Characterization of the Promoter/Regulatory Region of the Bovine CYP11A (P-450sec) Gene

BASAL AND CAMP-DEPENDENT EXPRESSION*

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The biosynthesis of steroid hormones in the adrenal cortex

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The promoter/regulatory region of the bovine CYP11A (P-450sec) gene was cloned from a bovine genomic library. One major start site of transcription was identified by primer extension analysis with a minor start site four nucleotides further upstream. A putative TATA box is located at position -31, and at position -68 resides a putative binding site for the transcription factor Sp1. Transient transfection of chimeric reporter gene constructs into mouse adrenal tumor Y1 cells was used to locate regions within the P-450sec 5'-flanking sequences that are important for basal and CAMP-dependent transcription of the reporter genes. While CAMP-dependent accumulation of mRNA derived from expression of the endogenous bovine P-450sec gene can be inhibited by protein synthesis inhibitors, transcription of reporter gene constructs containing the promoter/regulatory region of the P-450sec gene is not affected by cycloheximide following transient transfection of Y1 cells or primary bovine adrenocortical cells. Basal expression of these constructs as well as CAMP responsiveness is reduced upon deletion of sequences between -183 and -101, further deletion to -50 leading to loss of virtually all the remaining CAMP responsiveness. The sequence between -183 and -83 alone will direct both basal and CAMP-enhanced transcription when fused to a heterologous promoter and is equally active in either the correct or reverse orientation. No homology to the consensus CAMP-responsive element (CRE) or AP-2 binding site is found in this region whereas an activator protein 1-like sequence is found at position -116. It is concluded that the CAMP responsiveness of P-450sec gene expression is mediated by sequences different from canonical consensus regulatory elements. Whether or not there are sequences conferring CAMP responsiveness which are common both to P-450sec and the other steroidogenic P-450 genes remains to be established.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) J05245.

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* The abbreviations used are: ACTH, adrenocorticotropin; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; bp, base pairs; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)l-piperazineethanesulfonic acid; BAC, bovine adrenocortical; CRE, CAMP regulatory element; AP, activator protein.

1 F-450sec is the product of the CYP11A gene, F-450sec is the product of the CYP11B gene, P-450c11a is the product of the CYP11B gene, P-450c17 is the product of the CYP17 gene (Nebert et al., 1989).

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1987. Investigation of the cis-regulatory elements involved in cAMP-dependent expression of the murine CYP21 (P-450_{Ri}) and CYP11B (P-450_{11B}) genes indicates no such consensus sequences involved in P-450_{Ri} gene expression (Handler et al., 1988) and a functional CRE involved in P-450_{11B} gene expression (Mouw et al., 1989). In the present study, we have examined the 5'-flanking sequence of the bovine P-450_{mc} gene for regions required for both basal and cAMP-dependent transcription.

The complete structure of the human P-450_{mc} gene has been determined (Morohashi et al., 1987), and a 5.4-kilobase fragment of the 5'-flanking region of this gene coupled to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene has been used in transfection studies in mouse adrenal Y1 tumor cells. Treatment of such transfected cells with cAMP leads to an S-fold increase in CAT activity (Inoue et al., 1987). Investigation of the cis-regulatory elements involved

Cell Culture and Transient DNA Transfections—Mouse Y1 adrenocortical tumor cells (Yasumura et al., 1986) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and antibiotics. On the day before transfection, confluent dishes (100 mm) were split (1:6 each). The next morning the medium was changed, and 4 h later the DNA was added to the cells by the calcium-phosphate method (Graham and van der Eb, 1973) using 10 µg of plasmid/100-mm dish. After 4 h of exposure to the DNA precultures, the cells were glycerol-shocked (15% glycerol, 140 mM NaCl, 25 mM HEPES, 0.75 mM NaHPO_{4}) for 3 min at room temperature. The medium was changed the morning after transfection, and the cells were maintained in the presence or absence of 25 µM forskolin for the indicated time periods. Bac cells were prepared and maintained as described (Gospodarowicz et al., 1977), and their transfection was carried out using the same procedure.

