGGA ACAGGAAGGG

 -80  ACCTGAAGCA  AAGGTCAGAG CCACAGCAGA ACAAAGGACT GGAGTTGGGG

 -30  GCTATAAAAG  GCATATCAGG GCCCTCACAA  G
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Fig. 6. Nucleotide sequence of the *Cyp*-21 promoter. Nucleotide sequence is provided for the *Cyp*-21 promoter, from −330 to +1 relative to the transcriptional start site (arrow). This sequence has been submitted to geneBank/EMBL libraries under the accession number J05444.

−170, −230, −255 and −280 suggesting that these elements do not participate in cell-selective expression of *Cyp*-21. In contrast, the elements at −210, −140 and −65 produced prominent footprints with nuclear extracts from steroidogenic Y1 adrenal cells and MA10 testicular Leydig tumor cells but not with nuclear extracts from non-steroidogenic HeLa cells and PC12 adrenal medullary cells [24].

The interactions of nuclear proteins with the elements at −210, −140 and −65 were investigated further using a gel mobility shift assay. This assay provides an alternative method of analyzing DNA–protein interactions and can differentiate among different proteins that bind to the same sequence. As shown in Fig. 8, oligonucleotides corresponding to the elements at −210 and −65 produced similar shifted complexes when incubated with nuclear extracts from Y1 cells. One prominent complex (complex I) was seen with nuclear extracts from the steroidogenic Y1 and MA10 cells but not with nuclear extracts from the non-steroidogenic cell lines [24]. An oligonucleotide corresponding to the related element at −140 produced a single shifted complex with nuclear extracts from Y1 and MA10 cells and not with nuclear extracts from the non-steroidogenic cell lines (Rice and Parker, unpublished observations). This shifted complex, designated Complex I, had the same mobility as the cell-selective complexes obtained with the elements at −210 and −65 [Fig. 8(B)].

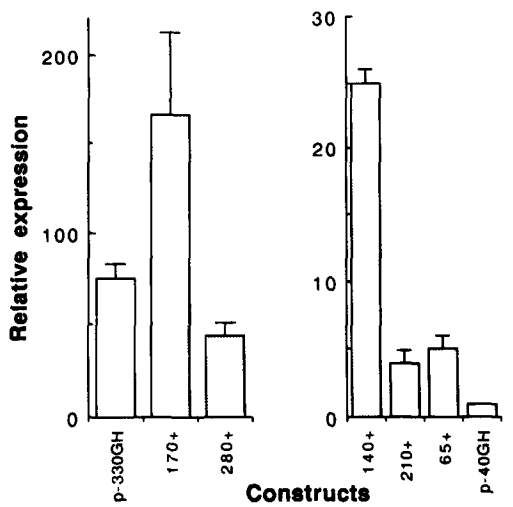

Fig. 7. *Cyp*-21 promoter elements enhance expression of a human growth hormone reporter gene. Plasmids containing single copies of the promoter elements centered at −65, −140, −170, −210 and −280 all in the correct orientation (+) were placed in the expression plasmid p-40$_{11\beta\text{-OH}}$GH ahead of the heterologous promoter, transfected into Y1 adrenal cells and analyzed for expression of growth hormone as described previously [22]. For comparisons, expression obtained using p-330GH and p-40$_{11\beta\text{-OH}}$GH (abbreviated p-40GH) also are included. Results are expressed as fold increases in growth hormone production over that obtained with p-40$_{11\beta\text{-OH}}$GH (B. P. Schimmer, A. Franklin and K. L. Parker, unpublished observations).

Based on the combined results of DNase I footprinting and gel mobility shift assays, the related elements at −210, −140 and −65 in the *Cyp*-21 promoter were the only elements identified that formed DNA–protein interactions specific to steroidogenic cell lines. We therefore suggest that these elements may contribute, at least in part, to the cell-selective expression of *Cyp*-21.

Effects of elements from the Cyp-21 promoter on cell-selective gene expression

In order to directly test the hypothesis that the elements containing the **AGGTCA**-like motif contributed to cell-selective expression of *Cyp*-21, we compared the ability of these elements to enhance the expression of a reporter gene in steroidogenic and non-steroidogenic cells [24].

