cAMP-dependent Transcription of the Human CYP21B (P-450c21)
Gene Requires a Cis-regulatory Element Distinct from the Consensus cAMP-regulatory Element*

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By utilizing chimeric genes constructed from 5'-flanking sequences of the human CYP21B (P-450c21) gene and reporter genes (chloramphenicol acetyltransferase or rabbit β-globin), a 34-nucleotide sequence has been found to be required for cAMP-dependent transcription. This sequence (−129/−96 base pairs) shows no homology to that of the consensus (CRE) cAMP-regulatory element. Gel retardation analysis shows that a protein-DNA complex is formed between this DNA sequence and nuclear proteins from mouse adrenal Y1 tumor cells or bovine adrenal cortical cells or human fetal adrenal tissue and that formation of this complex cannot be competed by DNA containing the consensus CRE sequence. Even though cAMP-enhanced accumulation of P-450c21 mRNA in primary cultures of bovine adrenocortical cells is inhibited by the protein synthesis inhibitor, cycloheximide, reporter gene transcription enhanced by the cAMP-responsive −129/−96 base pair fragment of the human CYP21B gene is not. We conclude that cAMP-dependent transcription of the human P-450c21 gene (CYP21B), an event required for maintenance of optimal steroidogenic capacity in the adrenal cortex, involves a stable transcription factor(s) distinct from the CRE-binding protein. Furthermore the cAMP-dependent cis-regulatory element of the human P-450c21 gene is distinct from those found associated with the other steroid hydroxylase genes, 11α-hydroxylase cytochrome P-450, cholesterol side chain cleavage cytochrome P-450, and 11β-hydroxylase cytochrome P-450, suggesting that each of these genes may require its own set of specific transcription factors for cAMP-dependent regulation.

Steroid 21-hydroxylase (P-450c21) is a member of the cytochrome P-450 superfamily of genes and appears to be expressed exclusively in the adrenal cortex, thereby playing an essential role in both glucocorticoid and mineralocorticoid synthesis. In humans and other species investigated to date, there are two closely related CYP21 genes (Nebert et al., 1989), one being the functional gene and the other being a pseudogene. These genes are located on human chromosome 6 (White et al., 1985), and upon their isolation and characterization (Higashi et al., 1986; White et al., 1986) it has been shown that human gene CYP21B encodes the active enzyme while human gene CYP21A is the pseudogene. In addition to the tissue-specific regulation of human CYP21B which appears to limit its expression to the adrenal cortex, its expression in cattle (and presumably humans) is regulated transcriptionally by adrenocorticotropic via CAMP (John et al., 1986a). This mechanism maintains optimal levels of 21-hydroxylase activity in the adrenal cortex and, consequently, optimal capacity for synthesis of glucocorticoids and mineralocorticoids. Furthermore, the cAMP-dependent increase in P-450c21 mRNA has been shown to require on-going protein synthesis in primary cultures of bovine adrenal cortical cells and to require several hours to become manifest following initiation of treatment (John et al., 1986b).

In the mouse it is found that Cyp21A is the functional gene and Cyp21B is the pseudogene (Chaplin et al., 1986). Parker, Schimmer, and colleagues have carried out detailed studies to identify the cis-regulatory elements associated with the mouse Cyp21A gene which are required for its transcription (Handler et al., 1989). These studies have shown that both tissue-specific and hormone-inducible expression of the mouse Cyp21A gene require sequences between −330 bp in the 5'-flanking region and the promoter. Deletion of sequences between −230- and −180-bp abolishes constitutive expression of this gene (Parker et al., 1986), while deletion of sequences between −330 and −156 bp abolishes cAMP-enhanced transcription (Handler et al., 1988). The precise sequences within these regions which are required for constitutive and cAMP-dependent transcription have not yet been defined. In the present study we have investigated the 5'-flanking region of the human CYP21B gene and have located a cis-regulatory element, 34 bp in length, containing the sequences required for its cAMP-dependent transcription. This sequence is unrelated to the consensus cAMP-regulatory element (CRE) (Montmery et al., 1986) and formation of protein-DNA complexes using this sequence (−129/−96) cannot be competed by DNA containing the consensus CRE. Apparently, then, P-450c21 gene expression is regulated by a trans-acting factor distinct from that (CREB) which binds to the CRE.