CAT Assays—Cell lysates were prepared at 10-50 µg and CAT assays executed as described (Gorman et al., 1982). Cell lysate (50 µg) was incubated with [3H]chloramphenicol (Amersham Corp.) and 0.4 mM acetyl coenzyme A (Sigma) for 30 min at 37°C. The reaction mixtures were extracted with ethyl acetate and analyzed by thin layer chromatography using a 95%/5% chloroform/methanol mobile phase. Following autoradiography the acetylated products were excised and counted in a liquid scintillation counter. Protein concentrations in the cell lysates were determined by the BCA assay (Pierce Chemical Co.) with bovine serum albumin as standard.

RNA Isolation and S1 Analysis—Cells were harvested using trypsin/EDTA and pelleted at 150 × g for 5 min. The cells were then washed with phosphate-buffered saline (PBS) once, and the cytoplasmic RNA was then isolated essentially as described by Westin et al. (1987). Briefly, the cell pellet from the PBS wash was resuspended in 1 ml of PBS and centrifuged for 10 s in a microcentrifuge. The resulting cellular debris was washed three times with 0.4 M lysis buffer (10 mM Tris-HCl, 140 mM NaCl, 1.5 mM MgCl_{2}, 0.5% Nonidet P-40, pH 8.6) and placed on ice for 5 min. The nuclei were removed by centrifugation for 5 min in a microcentrifuge, and the supernatant was treated for 30 min with 40 µl of 10% SDS and 10 µl of protease K (20 µg/µl) at 37°C. The samples were then extracted once with phenol/chloroform/isooamyl alcohol (25:24:1), once with chloroform/isooamyl alcohol (24:1), and the RNA was precipitated with ethanol.

Hybridization of 10-50 µg of cytoplasmic RNA was performed as described with a single stranded oligonucleotide probe extending between positions -18 and +75 on the noncoding strand in the rabbit β-globin gene followed by S1 nuclease analysis (Westin et al., 1987). Samples were analyzed on 10% polyacrylamide, 7.5 M urea gels. The gels were fixed in 10% trichloroacetic acid, dried, and subjected to autoradiography. After autoradiography the bands corresponding to correctly initiated transcripts were cut out from the gels, and the amount of radioactivity was determined by liquid scintillation counting.

RESULTS

Cloning and Nucleotide Sequence Determination of the 5'-End of the Bovine P-450_{mc} Gene—The promoter/regulatory region of the bovine P-450_{mc} gene was located in a genomic library screened with a 358-bp PvuII-BamHI fragment of the bovine P-450_{mc} cDNA. This fragment represents the 5'-end of the cDNA (Morohashi et al., 1984). One positive genomic clone was found in the initial screening, and this clone was plaque-purified and characterized by restriction analysis and Southern blotting. The clone contained a 4.2-kb fragment of

EXPERIMENTAL PROCEDURES

All enzymatic manipulations were done according to standard procedures (Maniatis et al., 1982). Restriction endonucleases were purchased from Bethesda Research Laboratories.

Cloning and Sequencing of the 5'-Flanking Region of the Bovine P-450_{mc} Gene—A bovine genomic library was constructed by digesting calf thymus DNA (Boehringer Mannheim) with EcoRI. The digested DNA was ligated with XhoI WES arms (Bethesda Research Laboratories), packaged in vitro (Packagene, Promega), and propagated in transient transfection of mouse adrenal Y1 cells, a 100-bp region within this 5'-flanking sequence has been found (Chung et al., 1989). In the present study we have elucidated the sequence of the 5'-end of the bovine P-450_{mc} gene including identification of the site of initiation of transcription and have coupled the 5'-flanking region to both the rabbit β-globin and CAT reporter genes. By deletion analysis and transient transfection of mouse adrenal tumor Y1 cells, a 100-bp region within this 5'-flanking sequence has been found which is required for both basal and cAMP-dependent transcription. Even though the P-450_{mc} gene is a member of the same P-450 gene family as the P-450_{11B} gene, no consensus CRE is found in the P-450_{mc} gene.
the P-450y gene, and the partial sequence of this fragment is shown in Fig. 1. Contained within this sequence is the first exon of the bovine P-450y gene, thus verifying its identity. Also contained within the sequence in Fig. 1 are 250 bp of the exon of the bovine P-450y gene, thus verifying its identity. Shown in Fig. 1. Contained within this sequence is the first intron and 900 bp of the 5′-flanking region. One nucleotide is different in the genomic sequence of the first exon compared with the cDNA, the last nucleotide in residue 9 being a C in the cDNA (Morohashi et al., 1984) and a T in the genomic sequence (Fig. 1), a change which does not alter the amino acid composition.

