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REGULATION OF EXPRESSION OF THE GENES ENCODING STEROIDOGENIC ENZYMES

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Summary—In recent years it has become apparent that tropic hormones involved in steroidogenesis act to regulate the expression of the enzymes involved in the various steroidogenic pathways. This is particularly evident in the ovary where the episodic secretion of steroids throughout the ovarian cycle is regulated largely by changes in the levels of the particular enzymes involved in each step of the steroid biosynthetic pathways. Recently, the genes for the various cytochrome $P450$ species involved in ovarian steroidogenesis, namely cholesterol side-chain cleavage $P450$ ($P450_{SCC}$), 17α-hydroxylase $P450$ ($P450_{17\alpha}$), and aromatase cytochrome $P450$ ($P450_{AROM}$) have been isolated and characterized, making it possible to study the regulation of expression at the molecular level. To this end, a series of chimeric constructs have been prepared in which fragments of the 5′-untranslated region of bovine $P450_{17\alpha}$ and $P450_{SCC}$ have been inserted upstream of the chloramphenicol acetyl transferase (CAT) and β-globin reporter genes. These constructs have been used to transfect primary cultures of bovine luteal and thecal cells. The results indicate that cAMP responsiveness lies within defined regions of genes which do not contain a classical CRE, similar to previous results utilizing adrenal cells in culture. Furthermore, although constructs containing both the $P450_{17\alpha}$ and $P450_{SCC}$ 5′-upstream regions are expressed in both luteal and thecal cell cultures, only those containing the $P450_{SCC}$ sequences are expressed in luteal cells. Studies on the expression of $P450_{AROM}$ indicate that the promoter which is responsible for its expression in human placenta is not operative in the corpus luteum. Thus estrogen biosynthesis may be regulated by the differential use of tissue specific promoters, thus accounting for the complexity and multifactorial nature of the expression of this activity.

INTRODUCTION

Steroid hormone biosynthesis in the ovary occurs in a highly coordinated and episodic fashion which is determined, in part, by the pattern of gonadotropin secretion. In the case of the bovine ovary, a dramatic switch in the pattern of steroid secretion occurs at the time of ovulation. Following the gonadotropin surge there is a marked increase in the secretion of progesterone by the developing corpus luteum and, at the same time, an equally dramatic decline in the secretion of androgens and estrogens. With the isolation and cloning of cDNA inserts complementary to mRNA species encoding these enzymes, and the subsequent characterization of the genes encoding these transcripts, it became possible to analyze the molecular mechanisms underlying these differential switches in steroid hormone secretion. Here, recent work from our laboratory to characterize the expression of genomic constructs derived from the 5′-end of the bovine cholesterol side-chain cleavage cytochrome $P450$ ($P450_{SCC}$) and 17α-hydroxylase cytochrome $P450$ ($P450_{17\alpha}$) genes will be described, as will the current efforts to define the regulatory elements responsible for expression of aromatase cytochrome $P450$ ($P450_{AROM}$), the enzyme responsible for estrogen biosynthesis.

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NORTHERN AND WESTERN ANALYSIS

Corpora lutea and follicles were dissected from bovine ovaries throughout the cycle and aliquots of the protein were submitted to Western blotting analysis utilizing antibodies raised against purified bovine $P450_{SCC}$, $P450_{17\alpha}$, and adrenodoxin [1]. It was observed that the expression of $P450_{SCC}$ and adrenodoxin was low in

follicles but increased abruptly and dramatically following the onset of luteinization. With the subsequent onset of luteolysis there was a precipitous decline in the levels of both of these proteins. By contrast, the expression of $P450_{17\alpha}$ was readily detectable in follicles but following ovulation declined abruptly to undetectable levels. On the other hand, the expression of control enzymes NADPH-cytochrome c reductase, a microsomal marker, and F1-ATPase, a mitochondrial marker, changed much less dramatically. Thus, it is clear that the differential expression of steroid biosynthesis throughout the bovine ovarian cycle can be explained, in part, by the differential expression of the enzymes responsible for their biosynthesis.