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The abbreviations used are: P-450c21, steroid 21-hydroxylase cytochrome P-450; P-450c, cholesterol side chain cleavage cytochrome P-450, P-450c21, 17α-hydroxylase cytochrome P-400; P-450c21, 11β-hydroxylase cytochrome P-450; CRE, cAMP-regulatory element; bp, base pairs; nt, nucleotides; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; GPT, xanthine-guanine phosphoribosyltransferase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
cAMP-regulatory Sequence in the Human P-450c21 Gene

MATERIALS AND METHODS

Restriction endonucleases were purchased from Bethesda Research Laboratories and New England Biolabs and used according to the manufacturers' instructions. Poly(A) mRNA was prepared according to Saiki et al. (1988) using a Perkin-Elmer/Cetus thermal cycler. All enzymatic manipulations were done according to standard procedures (Maniatis et al., 1982). All newly made plasmid constructs were confirmed by dideoxy chain-termination sequence analysis (Sanger et al., 1977).

Plasmids—The published numbering system of the 5'-flanking sequences of the human CYP21B gene begins with +1 being the first base to the 5'-side of the ATG initiation of translation (Higashi et al., 1986; White et al., 1986), while for the bovine P-450c21 gene (Chung et al., 1988) and the mouse CYP21A gene (Chaplin et al., 1986, 1988) it begins with the first nucleotide to the 5'-side of the initiation of transcription. Since the site of initiation of transcription of the human CYP21B gene is known to be nine nucleotides 5' from the ATG (Higashi et al., 1986, in this paper we have used the convention of numbering the first nucleotide to the 5'-side of this site of initiation of transcription as +1, and thus the numbering of the 5'-flanking sequences of the human, bovine, and mouse genes cited herein all begin at the same relative position.

The 5'-flanking region of the human CYP21B gene spanning from -1889 bp (BgiII) to -10 bp (ApaI) was used for construction of chimeric reporter genes to assess the biological significance of potential regulatory elements in its upstream region. This fragment was digested with BgiII and ApaI, gel purified from a C. Whitehead Un. Med. Coll. (New York, NY) (White et al., 1986). The BgiII/ApaI fragment was isolated, blunt-ended with T<sub>4</sub> polynu- merase, ligated with HindIII linkers, digested with HindIII, and inserted into the HindIII site of BSCAT, containing the bacterial chloramphenicol acetyltransferase (CAT) gene between the HindIII and BamHI sites of Bluescript (Strategene), thereby producing 1.7 CAT21. A series of plasmids containing different lengths of the CYP21B 5'-flanking sequence fused to the CAT gene were made from 1.7 CAT21, using the XhoI site in Bluescript and restriction sites contained within the CYP21B 5'-flanking sequence following blunt-ending with T<sub>4</sub> polynu- merase and ligating with T<sub>4</sub> ligase to produce 1.1 CAT21 (SpI), 380CAT21 (StuI), and 100CAT21 (KpnI). Plasmids in which the CAT gene was fused to the SV40 early promoter, SV2CAT (Gorman et al., 1982a) or to the 3' long terminal repeat of Rous sarcoma virus, R5VCAT (Gorman et al., 1982b), were used as transcription controls.

The plasmids OVEC and SV40-OVEC were kind gifts from Drs. Thomas Gerster and Walter Schaffner (University of Zurich) (Westin et al., 1987). The 3× CRE, which contains three tandemly repeated cAMP-responsive elements of the human chorionic gonado- tropin-α gene fused to the OVEC ß-globin sequences, was made by Johan Lund (Karolinska Institute, Huddinge, Sweden) as described previously (Lund et al., 1990). The fragment -93 bp to +108 bp was generated by digesting 1.7 CAT21 with KpnI, ligating the fragment into the KpnI site of pUC19, digesting the resulting plasmid with SalI and SacI, and cloning this fragment into the SacI/SalI sites of OVEC. The resulting fragment -840/-10 and -612/-10 bp, were synthesized and ligated with SalI and XhoI and was digested with KpnI and religated to yield the plasmid which contained the -210/-10 bp sequence of P-450c21. The fragment -210/-96 bp generated from this plasmid by digesting with KpnI was cloned into the KpnI site of pUC19 to yield 210pUC21. The fragment -250/-96 bp derived from p250BS21 by digestion with KpnI was digested with PstI or HindIII and the resulting fragments, -250/-180 and -250/-10 bp, were cloned between the KpnI and Smal site of pUC19 to yield 250/ 180pUC21, and 250/159pUC21, respectively. The plasmid which was digested with HindIII followed by digestion with SalI-31 and religated to yield 250/201pUC21. All these pUC constructs, 840pUC21, 612pUC21, 388pUC21, 250pUC21, 232pUC21, 210pUC21, 250/201pUC21, 250/180pUC21, and 250/159pUC21 were digested with SalI and SacI and the resulting fragments were cloned into the KpnI sites of OVEC vector to yield 840V21, 612V21, 388V21, 250V21, 232V21, 210V21, 250/201V21, 250/180V21, and 250/ 159V21, respectively.