Oligonucleotides corresponding to the promoter elements at −210 and −65 from the *Cyp*-21 gene were placed ahead of the thymidine kinase promoter and used to drive the

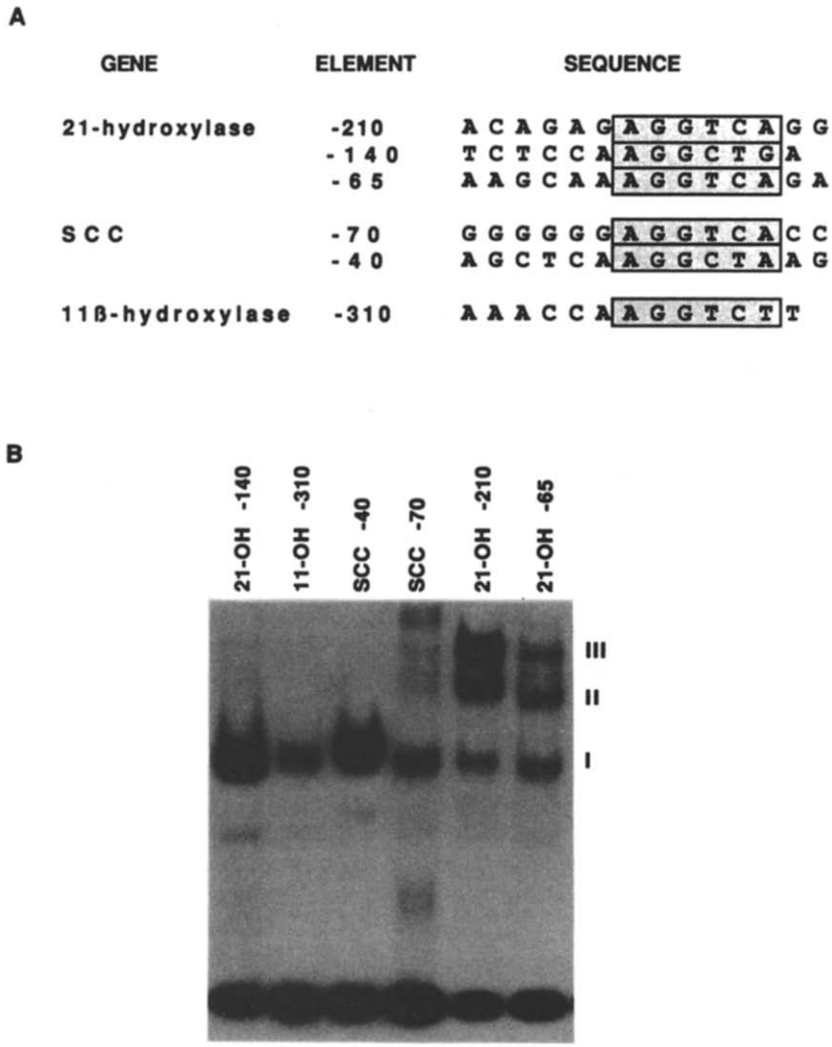

Fig. 8. Promoter elements from *Cyp*-21, *Cyp*11a, and *Cyp*11b have similar sequences and form similar complexes with adrenal nuclear extracts. (A) Sequences of related promoter elements from *Cyp*-21, *Cyp*11a, and *Cyp*11b that contain **AGGTCA**-like motifs (boxed) are shown. (B) Protein–DNA interactions between Y1 nuclear extracts and the elements indicated in (A) were examined in a gel mobility shift assay. Each element produced a shifted complex of similar mobility designated Complex I. Additional complexes, designated Complexes II and III, were seen only when certain of the elements were used as probes. Reprinted from [34] by courtesy of the Endocrine Society.

expression of the reporter gene, chloramphenicol acetyltransferase (CAT). As shown in Table 1, both elements significantly enhanced CAT expression over the level obtained with the thymidine kinase promoter alone. Neither element enhanced the activity of the CAT reporter gene in adrenal medullary PC12 cells, suggesting that these elements may contribute to the selective expression of *Cyp*-21 in cells of the adrenal cortex. We have not yet examined the activity of these elements in other steroidogenic cell lines like MA10 Leydig cells. The contribution of the related element at −140 in the *Cyp*-21 promoter to cell-selective expression also has not been examined; however, based on its similarity to the elements at −65 and −210 we predict that the −140 element would effect a similarly restricted pattern of expression.