**Determination of the Start Site of Transcription**—To locate the start site of transcription, a primer extension assay was employed utilizing a 32P-end-labeled synthetic oligonucleotide (27-mer) located 22 nucleotides downstream from the initiation codon (underlined in Fig. 1). The extended products were analyzed on a denaturing polyacrylamide gel, and one major start site of transcription was identified (Fig. 2) with an additional minor site four nucleotides upstream. This site is different in the genomic sequence as indicated by an asterisk.

**Consensus Binding Sites for Spl, AP-1, and OTF-1** are shown in bovine. The nucleotide sequence of the first exon has several interesting features. Upstream of the start site of transcription is a sequence TGAGTCT, identical at 6 out of 7 bases to the consensus binding site for the transcription factor Spl (Briggs et al., 1986). At position -261 located 66 bp upstream of the start site of transcription an octamer transcription factor binding site (Landolfi et al., 1986) is located. Also, relative to the start site of transcription an octamer transcription factor binding site (TGAGTCA) is found at position -116. In addition, the nucleotide sequence of the 5′-flanking region of the bovine P-450y gene was searched for several known consensus binding sites for other transcription factors (Jones et al., 1988). At position -261 relative to the start site of transcription an octamer transcription factor binding site (Landolfi et al., 1986) is located.

**Fig. 2. Site of initiation of transcription of the bovine P-450y gene.** Total RNA from either fetal bovine adrenocortical cells cultured in the presence of 1 µM ACTH, (lane 1) or from adult bovine adrenal cortex (lane 2) was hybridized to a 32P-end-labeled synthetic oligonucleotide (underlined in Fig. 1) and extended with reverse transcriptase. A sequence ladder obtained using the same primer is presented. The arrows indicate the major site of initiation of transcription and a minor site four nucleotides upstream. Also indicated in the figure is a putative TATA box.

**Fig. 1. Nucleotide sequence of the promoter/regulatory region, first exon, and part of the first intron of the bovine P-450y gene.** The nucleotide sequence was determined as described under “Experimental Procedures.” Underlined are primers used for primer extension, PCR, and sequencing. The major start site of transcription is indicated as +1. A putative TATA box and the consensus binding sites for SP1, OTF-1, and AP-1 are shown in boxes. The nucleotide in exon 1 that is different in the genomic sequence as compared with the cDNA is indicated with an asterisk.
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promoter and structural gene, illustrating that these constructs utilize transient transfections. A, an outline of OVEC constructs where P-450₄₀α (Westin et al., 1987). In the plasmid -896/CATscc expression initiated from the P-450₄₀α, putative TATA box and start site of transcription. The P-450₄₀α, sequences have been fused to the rabbit β-globin minimal promoter is measured transcripts to be distinguished (93 nucleotides, and 88 respectively) (Westin et al., 1987). In the plasmid -896/CATscc (Fig. 3B), P-450₄₀α sequences between -896 and +12 are inserted upstream of the CAT structural gene, and the CAT expression initiated from the P-450₄₀α promoter is measured enzymatically in lysates of transfected cells.