Two complementary oligonucleotides containing the CYP19B1 se- quence -250/-225 bp, having an XhoI site at the 5'-end and a SacI site at the 3'-end were synthesized, annealed, ligated to make multi- copy fragments, and digested with SalI and XhoI to yield tandemly repeated multiplicity fragments. The resulting fragments were cloned into the SacI and the plasmids which contained a single or tandemly duplicated -250/-225 bp fragments were selected and digested with SacI and SalI. The resulting fragments were cloned into the KpnI site of OVEC, giving 1×250, 2×250, 3×250, 4×250, and 5×250, respectively. The 210bp fragment was used as described above to yield 1×210V21 and 2×210V21. The fragment -129/-30 bp was synthesized by polymer- erase chain reaction using specific oligonucleotides having an XhoI site at 5'-end and SacI site at 3'-end and was digested with KpnI and SacI. The resulting fragment of -230/-225 bp was cloned into the KpnI site and SacI site of pUC19 to yield 100/300V21. This was then cloned into OVEC following SacI/SalI digestion to produce 100/ 300V21.

Cell Culture and Transfection—The mouse adrenocortical tumor cell line Y1 (Yasumura et al., 1980) was cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal cell serum. Primary cultures of bovine adrenocortical cells were prepared as described previously (Gospodarowicz et al., 1973). The acetylated products of [14C]chloramphenicol (Amersham Corp.) were analyzed on thin layer chromatography plates (PE SIL G, Whatman). After autoradiog- phy the spots were excised, and the amount of radioactivity was determined by liquid scintillation counting. The plasmid RSV-GPT in which the 3'-long terminal repeat of the RSV was fused to the GPT gene was cotransfected with the CAT constructs to normalize the transection efficiencies using the GPT assay (Chu and Berg, 1986).
shift analysis were either synthetic double-stranded oligonucleotides or fragments generated by restriction digestion. Such fragments were labeled with \( \gamma^{-32} \)P]ATP using T<sub>4</sub> polynucleotide kinase or with [\( \alpha^{-32} \)P]dCTP using Klenow fragment. Gel shift analysis was performed using 10 \( \mu \)g of nuclear protein, 4 \( \mu \)g of poly(dI-dC), and 2000–5000 cpm \( ^3P \)-labeled DNA fragment in 20 mM Hepes (pH 7.8), 80 mM KCl, 4 mM MgCl\(_2\), 500 \( \mu \)g/ml yeast tRNA, and 4% Ficoll. The protein-DNA complexes were analyzed on a pre-electrophoresed 5% polyacrylamide gel with 0.5 x Tris-buffered saline (Maniatis et al., 1982) KCl. 4 mM MgCl\(_2\), 500 \( \mu \)g/ml yeast tRNA, and 4% Ficoll. The protein-

**RESULTS**

**Transient Expression of Human P-450\(_{c21}\) Reporter Gene Fusions**—In order to investigate the transcriptional regulation of the P-450\(_{c21}\) gene, we constructed two series of plasmids, OVEC and CAT. In experiments utilizing the OVEC constructs, the rabbit \( \beta \)-globin TATA box (promoter) was used to initiate transcription. All of the CAT constructs contain P-450\(_{c21}\) sequences beginning from -10 bp at 3' end in order to utilize the TATA box (promoter) of the P-450\(_{c21}\) gene. Mouse adrenal cortical tumor Y1 cells were transfected with both the OVEC and CAT plasmids, and cells were incubated with 25 \( \mu \)M forskolin which raises intracellular cAMP levels (Seamon et al., 1981). When the plasmid 1.3OV21 was transfected into Y1 cells, a 4-fold increase in correctly initiated globin transcripts is observed upon forskolin treatment (Fig. 1). Transfection with the CRE-containing OVEC plasmid leads to a 13-fold increase in correctly initiated transcripts upon forskolin treatment. From cells transfected with OVEC (OV, containing only the minimal globin promoter) very low levels of protected correctly initiated transcripts are observed, both in the presence and absence of forskolin treatment. The plasmid SV40-OVEC in which the SV40 enhancer is inserted upstream of the minimal globin promoter (Westin et al., 1987) was used as a positive transfection control, and this plasmid yields high levels of globin transcripts both in the presence and absence of forskolin treatment. A 6-h treatment with forskolin was shown to be optimal for cAMP-enhanced transcription directed by the CYP21B 5'-flanking region (data not shown) just as shown previously for that directed by the 5'-flanking region of the bovine P-450\(_{ sarc}\) gene (Ahlgren et al., 1990).