In order to investigate whether the levels of these enzymes were in turn a reflection of the levels of the mRNA species encoding these enzymes, Northern analysis [2] was conducted on RNA extracted from follicles and corpora lutea throughout the bovine ovarian cycle, using as probes cDNAs complementary to mRNAs encoding $P450_{SCC}$, $P450_{17\alpha}$, adrenodoxin, and the LDL receptor. It was found that the levels of mRNA encoding $P450_{SCC}$ (Fig. 1A), adrenodoxin, and the LDL receptor were low in follicles but increased dramatically following ovulation, then decreased equally abruptly with the onset of luteolysis. Thus, the pattern of expression of all three of these proteins which are intimately involved in progesterone biosynthesis follows very closely the pattern of progesterone secretion throughout the bovine ovarian cycle. The activity of HMG CoA reductase was also determined and, again, closely paralleled that of these proteins. Thus, it appears that the pattern of secretion of progesterone is explicable, to a high degree, by the pattern of expression of the enzymes responsible for its biosynthesis, together with the proteins required to optimize the supply of cholesterol to the steroidogenic machinery required for progesterone biosynthesis. By contrast, when the levels of mRNA encoding $P450_{17\alpha}$ were determined (Fig. 1B), they were found to be detectable in follicles, but promptly disappeared with the onset of ovulation and were undetectable following luteinization, thus again mimicking the pattern of androgen secretion in the bovine ovary.

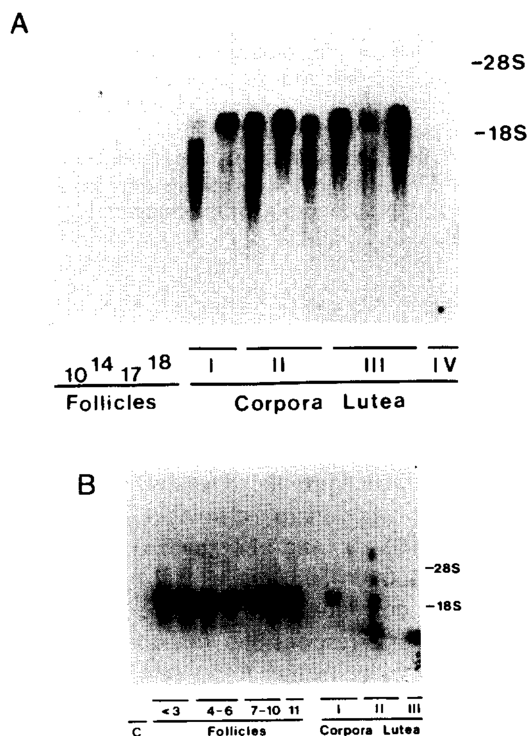

Fig. 1. (A) Relative levels of $P450_{SCC}$ mRNA in bovine follicles and corpora lutea as determined by Northern blot analysis of total RNA (20 μg) from individual follicles of differing diameter (10, 14, 17, 18 mm) and individual corpora lutea of differing stages of development (I, II, III, IV) [11]. Positions of 18S and 28S ribosomal RNA are indicated. (B) Relative levels of $P450_{17\alpha}$ mRNA in bovine follicles and corpora lutea as determined by Northern blot analysis of poly(A)$^+$ RNA (5 μg) from ovarian cortex (C) and individual (11 mm) and pools (<3, 4–6, 7–10 mm) of follicles and individual corpora lutea of differing stages of development (I, II, III) [11].

FACTORS REGULATING EXPRESSION OF STEROIDOGENIC ENZYMES IN THE OVARY

Based on these results, we can conclude that the pattern of steroid hormone secretion throughout the ovarian cycle is explicable, to a high degree, on the basis of the differential expression of the various enzymes involved in the steroidogenic pathway. The next question which arises, therefore, is to determine what factors are responsible for regulating the expression of these enzymes and, in particular, which are the mRNA levels encoding these enzymes. We, like others, have been interested in the possibility that a number of growth factors and cytokines produced locally within the ovary, some in response to gonadotropins, may differentially regulate the expression of steroidogenic enzymes in either a paracrine or autocrine fashion. In particular, in the case of 17α-hydroxylase expression by thecal cells, it has been established in our laboratory, using cultures of human thecal cells [3] as well as bovine thecal cells [4], that a number of growth

factors are inhibitory of 17α-hydroxylase activity. These include bFGF, TGF-β, EGF, as well as the phorbol ester, TPA. Because the presence of bFGF has previously been characterized in the bovine ovary [5], particularly in the corpus luteum, we decided to examine the expression of this growth factor in the bovine ovary. Using a riboprobe technique and RNA extracted from follicles and corpora lutea from bovine ovaries throughout the ovarian cycle, it was established that the expression of bFGF was undetectable in follicles but that it was expressed to a high level in corpora lutea throughout the luteal phase of the cycle, and in fact was also expressed in corpora lutea undergoing luteolysis [6]. This result suggested that the expression of bFGF in the bovine ovary might occur, at least in part, in response to gonadotropins. Consequently, bovine luteal cells were placed in primary culture and the expression of bFGF by these cells was examined. It was found that the expression was extremely low in control cells but was induced markedly in the presence of either hCG, LH, or dibutyryl cAMP [6, 7]. Thus, it is possible to present a reasonably coherent hypothesis that bFGF formed in luteinizing cells as a consequence of the gonadotropin surge serves not only as an angiogenic factor, but also to suppress the expression of 17α-hydroxylase and thus contributes to the differential expression of the steroidogenic enzymes which occurs at the time of ovulation in the bovine ovary.