Our finding that the −65, −140 and −210 elements exhibit similar interactions with nuclear proteins from Y1 adrenal cells and MA10 Leydig cells nonetheless raise some interesting questions about the extent of their contributions to cell-selective gene expression. One possibility is that the three elements confer selectivity to steroidogenic tissues and additional factors are required to restrict gene expression to adrenal cells. These additional factors might include other positive or negative control elements within the promoters, or selective *cis* modifi-

Table 1. Effects of *Cyp*-21 elements on expression of pTKCAT

Cell line	Plasmid	Expression (cpm/mg protein)	Stimulation (-fold)
Y1	PTKCAT	1599 ± 387	
	pTKCAT(−210)	5213 ± 33	3.3
	pTKCAT(−65)	36,300 ± 3800	22.7
PC12	pTKCAT	5860 ± 465	
	pTKCAT(−210)	4553 ± 758	0.8
	pTKCAT(−65)	6517 ± 674	1.1

Y1 mouse adrenocortical tumor cells and rat PC12 pheochromocytoma cells were transfected with plasmid DNA, and cell lysates were assayed for CAT activity. Results are expressed as cpm of chloramphenicol acetylated per mg protein ± SD ($n = 3$).
Reprinted from [24] with permission.

cations of the genes (e.g. methylation) in non-expressing tissues [19, 30]. Alternatively, the protein–DNA interactions observed with extracts from MA10 cells may differ from those seen using adrenal cell extracts and may not reflect transcriptionally important complexes. For example, the proteins from MA10 Leydig cells involved in these interactions may not be transcriptionally active, they may be different from those in Y1 adrenal cells, or they may be present in lesser abundance and have only marginal effects on transcription. In order to fully understand the contributions of these elements to the adrenal cell-selective expression of the steroidogenic *P*450 genes, it is clear that further characterization of these elements and the proteins with which they interact is required.

Shared promoter elements regulate the expression of three adrenal steroidogenic cytochrome P450s

In extensions of our studies on *Cyp*-21 expression, we isolated the promoter regions from the mouse *Cyp*11a and *Cyp*11b genes* and defined minimum promoter sequences required for constitutive and cAMP-induced expression in Y1 adrenocortical cells [28, 31–33]. DNase I footprinting experiments identified sequences in each promoter that interacted either solely or predominantly with nuclear extracts from steroidogenic Y1 adrenal and MA10 Leydig cells. In the *Cyp*11a promoter, these sequences were centered at −40 and −70; in the *Cyp*11b promoter, this sequence was centered at −310 [31, 33]. The importance of each of these elements in gene regulation has been demonstrated by mutational analysis [31, 33]. Furthermore, in direct experiments, the sequences at −70 in *Cyp*11a and at −310 in *Cyp*11b acted as enhancers when placed upstream of p-40$_{11\beta\text{-OH}}$GH (Table 2). Consistent with its role as an enhancer, the −310 element from *Cyp*11b enhanced the expression of p-40$_{11\beta\text{-OH}}$GH in either the correct or reverse orientation. Inspection of the sequences defined by these footprints suggested that they contained variations of the **AGGTCA** motif found in the *Cyp*-21 promoter (Fig. 8). The relationship of the elements in the *Cyp*11a and *Cyp*11b promoters to the **AGGTCA**-like sequences from *Cyp*-21 was further explored by comparing the gel mobility shift patterns formed by the various elements with nuclear extracts from Y1 adrenal cells. The gel shift patterns obtained (Fig. 8) fell into two classes. All six of the elements produced a prominent Complex I that migrated at approximately the same position in the gel. The −210 and −65 elements from *Cyp*-21 and the −70 element from *Cyp*11a also formed complexes of slower mobility (Fig. 8).

Oligonucleotide competition experiments, using the −140 and −210 elements from *Cyp*-21 as probes, suggested that the six **AGGTCA**-like elements from the *Cyp*-21, *Cyp*11a and *Cyp*11b promoters had similar protein binding specificities and likely were related [34]. In order to determine if the Complex I formed using the promoter elements from the three *Cyp* genes reflected interactions with the same protein, Y1 nuclear extracts were fractionated on CM-sepharose and the elution profiles of the DNA-binding proteins were compared. Using either the *Cyp*-21 −140 or −210 element as a probe, Complex I forming activity eluted in the same fractions. The Complex II forming activity associated with the −210 element had a distinct elution profile, while Complex III formation was only seen in those fractions capable of forming both Complex I and II. Elution profiles