**Identification of Regulatory Elements between -1697 and -30 bp of the CYP21B Sequences**—The location of the cAMP-regulatory region(s) was identified using a series of plasmids deleted from the 5' end as described under "Materials and Methods." When the sequences from -1305 to -389 bp are removed from 1.3OV21, the level of expression of the globin gene induced with forskolin was reduced gradually as was the basal level of expression, although the fold stimulation by forskolin was maintained at approximately the same level (Fig. 2). The plasmid 250OV21 in which the sequences from -1305 to -250 bp were deleted from 1.3OV21 gives the highest levels of both basal and induced expression without any significant change of the stimulation ratio. These results suggest that there may be one or more weak negative transcriptional regulatory sequences between the positions -1305 to -250 bp. When an additional 18 bp are removed from 250OV21, producing 323OV21, the expression of the globin gene is reduced substantially both in the absence and presence of forskolin although moderate levels of expression remain and the fold stimulation by forskolin remains unchanged. This substantial change in both the basal and cAMP-enhanced expression indicates that the deletion from -250 to -232 bp removes sequences which enhance human CYP21B expression.

When the plasmid 129OV21 containing the P-450\(_{c21}\) sequences from -129 to -96 bp is transfected into Y1 cells, the expression upon forskolin treatment again increases, while the basal expression does not show a significant increase. The resulting stimulation ratio, therefore, is increased up to 10-fold. This observation locates a cAMP-responsive cis-regulatory element of the human CYP21B gene between -129 and -96 bp. The plasmid 100/300OV21 containing the P-450\(_{c21}\) sequences from -100 to -30 bp fused to \( \beta \)-globin gene yields levels of expression as low as those with OVEC alone which only contains a minimal globin promoter. No enhancement of transcription by forskolin treatment is observed with 100/300OV21 indicating that the major cAMP-responsive element in this gene is located between -129 and -96 bp.

In the experiments involving transient transfection of the OVEC constructs, the effects of P-450\(_{c21}\) sequences to enhance transcription by the globin promoter are analyzed by measuring the expression of globin RNA using an S1 nuclease protection assay. In order to compare the results using the globin promoter with results using the P-450\(_{c21}\) promoter, we...
also investigated the transient transfection of CAT constructs in which CYP21B sequences containing P-450cZ1, TATA box are fused to the CAT gene. After transfection, cells were treated with 25 μM forskolin for 14 h, and the CAT enzymatic activities of the cell lysates were analyzed. As shown in Table I, a series of plasmids containing the P-450cZ1-CAT fusion genes gives a similar pattern of cAMP enhancement as does the series of OVEC constructs. Sequences required for cAMP-induced CAT activity are found to be located between −389 and −100 bp. In contrast to the results from the series of OVEC constructs, all of the basal activities of the CAT constructs are as low as that of BSCAT which was used as a negative control. This difference in basal expression between the OVEC constructs and the CAT constructs may arise from the different sensitivities of the assay methods or from the fact that they utilize different promoters.

From this series of experiments, the presence of two short sequences in the 5′-flanking region of the human P-450cZ1 which serve as positive regulatory elements of expression is suggested. Because removal of sequences between −250 and −232 bp from 250OV21 yields a significant decrease of expression in the presence and absence of forskolin treatment, it is suggested that the sequence between −250 to −232 bp includes a positive regulatory element. This element may only be involved in basal expression since the fold increase observed upon forskolin treatment is not changed between 250OV21 and 232OV21. On the other hand, comparison of the results obtained using the plasmids 129OV21 and 100/30OV21 suggests the presence of a cAMP-responsive cis-acting element within the sequence from −129 to −96 bp.

Nevertheless, since the removal of the sequence between −250 and −232 from 250OV21 results in significant decreases of expression in the presence and absence of forskolin treatment, we then tested whether this region alone might confer a cAMP responsiveness to a globin promoter. We constructed the plasmids 1X250/225OV21 and 2X250/225OV21, containing one copy of the −250/−225-bp sequence and two tandemly repeated copies of this sequence, respectively, upstream of the globin promoter. The plasmids 1X129/96OV21 and 2X129/96OV21 which contain one copy and two tandemly repeated copies of the −129/−96 bp sequences, respectively, were also tested. As can be seen in Fig. 2, the plasmids 1X250/225OV21 and 2X250/225OV21 do not yield any significant increase on the expression of globin gene upon forskolin treatment. Unexpectedly, the basal level of expression in the presence of these sequences was the same as with the OVEC plasmid and considerably less than with 250OV21. In order to see whether the sequence from −250 to −225 bp might not contain a complete cAMP-responsive sequence, the plasmids 250/201OV21, 250/180OV21 and 250/159OV21 which contain the sequences from −250 to −201, −250 to −180, and −250 to −159 bp, respectively, but not the cAMP-responsive sequence between −129/−96, were tested. These plasmids also yield a background level of expression and no significant increase upon treatment with forskolin (data not shown). These results suggest that the sequence from −250 to −232 functions in a positive fashion on basal transcriptional regulation only in the presence of the cAMP-responsive element −129/−96. The plasmid 2X129/96OV21 gives higher levels of globin gene expression than does the plasmid 1X129/96OV21. The 2X129/96OV21 construct was also tested in primary cultures of human fetal adrenal cells. As seen in Fig. 2, forskolin treatment dramatically enhances correctly initiated globin transcripts from this construct in human fetal adrenal cells, just as it does in mouse Y1 tumor cells and primary bovine adrenocortical cells (data not shown).