REGULATION OF THE GENES ENCODING STEROIDOGENIC ENZYMES

Of course, bFGF is only one of many locally produced factors that might regulate the expression of the steroidogenic enzymes, and such experiments do not necessarily provide information as to which may be the most important physiologically. A different approach, then, to understanding the regulation of these enzymes is to examine the factors that influence the transcription of the genes encoding these enzymes with the hope that definition of the *cis*-acting and *trans*-acting elements that are responsible for the regulation will not only provide important information in its own right, but will eventually lead back to the cell surface and a complete picture of the regulatory pathway. To this end (Fig. 2), a series of chimeric constructs containing fragments of the 5′-end of the bovine $P450_{SCC}$ and $P450_{17\alpha}$ genes fused upstream of

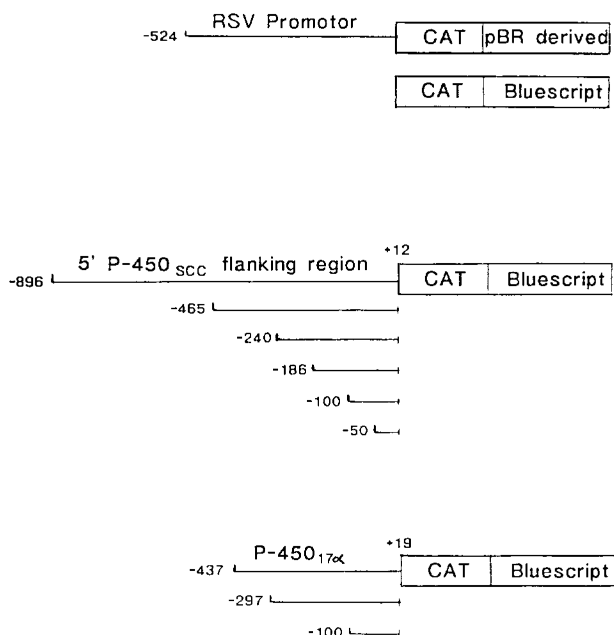

Fig. 2. Schematic diagram of reporter genes used in transient transfection. Sequences of the 5′ regulatory regions of $P450_{SCC}$ and $P450_{17\alpha}$ genes have been fused to the CAT reporter gene. These constructs contain the respective putative $P450_{SCC}$ and $P450_{17\alpha}$ TATA boxes and start sites of transcription. RSV–CAT; this construct was used as a positive control and contains part of the Rous sarcoma virus, leading to constitutive high expression. CAT; this construct lacks specific promoter sequences and was used to monitor non-specific CAT activity.

the chloramphenicol acetyl transferase (CAT) gene were prepared [8, 9]. These were transfected into bovine luteal cells and bovine thecal cells by means of the technique of electrophoration [10]. When the series of $P450_{SCC}$ constructs were transfected into bovine luteal cells, it was observed that deletion from −186 to −100 drastically reduced both the basal- and forskolin-stimulated activity (Table 1), indicating that this region contained perhaps a basal enhancer and a cAMP-responsive element. A feature of these steroidogenic genes, however, is that no classical cAMP-responsive element such as TGACGTCA has been detected. An identical pattern of expression has been observed when this set of constructs was transfected into mouse Y1 adrenal tumor cells [8], indicating that the regulation of the $P450_{SCC}$ gene in adrenal cells and luteal cells appears to occur via similar mechanisms. Interestingly however, when the corresponding $P450_{17\alpha}$ constructs were transfected into bovine luteal cells, no expression of CAT was observed either in the presence or absence of forskolin (Table 1). Thus, the expression of the transfected constructs appears to follow that of the endogenous expression. On

Table 1. Relative expression of CAT reporter gene constructs after transfection into bovine luteal and thecal cells in primary culture