Table 2. Effects of **AGGTCA**-like elements from *Cyp*11a and *Cyp*11b on gene expression

Plasmid	Expression (ng growth hormone/plate)	Stimulation (-fold)
p-40$_{11\beta\text{-OH}}$GH	2 ± 0.2	
P-40$_{11\beta\text{-OH}}$GH(−70+)	28 ± 0.6	14.0
P-40$_{11\beta\text{-OH}}$GH(−310+)	35 ± 1.1	17.5
P-40$_{11\beta\text{-OH}}$GH(−310−)	28 ± 1.3	14.0

The effects of the −70 element from *Cyp*11a and the −310 element from *Cyp*11b on expression of P-40$_{11\beta\text{-OH}}$GH in Y1 cells were evaluated in transient transfection assays. The activity of the −70 element was evaluated in the correct orientation (+); the activity of the −310 element was evaluated both in the correct (+) and in the reverse (−) orientation. Values for growth hormone production are expressed as means ± SEM ($n = 3$).
B. P. Schimmer, A. Franklin and K. L. Parker, unpublished observations.

*Recent observations [46] indicate that the mouse contains two closely related *Cyp*11b genes. The *Cyp*11b gene described herein directs the synthesis of aldosterone when transfected into COS cells and is most likely equivalent to the mouse aldosterone synthetase (*Cyp*11b2).

obtained using the −70 element from *Cyp*11a and the −310 element from *Cyp*11b as probes were essentially identical to the profiles obtained with the *Cyp*-21 −210 and −140 elements, respectively. Fractionation of Y1 nuclear extracts over Sephacryl S-300, phosphocellulose, and sequentially over heparin- and DEAE-sepharose failed to distinguish the Complex I forming activities or Complex II forming activities of the different probes [34].

While the identity of the nuclear factors responsible for Complex I formation are unknown, recent experiments suggest that the chicken ovalbumin upstream promoter transcription factor (COUP-TF) is involved in formation of complexes II and III (e.g. Fig. 8). COUP-TF is an orphan member of the nuclear hormone receptor family that recognizes a subset of promoter elements containing the **AGGTCA** motif [35]. Antibodies against COUP-TF, when added to the reaction of Y1 nuclear extracts with *Cyp* promoter elements, interfered with the formation of Complexes II and III without affecting Complex I [34].

Taken together, our results suggest that the regulatory elements bearing **AGGTCA**-like motifs in the *Cyp*-21, *Cyp*11a and the *Cyp*11b promoters interact with the same or highly related nuclear protein(s) to form Complex I, and that this complex may contribute to the cell-selective expression of these genes. Our results further suggest that Complex II results from the interaction of a subset of these elements with a different protein that is antigenically related to COUP-TF, and that Complex III forms as the result of an interaction with two DNA-binding proteins—the Complex I- and II-forming proteins. Several lines of evidence support this latter conclusion: (a) Complex III forms only in those chromatographic fractions that support both Complex I and II formation; (b) Complex III formation is inhibited by the oligonucleotide competitors that do not themselves form this complex, but do form Complex I; and (c) Complex III formation, like Complex II formation, is affected by an antiCOUP-TF antibody. Whereas the protein responsible for Complex I formation is implicated in cell-selective gene expression, the importance of COUP-TF or a related protein in regulating adrenal steroidogenic enzyme synthesis remains to be defined. Inasmuch as COUP-TF is a member of the nuclear hormone receptor family, it is attractive to speculate that steroids might modulate the activity of COUP-TF and thus auto-regulate the expression of the steroidogenic enzymes. Alternatively, the involvement of COUP-TF might reflect the participation of a second messenger-dependent regulatory pathway [36].

cAMP-DEPENDENT ELEMENTS IN *Cyp*-21

An intact cAMP-dependent protein kinase is required for the constitutive expression of Cyp-21