The Effect of Cycloheximide Treatment in Transient Transfection—Cycloheximide, an inhibitor of translation, has been shown to inhibit the adrenocorticotropic-induction accumulation of bovine P-450cZ1 RNA in bovine adrenocortical cells in primary culture (John et al., 1986b). As noted above, the two sequences −250/−232 and −129/−96 bp are suspected to confer basal expression and cAMP responsiveness, respectively, to the human CYP21B gene. We therefore examined the effect of cycloheximide on the transient expression of the globin gene in Y1 cells transfected with 250OV21 or 129OV21. The increased expression of globin transcripts upon forskolin treatment is not inhibited by cycloheximide in Y1 cells transfected with either plasmid but rather was moderately increased (Fig. 3). Treatment with cycloheximide alone also moderately increases the basal expression of the globin gene.

**Table I**

| Plasmid   | Relative activity | Fold induction |
|-----------|------------------|----------------|
| 1.7CAT21  | 1.1              | 7.2            |
| 1.1CAT21  | 1.0              | 4.5            |
| 888CAT21  | 1.1              | 5.9            |
| 100CAT21  | 1.0              | 1.1            |
| BSCAT     | 0.9              | 0.9            |

**FIG. 3.** Effect of cycloheximide on transient globin gene expression. Y1 cells were transfected with 1 × 129/96OV21 and 250OV21 and incubated for 6 h without any treatment or with 25 μM forskolin or 40 μM cycloheximide or with 25 μM forskolin and 40 μM cycloheximide. S1 nuclease protection analysis was performed on the cytoplasmic RNA isolated from transfected cells, i.e., incorrectly initiated transcripts; c.i., correctly initiated transcripts.
Forskolin-Y1 nuclear proteins binding to a labeled fragment containing the -129/-96 bp region. The complex formation between a sequence containing -129/-96 (data not shown). We have also observed that -250/-225 does not inhibit protein binding to transcriptional regulation by CAMP. The sequence from -129 to -96 bp of the P-450_{c21} gene shows no apparent similarity to the CRE consensus sequence, tested whether an unlabeled DNA fragment containing the CRE of the human n-chorionic gonadotropin gene (Deutsch et al., 1987) or the CAMP-responsive -243/-225-bp fragment would compete for binding of nuclear proteins to this sequence (Lund et al., 1990). The P-450_{c21} -243/-225-bp sequence shows no apparent similarity to the sequence -129/-96 bp of human P-450_{c21}, and as seen in Fig. 4B it does not inhibit the DNA-protein complex formation of this sequence of human P-450_{c21}. Thus, the CAMP-dependent transcription of the P-450_{c21} gene is regulated by a trans-acting protein factor which is different from that of the other adrenocortical microsomal steroid hydroxylase, P-450_{c11}.

The sequence within -129/-96 bp of the human CYP21B gene is somewhat conserved at approximately the same position in the mouse and bovine genes (Fig. 5). From gel shift assays, the DNA-protein complexes formed between this sequence and nuclear extracts from mouse Y1 cells, bovine adrenocortical cells, and human fetal adrenal tissue show about the same mobility (Fig. 4A). Also, the 5'-flanking sequence of the human P-450_{c21} gene confers increased expression to a reporter gene in primary cultures of both bovine adrenal cells and human fetal adrenal cells, as well as in mouse Y1 cells. Thus, it can be suggested that this region of the P-450_{c21} gene is involved in CAMP responsiveness in mouse, bovine, and human adrenals. Studies by Parker, Schimmer, and colleagues have indicated that the sequence required for adrenocorticotropic (CAMP)-dependent enhanced transcription resides between -330/-156 bp of the mouse P-450_{c21} gene (Hindler et al., 1988; Handler et al., 1989), although the precise location of the CAMP-responsive element within this sequence remains to be elucidated. These results argue against the mouse sequence in Fig. 5, which is related to the -129/-96, being the CAMP-responsive element in the mouse Cyp21A gene. Therefore, differences may exist between the human CYP21B and the mouse Cyp21A gene, with respect to CAMP-dependent regulation. It must be noted, however, that the CAMP-responsive element in the human gene (<129/-96 bp) has been localized by analysis of RNA transcripts using a heterologous promoter while localization