**Transient Expression of Bovine P-450₄₀α Fusion Genes in Y₁ Cells**—After transfection of the mouse adrenal tumor cell line Y₁ with -896/OVscc, correctly initiated β-globin transcripts are observed in cytoplasmic RNA from cells maintained in the absence or presence of 25 μM forskolin, which elevates intracellular levels of cAMP (Seamon et al., 1981). The time course of globin expression is shown in Fig. 4, A and B. Cells treated with forskolin for 1.5 h show an increased level of β-globin transcripts when compared with control cells, and the cAMP-stimulated globin expression increases with time up to 6 h of treatment when maximal expression is reached (9-fold stimulation). Longer treatment results in a decrease in cAMP-stimulated transcription, and at 24 h of treatment with 25 μM forskolin only a 1.5-fold stimulation is observed. The basal globin expression also increases over time and reaches a maximum level at 6 h but remains at this level at 12 and 24 h of culture (Fig. 4, A and B).

When OVEC containing only the minimal β-globin promoter is transfected into Y₁ cells essentially nondetectable levels of transcripts are obtained from control cells, with a slight increase upon treatment with 25 μM forskolin for 6 h occasionally being observed (Fig. 7). As a positive control for transfection we used a plasmid containing the SV40 enhancer inserted upstream of the minimal β-globin promoter (Westin et al., 1987). This plasmid gives high levels of β-globin transcripts in Y₁ cells both in the presence and absence of 25 μM forskolin (Fig. 7).

The plasmid -896/CATscc, which contains the homologous TATA box and start site of transcription, was also transfected into Y₁ cells followed by maintenance of the cells in the absence or presence of 25 μM forskolin for 24 h (Fig. 5). This plasmid gives both basal and cAMP-induced CAT activity with a 2.9-fold increase upon treatment. When a plasmid containing only the CAT structural gene is transfected into Y₁ cells, very low CAT activity is observed in both control and treated cells (Fig. 5). A plasmid with the Rous sarcoma virus promoter coupled to the CAT gene yields high CAT activity in both control cells and forskolin-treated cells, with a slight increase in CAT activity in the treated cells (Fig. 5).

**Effect of Cycloheximide in Transient Transfection of -896/OVscc**—Previous studies have shown that the increased level of P-450₄₀α mRNA observed in BAC cells treated with CAMP or ACTH is inhibited by cycloheximide (John et al., 1986a). We therefore examined whether cycloheximide had the same effect on cells transiently transfected with -896/OVscc. Primary BAC cells and Y₁ cells transfected with -896/OVscc
Fig. 5. Transient expression of CAT constructs in Y1 cells. Y1 cells were transfected with the reporter plasmids -896/CATcco, RSV-CAT, and pCAT. The cells were maintained in the presence (+) or absence (-) of 25 μM forskolin for 24 h. The level of CAT expression was determined enzymatically in cell lysates.

Fig. 6. Effect of cycloheximide on reporter gene expression upon transfection of -896/OVscc into Y1 cells and primary bovine adrenocortical cells. The plasmid -896/OVscc was transfected into Y1 cells and BAC cells. The cells were treated for 6 h in the presence or absence of 25 μM forskolin and concomitantly with or without 40 μM cycloheximide (CHX). S1 nuclease mapping was performed on the cytoplasmic RNA.

Fig. 7. Effect of 5'-deletions of P-450<sub>sec</sub> sequences on relative β-globin expression. The 5'-deletions of -896/OVscc were transiently expressed in Y1 cells which were maintained in the presence (+, solid bars) or absence (-, open bars) of 25 μM forskolin for 6 h. The cells were harvested, and the relative expression of globin was determined by S1 nuclease mapping on cytoplasmic RNA. A, the autoradiogram from one experiment (ci, correctly initiated transcripts; ii, incorrectly initiated transcripts). B, the histogram shows the mean ± S.D. of three transfections except for [-183/-83]OVscc and the [-83/-183]OVscc data which represent the mean of two transfections. The relative expression of -896/OVscc in the absence of forskolin is set arbitrarily as 1.00.
Fig. 8. Effect of 5′ deletions of P-45017α sequences on relative CAT expression. Y1 cells were transfected with the 5′ deletions of −896/CATcc and maintained in the presence (solid bars) or absence (open bars) of 25 μM forskolin for 24 h. The cells were then harvested, and the relative expression of CAT was determined enzymatically in cell lysates. The relative expression of −896/CAT in the absence of forskolin treatment is set arbitrarily as 1.0. The histogram shows the mean and standard deviations from five transfections except for −50/CATcc data which represent the mean of two transfections.