The role of cAMP as a second messenger in ACTH-stimulated transcription of *Cyp*-21 is well documented [1, 2]. Inasmuch as most effects of cAMP involve activation of cAMP-dependent protein kinase, it seems likely that ACTH and cAMP stimulate transcription of *Cyp*-21 through an event requiring protein phosphorylation. Studies from our laboratories, however, demonstrated that cAMP and cAMP-dependent protein kinase are also required for the basal expression of *Cyp*-21. As shown in Fig. 1, Y1 cells transfected with a cosmid clone containing genomic DNA encoding *Cyp*-21 constitutively expressed large amounts of *Cyp*-21 mRNA and *Cyp*-21 expression was further increased by ACTH. When similar experiments were carried out using a Y1 mutant harboring a cAMP-resistant protein kinase (clone Kin-8) [37], both constitutive and ACTH-induced expression of *Cyp*-21 were markedly reduced. This requirement for cAMP-dependent protein kinase activity in constitutive gene expression was also observed with the p-330GH construct. Transfection of Y1 cells with p-330GH led to a 40-fold enhancement of gene expression. In contrast, gene expression from this construct was 85% less efficient in the mutant, Kin-8 and approached background levels (Fig. 9). The requirement for cAMP-dependent protein kinase in constitutive gene expression must be at the level of gene transcription, since this requirement could be demonstrated both with genomic *Cyp*-21 DNA and with constructs containing a reporter gene driven by *Cyp*-21 5′-flanking DNA. In the same experiments, constucts containing the human growth hormone gene driven by the mouse metallothionein 1 promoter [22] were expressed equally well in Y1 and Kin-8 mutants cells indicating a degree of specificity of the effect of the protein kinase mutation on the expression of steroidogenic cytochrome *P*450 genes. Similar requirements for a functional cAMP-dependent protein kinase in constitutive

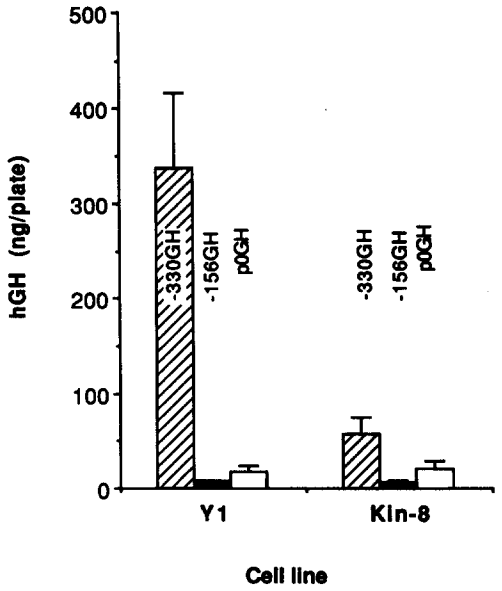

Fig. 9. *Cyp*-21 promoter activity in protein kinase-defective Kin-8 mutants. Y1 and mutant Kin-8 cells were transfected with the promoterless expression plasmid, pOGH or with plasmids containing either 330 bp (p-330GH) or 156 bp (p-156GH) of *Cyp*-21 5′-flanking DNA ahead of the promoterless reporter gene. Culture medium was assayed for hGH production 48 h after transfection. Results are expressed as means ± SEM ($n = 5$). Reprinted from [22] by courtesy of the American Society for Biochemistry and Molecular Biology.

gene expression have been observed for a *Cyp*11b gene, and to a lesser extent the *Cyp*11a gene [12, 31]. These results suggest that specific elements within the *Cyp*-21 promoter enhance constitutive expression of the *Cyp*-21 gene in a manner strictly dependent upon cAMP-dependent protein phosphorylation. As a working hypothesis, we further suggest that these same elements participate in the induction of *Cyp*-21 expression by ACTH via its second messenger, cAMP.

A consensus cAMP-response element (CRE) is not involved in ACTH and cAMP actions on the Cyp-21 promoter

Despite their similarity to the CRE consensus, the *Cyp*-21 elements at −65 and −140 and −210 clearly do not function as classical CREs. The footprint interactions on the *Cyp*-21 promoter at −65, −140 and −210 obtained with Y1 nuclear extracts were not displaced with synthetic oligonucleotides encoding bona-fide CREs [24]. In the reciprocal experiment, footprint interactions with a bone-fide CRE were not displaced by synthetic oligonucleotides corresponding to the sequences at −65 and −210 in the *Cyp*-21 promoter [28]. Furthermore, other consensus cAMP-responsive elements such as AP1 or AP2 were not detected within the *Cyp*-21 promoter [24], indicating that novel cAMP-response elements are involved in *Cyp*-21 expression. Similarly, novel cAMP-responsive elements appear to be involved in ACTH- and cAMP-stimulated expression of the human *CYP*-21 gene [25], the mouse and bovine *CYP*11A genes [31, 38] and the bovine 17α-hydroxylase (*CYP*-17) gene [39] in adrenocortical cells. The mechanisms involved in ACTH- and cAMP-stimulated expression of *Cyp*-21, *CYP*11A and *CYP*-17 contrast sharply with the mechanisms involved in the regulated expression of the mouse *Cyp*11b gene. For the mouse *Cyp*11b gene, the experimental evidence clearly points to a consensus cAMP-response element containing the specific sequence **5′-TGACGTGA-3′** being responsible for cAMP-stimulated gene expression [28, 32].