DISCUSSION

In this paper we report the sequences within the 5'-flanking region of the human CYP21B gene which are involved in its transcriptional regulation by cAMP. The sequence from -129 to -96 bp of the P-450_{c21} gene can confer increased CAMP responsiveness to a reporter gene. This sequence, 5'-GGCCACCTCTGGGCGGCTCGGTGGGAGGTACC-3', shows no apparent similarity to the CRE consensus sequence, TGACGTCA (Montminy et al., 1986; Short et al., 1986) or the AP-2-binding sequence, CCACCAGGC, (Imagawa et al., 1987; Roessler et al., 1988). A series of experiments by Parker, Schimmer, and their colleagues examining the expression of the mouse Cyp21A gene also indicates the absence of known cAMP-responsive sequences in the 5'-flanking region of the functional P-450_{c21} gene in this species (Parker et al., 1986; Chaplin et al., 1986; Handler et al., 1988). Additionally, the consensus CRE sequence does not inhibit the DNA-protein complex formation between the sequence -129/-96 bp of P-450_{c21} and nuclear extracts from Y1 cells (Fig. 4B). Thus, it is concluded that the P-450_{c21} gene is regulated by a mechanism involving transcription factors which are different from those required for genes involving consensus CRE sequences. However, it should be noted that the sequence -243/-225 bp which confers CAMP responsiveness to the bovine P-450_{c11} gene is also without any apparent similarity to the consensus CRE sequence, yet the CRE from the human alpha-chlorionic gonadotropin gene competes binding of nuclear proteins to this sequence (Lund et al., 1990). The P-450_{c11} -243/-225-bp sequence shows no apparent similarity to the sequence -129/-96 bp of human P-450_{c11}, and as seen in Fig. 4B it does not inhibit the DNA-protein complex formation of this sequence of human P-450_{c11}. Thus, the CAMP-dependent transcription of the P-450_{c21} gene is regulated by a trans-acting protein factor which is different from that of the other adrenocortical microsomal steroid hydroxylase, P-450_{c11}.

The sequence within -129/-96 bp of the human CYP21B gene is somewhat conserved at approximately the same position in the mouse and bovine genes (Fig. 5). From gel shift assays, the DNA-protein complexes formed between this sequence and nuclear extracts from mouse Y1 cells, bovine adrenocortical cells, and human fetal adrenal tissue show about the same mobility (Fig. 4A). Also, the 5'-flanking sequence of the human P-450_{c21} gene confers increased expression to a reporter gene in primary cultures of both bovine adrenal cells and human fetal adrenal cells, as well as in mouse Y1 cells. Thus, it can be suggested that this region of the P-450_{c21} gene is involved in CAMP responsiveness in mouse, bovine, and human adrenals. Studies by Parker, Schimmer, and colleagues have indicated that the sequence required for adrenocorticotropic (CAMP)-dependent enhanced transcription resides between -330/-156 bp of the mouse P-450_{c21} gene (Hindler et al., 1988; Handler et al., 1989), although the precise location of the CAMP-responsive element within this sequence remains to be elucidated. These results argue against the mouse sequence in Fig. 5, which is related to the -129/-96, being the CAMP-responsive element in the mouse Cyp21A gene. Therefore, differences may exist between the human CYP21B and the mouse Cyp21A gene, with respect to CAMP-dependent regulation. It must be noted, however, that the CAMP-responsive element in the human gene (-129/-96 bp) has been localized by analysis of RNA transcripts using a heterologous promoter while localization

**Fig. 4.** Nuclear protein binding to the sequences within -250/-225 and -129/-96 bp of the human CYP21B gene. Double-stranded DNA fragments containing the regions -250/-225 and -129/-96 bp were labeled with 32P. In panel A, the 32P-labeled fragments were incubated with nuclear extracts (10 μg/reaction mixture) from Y1 cells, bovine primary adrenocortical cells in culture (BAC), or human fetal adrenal tissue (HFA). In panel B, nuclear extracts from Y1 cells without forskolin treatment were incubated with the 32P-labeled -129/-96 bp fragment in the presence of various amounts of unlabeled fragments containing the CRE, the -129/-96 bp of human P-450_{c21} gene, or -243/-225 bp of the bovine P-450_{c21} gene.

Also that the protein factors are conserved among these three different species.

Since the sequences between -129/-96 bp of the P-450_{c21} gene confer CAMP responsiveness to the globin promoter we tested whether an unlabeled DNA fragment containing the CRE of the human alpha-chlorionic gonadotropin gene (Deutsch et al., 1987) or the CAMP-responsive -243/-225-bp fragment of bovine P-450_{c21} gene (Hindler et al., 1988) would compete for Y1 nuclear proteins binding to a labeled fragment containing the -129/-96 bp region. The complex formation between a protein factor and the -129/-96-bp fragment was not inhibited by either of these sequences even though 200-fold excess amounts of unlabeled fragments were used (Fig. 4B). Also we have observed that -250/-225 does not inhibit protein binding to -129/-96 (data not shown).