450α sequences between −183 and −83 bp fused in the correct or reverse orientation to the minimal β-globin promoter were prepared (−183/−83)OVsc and (−83/−183)OVsc, respectively) to test whether these sequences were sufficient to give basal and cAMP-enhanced expression in Y1 cells. The results of transfections with these constructs are shown in Fig. 7. When the plasmid (−183/−83)OVsc is transfected into Y1 cells and the cells are treated with 25 μM forskolin for 6 h, essentially the same level of β-globin expression as that obtained with the −186/OVsc construct is observed. The orientation of the region between −183 and −83 bp seems to have no effect on cAMP-enhanced transcription since the plasmid (−83/−183)OVsc yields the same high level of expression upon forskolin treatment as does (−183/−83)OVsc. This suggests that in the region between −183 and −83 bp in the P-450α gene is located an element that can confer cAMP responsiveness to a heterologous promoter independent of its orientation, one of the characteristics of an enhancer.

**Discussion**

In the present study we have shown that the bovine P-450α promoter/regulatory region between −896 and −32 fused to a minimal β-globin reporter gene promoter and structural gene can direct basal and cAMP-stimulated expression in transiently transfected Y1 cells. By creating a series of deletion constructs, a region between −186 and −101 bp has been identified as being important for basal expression. Deletion of the region between −186 and −101 bp also reduces cAMP-dependent expression, although a 9-fold increase in response to cAMP remains. By deletion to −50 bp, cAMP-dependent expression is essentially lost. Perhaps two cAMP-responsive regions are present between −186 and −50 bp or alternatively −101 bp is very close to a single cAMP-responsive element such that deletion to this point leads to partial reduction of cAMP responsiveness. Furthermore we have shown that sequences between −183 and −83 bp of the 5′-flanking region of the P-450α gene can confer cAMP responsiveness when coupled to the heterologous β-globin promoter in either the correct or reverse orientation.

Accumulation of steroid hydroxylase mRNA (including P-450α mRNA) in primary BAC cells in response to ACTH or dibutyryl-cAMP is inhibited by cycloheximide (John et al., 1986a). However, upon transient transfection of Y1 cells or primary BAC cells with −896/OVscc, cycloheximide has no inhibitory effect on forskolin-stimulated reporter gene transcription. A similar discrepancy between the effect of cycloheximide on endogenous gene expression and transient reporter gene expression has been observed in a study of the bovine P-45017α gene (Lund et al., 1990). This suggests that in transient transfections of Y1 and BAC cells, cAMP exerts its effect on reporter gene expression by modifying pre-existing transcription factors rather than enhancing their synthesis. Further investigation is required to elucidate the basis of the difference between cycloheximide effects on the accumulation of P-450α mRNA from the endogenous gene and the accumulation of globin mRNA from the reporter gene following transient transfection.