Identification of cAMP-dependent elements within the Cyp-21 promoter

In an attempt to identify more specifically the promoter regions responsible for cAMP-dependent expression of *Cyp*-21, the ability of selected elements from the *Cyp*-21 promoter to confer cAMP-inducible expression to p-$40_{11\beta\text{-OH}}$GH was examined in the Y1 cell line. The sequences analyzed included the related elements at −65, −140 and −210, the potent regulatory element at −170 and the sequence centered at −280 that resembled the cAMP-response sequence of the human *CYP*-21 gene [25]. The results of these studies are summarized in Table 3. Whereas the construct containing the 330 bp of 5′-flanking DNA from the *Cyp*-21 gene rendered the expression of the human growth hormone gene responsive to

Table 3. cAMP-dependent elements in the *Cyp*-21 promoter

Plasmid	Induction by 8-bromo-cAMP	Expression in Kin-8
p-330GH	+ + +	↓↓↓
P-$40_{11\beta\text{-OH}}$GH(−280)	−	−
P-$40_{11\beta\text{-OH}}$GH(−210)	−	↓
P-$40_{11\beta\text{-OH}}$GH(−170)	−	↓↓↓↓
P-$40_{11\beta\text{-OH}}$GH(−140)	−	↓↓
P-$40_{11\beta\text{-OH}}$GH(−65)	−	↓

Parental Y1 cells and mutant Kin-8 cells were transfected with the expression plasmid P-$40_{11\beta\text{-OH}}$GH containing the *Cyp*-21 elements at −280, −210, −170, −140 and −65 ahead of the heterologous promoter or with p-330GH (an expression plasmid driven by 330 bp from the *Cyp*-21 promoter). For induction experiments, cells were incubated with or without 3 mM 8-bromo-cAMP and analyzed for growth hormone production. Results are expressed qualitatively—relative fold induction of gene expression by 8-bromo-cAMP (+); relative inhibition of gene expression in Kin-8 (↓); no difference from the untreated Y1 control (−).

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cAMP, none of the individual elements tested (either in single or multiple copies) were capable of rendering the reporter gene responsive to the cyclic nucleotide. Our failure to detect a cAMP-responsive sequence in this manner might indicate: (a) that another element on the *Cyp*-21 promoter is primarily responsible for cAMP-regulated gene expression, (b) that a combination of elements is required, (c) that the element must be placed an appropriate distance from the promoter, or (d) that cAMP responsiveness from that *Cyp*-21 cAMP-response element is not supported by the *Cyp* 11b early promoter.

As an alternative strategy to identify the elements responsible for cAMP-dependent expression of *Cyp*-21, we compared the abilities of the various *Cyp*-21 elements to enhance gene expression in parental Y1 cells and in the protein kinase-defective Y1 mutant, Kin-8. This alternate strategy was based on our earlier finding that expression of *Cyp*-21 was markedly dependent upon the integrity of the cAMP-dependent protein kinase [17, 22]. The Kin-8 mutant arose as the result of a single amino acid change in the cAMP-binding domain of the regulatory subunit of the type 1 cAMP-dependent protein kinase isozyme [40]. As a consequence of this mutation the regulatory subunit exerts a dominant inhibitory effect on the protein kinase catalytic subunit and renders the cell line resistant to ACTH and cAMP [37, 41, 42]. Using this mutant, we have identified two different elements that were significantly dependent upon an intact cAMP-dependent protein kinase for enhancer function—i.e. the element at −170 and the variations of **5′-AGGTCA-3′** found at −65, −140 and −210 (Table 3). The degree of dependence of the various elements on cAMP-dependent protein kinase activity paralleled their enhancing potential—i.e. $-170 \gg -140 > -210 \geqslant -65$. The element at −280 functioned equally well in parental Y1 cells and in the Kin-8 mutant, and thus showed no requirement for a functional cAMP-dependent protein kinase. On the basis of these observations we suggest that the requirement for a functional cAMP-dependent protein kinase in the constitutive expression of *Cyp*-21 is targeted to the element at −170, and to the related elements at −140, −210 and −65. Furthermore, it is likely that these same elements, perhaps in combination, participate in the ACTH- and cAMP-induced expression of *Cyp*-21. This latter possibility is currently under investigation.