**Fig. 5.** The corresponding regions in the bovine (Chung et al., 1986) and mouse (Chaplin et al., 1986) P-450_{c21} genes having greatest homology to the CAMP-responsive sequence -129 to -96 of the human P-450_{c21} gene. The dashes indicate identical bases, and the asterisks indicate gaps.
of the region of the mouse gene containing the cAMP-responsive element (−230/−156 bp) involved use of the homologous promoter and measurement of protein products (growth hormone or CAT). Also, constitutive expression of the human CYP21B gene and the mouse Cyp21A is clearly different when comparing results of Parker et al. (1986) and those herein. Deletion of sequences between −230 and −100 bp in the mouse Cyp21A gene dramatically reduced constitutive expression and suggested the presence of an enhancer for constitutive expression within this sequence. The results in Fig. 4 show no evidence for such a sequence in the human CYP21B gene. Finally, Parker et al. (1986) demonstrated the presence of a 40 bp highly conserved sequence in the 5′-flanking region of the P-450c17 gene among different species (mouse, bovine, and human). This sequence is located between −231 and −191 bp in the human P-450c17 gene (nucleotide numbering system used herein) and is not found to be required for either basal or cAMP-dependent expression in the present study. Thus, there appear to be differences in regulatory elements associated with both basal and cAMP-dependent expression between the human and mouse P-450c17 genes which cannot be explained simply by the fact that experiments have been carried out in different laboratories. At present we can only conclude that the sequence −129/−96 bp of the human CYP21B gene contains within it a cAMP-responsive element which serves to enhance transcription of a reporter gene in human, bovine, and mouse adrenal cells. How this element relates to similar elements in the mouse and bovine P-450c17 genes is not clear at this time. The sequence −129/−96 in the human P-450c17 gene contains within it an almost perfect Sp1-binding site (G/TGCGCGGG/AG/AC/T; Kadonaga et al., 1986), and the corresponding sequence in the bovine P-450c17 gene contains a perfect Sp1-binding site. However, the relationship between an Sp1-binding site and cAMP-dependent transcription is not yet apparent.

Although the sequence −250/−225 bp in human CYP21B forms a DNA-protein complex with nuclear extracts from Y1 and bovine adrenocortical cells, and human fetal adrenal tissue, the function of this sequence remains unknown. Deletion of this sequence from 250OV21 reduces the basal expression dramatically. However, the plasmids 1x250/225OV21 and 2x250/225OV21 show neither elevated basal expression nor CAMP responsiveness suggesting that this sequence requires the other cis-elements to function. As shown in Fig. 2, the plasmid 250OV21 produces maximum expression both in the presence and absence of forskolin treatment, while 232OV21 and 210OV21 produce lower levels of cAMP-dependent expression than does 129OV21 which contains only the sequence −129/−96 bp of P-450c17. Perhaps there is a modest negative regulatory element with respect to cAMP-responsive transcription located between −232 and −129 bp. If so, we could speculate that the protein factor(s) binding to the sequence −250/−225 bp release the negative regulatory effect located between −232 and −129 bp. Clearly, further investigation is required to elucidate the function of the sequence between −250 and −232 bp in transcription of the human CYP21B gene.

In addition to the P-450c17 gene, transcription of the P-450c17 gene, the P-450c18 gene, the P-450c19 gene, and the adrenodoxin gene is responsive to cAMP (John et al., 1986a). The mouse P-450c18 gene has been found to be regulated by a modified CRE, TGACGTGA (Mouw et al., 1989; Rice et al., 1989). The bovine P-450c18 gene is found to contain two cAMP-responsive sequences, neither of which is homologous to the other nor to the consensus CRE (Lund et al., 1990). Within a 100-bp sequence (−185/−80 bp) of the bovine P-450c18 gene a cAMP-responsive sequence is also found (Ahlgren et al., 1990). There is some sequence relatedness between a segment within this 100 bp region of the P-450c18 gene and the −213/−225 bp sequence of the P-450c19 gene, but it is not yet known whether this region of homology is required for cAMP-dependent P-450c18 expression. No consensus CRE is found in the P-450c18 gene (−183/−85 bp sequence). We now report another cAMP-responsive sequence, this from the human P-450c18 gene (−125/−96 bp), which does not contain a consensus CRE and is apparently not related to the other cAMP-responsive sequences associated with the steroid hydroxylase genes. It is becoming evident that each steroid hydroxylase gene contains its own set of cAMP-responsive sequences, and presumably, there exist a number of different transcription factors, each conferring cAMP responsiveness to a particular sequence element on a particular steroid hydroxylase gene.