In a number of genes that are regulated by cAMP, a conserved 8-bp sequence motif (CRE) is found, namely TGACGTCA (for review see Roessler et al., 1988; Karin, 1989). A CRE-binding protein has been identified which upon binding to the CRE activates transcription in vitro (Montmmy and Bilezikjian, 1987). Another transcription factor, AP-2, has also been implicated in cAMP-dependent transcription (Imagawa et al., 1987). Neither the consensus CRE sequence nor the consensus AP-2 binding sequence (TCCCCANGCG) is present in the region between −183 and −83 bp in the bovine P-450α gene. In addition to the P-450α gene, the genes encoding P-45017α (Zuber et al., 1986), P-45021α (John et al., 1986b), and P-45021b (John et al., 1986c) are responsive to cAMP. In the bovine P-45017α gene, two independent regions have recently been identified that confer cAMP responsiveness when coupled to a heterologous promoter (Lund et al., 1990). Like the P-450α cAMP-responsive region described herein, neither of these P-45017α elements contains a consensus CRE or AP-2 binding site. In the murine P-45021α gene the 5′-flanking cAMP-responsive element has also been found not to contain known cAMP-responsive sequences (Parker et al., 1986; Chaplin et al., 1986; Handier et al., 1988). Of the steroid hydroxylase genes, only the murine P-45017α gene is reported to contain a functional CRE in the 5′-flanking region (Mouw et al., 1989). This suggests that a variety of cis-acting elements can mediate the chronic response to cAMP in the steroidogenic pathways. One area of homology is found between the bovine P-45017α gene and the bovine P-45021α gene.

This is particularly intriguing because a portion of this sequence (TTGATG) has been found within one of the 5′ cAMP-responsive elements of the bovine P-45017α gene (Lund et al., 1990). However, we do not know which, if any, of these nucleotides are required for P-45017α expression or P-45021α expression and accordingly do not yet know whether this sequence homology has any functional significance.

The consensus binding site for AP-1 (TGAGTCGTA) has been reported to, in some instances, mediate response to cAMP in addition to phorbol esters (Deutsch et al., 1988; Hoeflter et al., 1989; for review see Karin, 1989). The region of the P-450α gene that we have identified to be responsive to cAMP in this study contains an AP-1-like sequence, and it cannot be excluded that this sequence can mediate cAMP responsiveness. Certainly more detailed analysis of the region between −183 and −83 bp is required. Interestingly, it has been shown that the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, has a modest stimulatory effect on progesterone for-
mation in cultured rat granulosa cells, and it also measures the stimulatory effect of cAMP on P-450αc mRNA accumulation in the same cells (Trzeciak et al., 1987). The 5′-flanking sequence of the bovine P-450αc gene also contains the sequence CCGGCC, which resembles the consensus binding site for the transcription factor Sp1. Sp1 has been shown to activate transcription from a number of viral and cellular promoters (Kadonaga et al., 1986) and is found in some housekeeping genes such as hydroxymethylglutaryl-CoA reductase and the low density lipoprotein receptor (Osborne et al., 1988; Dawson et al., 1988). The human P-450αc gene has a putative CAAT box upstream of its putative TATA box (Morohashi et al., 1987) instead of the Sp1 site, which might suggest interesting differences in regulation of expression of the P-450αc gene in different species.

While the common and distinct features of cAMP-dependent genes and constitutive expression of the different steroid hydroxylase are not yet evident, certain interesting parallels between the bovine P-450αc gene and the murine P-450αc gene exist. The 5′-flanking region of the murine P-450αc gene contains closely associated constitutive and cAMP-responsive cis-elements within ~300 bp of the start site of transcription (Handler et al., 1988). A similar close association of constitutive and cAMP-dependent regulation of the bovine P-450αc gene is found in the present studies. Needless to say, the degree to which the regulation of expression of these two genes and the other steroid hydroxylase genes share common features is not clear. Characterization of the trans-acting factors and the detailed analysis of cis-elements associated with constitutive and cAMP-dependent transcription of the different steroid hydroxylase genes will help clarify this point.

It is now apparent that the P-450αc gene (CYP11A) and the P-450αc gene (CYP11B) are members of the same P-450αc gene family (Nebert et al., 1989). In this respect it is interesting to note that the murine P-450αc gene can be regulated by cAMP via a consensus CRE while the bovine P-450αc is not. Thus, in addition to the obvious consequence of evolution, namely two different forms of mitochondrial P-450 catalyzing very distinct steroid hydroxylations, two distinct cis-elements for cAMP-dependent transcription of these genes appear also to have developed over evolutionary time.

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