SUMMARY AND PROSPECTS

This review has examined the molecular basis for the regulated expression of the mouse *Cyp*-21 gene in Y1 adrenocortical tumor cells; a study that has been greatly facilitated by the availability of the homologous, ACTH-responsive, steroid secreting Y1 cell line and its mutant derivatives. Using the Y1 adrenal cell system, we have demonstrated that 330 bp of 5′-flanking DNA from the *Cyp*-21 gene is sufficient for cell-selective and ACTH-induced expression of *Cyp*-21, and that this promoter region comprises multiple, closely spaced enhancer elements each of which is required for promoter function. Within this promoter, we have identified three related elements that contain variations of an **AGGTCA** motif and that contribute to the expression of *Cyp*-21 in Y1 adrenocortical tumor cells. The extent of their cell-selectivity, however, has not yet been fully evaluated and requires further investigation. Our finding that these elements are recognized by nuclear extracts from steroidogenic adrenal and Leydig cell lines raises the possibility that they restrict gene expression to steroidogenic cell types; it follows, then, that additional factors may be required for further restriction of gene expression to cells of the adrenal cortex. Of further interest is our finding that variations of these same **AGGTCA**-bearing elements are involved in the expression of *Cyp* 11a and *Cyp* 11b in Y1 adrenocortical cells. These latter observations suggest that shared elements contribute to the adrenal cell-selective expression of at least three steroidogenic cytochrome *P*450 genes in the mouse and possibly account, in part, for the coordinate expression of these genes.

The elements responsible for ACTH- and cAMP-regulated expression of *Cyp*-21 have been more elusive. None of the elements identified within the *Cyp*-21 promoter correspond to previously defined consensus cAMP-response elements and none of the elements individually are capable of conferring cAMP-responsiveness to a reporter gene driven by a heterologous promoter. The protein kinase-defective mutant, Kin-8, provided a novel system to identify elements responsible for cAMP-dependent expression of *Cyp*-21. We demonstrated that gene expression from the *Cyp*-21 promoter requires an intact cAMP-dependent protein kinase, and that two groups of elements contribute to this cAMP-dependence—i.e. the **AGGTCA**-like elements implicated in cell-selective expression of

Cyp-21 and a second element at −170 in the *Cyp*-21 promoter. As a working hypothesis, we suggest that these same elements, possibly acting in combination, are required for ACTH- and cAMP-induced gene expression. It is interesting that these cAMP-dependent elements bear no resemblance to the sequences implicated in cAMP-induced expression of the human *CYP*-21 gene ([25] and see the review by Waterman *et al.* in this volume for further discussion of the cAMP-responsive sequences of the bovine *CYP*17 and *CYP*11A and human *CYP*11B genes). On the other hand, these two groups of elements resemble a pair of elements (designated AD3 and AD4) required for cAMP-induced expression of the bovine *CYP*11A gene [43].

Most of the studies dealing with expression of *Cyp*-21 have dealt with identification of the *cis*-acting elements responsible for cell-selective and ACTH-induced gene expression. DNase I protection and gel mobility shift assays indicate that these elements interact with specific proteins some of which are restricted in their tissue distribution and some of which resemble the transcription factor, COUP-TF. In order to further understand the molecular basis for regulated expression of *Cyp*-21, these DNA-binding proteins will have to be isolated, and their contributions to transcription determined in direct, functional experiments. Towards these ends, Zanger *et al.* [44] have partially purified a protein from Y1 cells that interacts with a cAMP-response sequence from the bovine *CYP*-17 gene and Rice and Parker [34] have partially purified a protein from Y1 cells that interacts with the **AGGTCA**-like motifs in the *Cyp*-21 promoter.

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