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REFERENCES

Ahlgren, R., Simpson, E. R., Waterman, M. R., and Lund, J. (1990) J. Biol. Chem. 265, 3313–3319

Chaplin, D. D., Galbraith, L., Seidman, J. G., White, P. C., and Parker, K. L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9601–9605

Chu, G., and Berg, P. (1986) Nucleic Acids Res. 13, 2921–2930

Chung, B., Matteson, K. J., and Miller, W. L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4243–4247

Deutsch, P. J., Jameson, J. L., and Habener, J. F. (1987) J. Biol. Chem. 262, 12169–12174

Dgnam, J. D., Lehovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489

Funkenstein, B., McCarthy, J. L., Dus, K. M., Simpson, E. R., and Waterman, M. R. (1983) J. Biol. Chem. 258, 5938–9405

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982a) Mol. Cell. Biol. 2, 1044–1051

Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I., and Howard, B. H. (1982b) Proc. Natl. Acad. Sci. U. S. A. 79, 6777–6781

Gospodarowicz, D., Ill, C. R., Hornsby, P. R., and Gill, G. N. (1977) Endocrinology 100, 1080–1089

Graham, F. L., and van der Eb, A. J. (1973) Virology 52, 456–467

Handler, J. D., Schimmer, B. P., Flynn, T. R., Styf, M., Seidman, J. G., and Parker, K. L. (1988) J. Biol. Chem. 263, 13065–13073

Handler, J. D., Schimmer, B. P., Flynn, T. R., Styf, M., Rice, D. A., and Parker, K. L. (1989) Endocrine Res. 15, 31–47

Higashi, Y., Yashiohka, H., Yamane, M., Gotoh, O., and Fujii-Kuriyama, Y. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2841–2845

Imagawa, M., Chiu, R., and Karin, M. (1987) Cell 51, 251–260

John, M. E., John, M. C., Boggaram, V., Simpson, E. R., and Waterman, M. R. (1986a) Proc. Natl. Acad. Sci. U. S. A. 83, 4715–4719

John, M. E., Okamura, T., Dee, A., Adler, B., John, M. C., White, P. C., Simpson, R. F., and Waterman, M. R. (1986b) Biochemistry 25, 2846–2853

Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) Trends Biochem. Sci. 11, 20–23

Lund, J., Ahlgren, R., Wu, D., Kagimoto, M., Simpson, E. R., and Waterman, M. R. (1990) J. Biol. Chem. 265, 3304–3312

Maniatis, T., Fritsh, E. F., and Sambrook, J. F. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Masou, J. I., Rainey, W. E., and Carr, B. R. (1985) in Lipoprotein and Cholesterol Metabolism in Steroidogenic Tissues (Strauss, J. F., and Menon, M. J., eds) pp. 45–46, G. F. Stickley Co., Philadelphia

McManus, J. T., Fritsh, E. F., and Sambrook, J. F. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Mouw, A. R., Rice, D. A., Meade, J. C., Chua, S. C., White, P. C., Schimmer, B. P., and Parker, K. L. (1989) J. Biol. Chem. 264, 1305–1309
CAMP-regulatory Sequence in the Human P-450<sub>C11</sub> Gene

Neubert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Guzmán, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., and Waterman, M. R. (1989) DNA 8, 1-13

Parker, K. L., Schimmer, B. P., Chaplin, D. D., and Seidman, J. G. (1986) J. Biol. Chem. 261, 15353-15355

Rice, D. A., Aitken, L. D., Vandenbark, G. R., Mouw, A. R., Franklin, A., Schimmer, B. P., and Parker, K. L. (1989) J. Biol. Chem. 264, 14011-14015

Roeeler, W. J., Vandenbark, G. R., and Hanson, R. W. (1988) J. Biol. Chem. 263, 9063-9066

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Science 239, 487-491

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467

Seamon, K. B., Padgett, W., and Daly, J. W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3363-3367

Short, J. M., Wynshaw-Boris, A., Short, H. P., and Hanson, R. W. (1986) J. Biol. Chem. 261, 9721-9726

Westin, G., Gerster, T., Muller, M. M., Schaffner, G., and Schaffner, W. (1987) Nucleic Acids Res. 15, 6787-6797

White, P. C., Grossberger, D., Onuffer, B. J., Chaplin, D. D., New, M. I., Dupont, B., and Strominger, J. L. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1099-1093

White, P. C., New, M. I., and Dupont, B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5111-5115

Yasumura, Y., Buonassisi, V., and Sato, G. (1988) Cancer Res. 48, 529-535
cAMP-dependent transcription of the human CYP21B (P-450C21) gene requires a cis-regulatory element distinct from the consensus cAMP-regulatory element.

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