DNA construct	CAT activity (% conversion)		Cell type
	−Forskolin	+Forskolin	
RSV-CAT	98.5	98.7	Luteal
CAT	8.8	9.5	Luteal
SCC-896	8.3	14.3	Luteal
SCC-465	15.2	26.7	Luteal
SCC-240	47.3	53.5	Luteal
SCC-186	15.0	56.8	Luteal
SCC-100	2.4	3.9	Luteal
SCC-50	4.4	3.1	Luteal
17α-437	2.0	4.8	Luteal
17α-297	4.1	4.2	Luteal
17α-100	2.5	2.4	Luteal
RSV-CAT	71.9	67.2	Thecal
CAT	1.7	4.9	Thecal
SCC-186	9.8	24.8	Thecal
17α-297	25.5	34.8	Thecal

Numbers represent % conversion of [^{14}C]chloramphenicol to acetylated products and are calculated from one single experiment. Similar relative CAT reporter gene expression has been obtained in two other independent experiments. Conditions for electrophoration and for determination of CAT activity are described [18].

the other hand, when the $P450_{SCC}$ (−186 to +12 bp) and $P450_{17\alpha}$ (−297 to +19 bp) constructs were transfected into bovine thecal cells in culture, both of these were expressed and the expression appeared to be stimulated by forskolin [4]. Thus again, in these cells the expression of the transfected constructs parallels closely that of the endogenous expression.

It appears, therefore, that primary bovine luteal and thecal cells in culture provide a model system to study the differential expression of the genes encoding $P450_{SCC}$ and $P450_{17\alpha}$. In particular, the failure of the luteal cells to express constructs containing the 5′-end of the $P450_{17\alpha}$ gene, strongly indicates that during the process of luteinization either a positive transcription factor required for expression of $P450_{17\alpha}$ has been lost, or else a negative transcription factor shutting off the expression of $P450_{17\alpha}$ has been expressed. Use of these cells, therefore, should provide the means of defining the *trans*-acting elements responsible for the differential expression of these two genes and thus, ultimately, the mechanism underlying the dramatic switch in the steroidogenic profile which takes place in the bovine ovary at the time of ovulation.

STRUCTURE OF THE HUMAN AROMATASE CYTOCHROME *P*450 GENE

The gene encoding $P450_{AROM}$ is similar to that of other cytochrome *P*450 species, in that the coding region comprises nine exons (most cytochrome *P*450 genes contain between 8 and 10 exons); the heme binding region and the entire 3′-untranslated region are encoded by the last exon (Fig. 3) [12]. However, the gene is much larger than those of other steroidogenic cytochromes *P*450 and may in fact be the largest cytochrome *P*450 gene analyzed at this time. The entire gene spans at least 70 kb, and since there is a region where the clones do not overlap, the actual size is unknown. By comparison, the genes for the other two microsomal steroidogenic cytochrome *P*450s, namely $P450_{C21}$ and $P450_{17\alpha}$ are 3.7 and 6.5 kb, respectively. Of the mitochondrial steroidogenic cytochrome *P*450 species, the gene for bovine 11β-hydroxylase *P*450 ($P450_{11\beta}$) is 8 kb [15] long, whereas that for human $P450_{SCC}$ is at least 20 kb long [16] and this also has an intron in which the clones do not overlap.

Analysis of the intron–exon boundaries reveals rather poor correlation with other

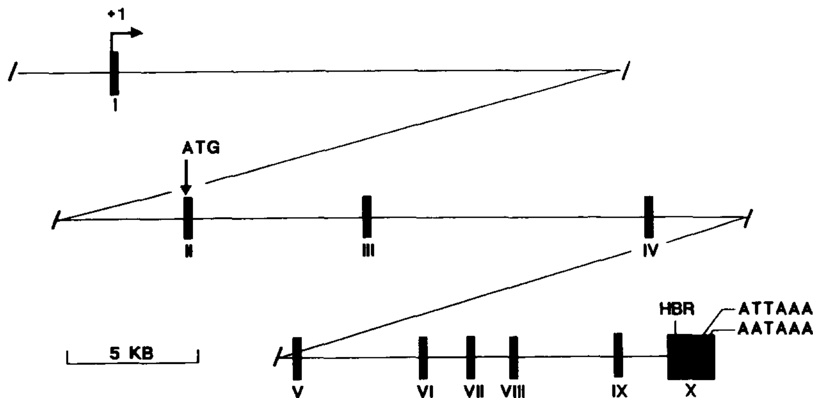

Fig. 3. Schematic of the gene encoding human cytochrome $P450_{AROM}$. The gene spans four clones as indicated, the first containing an untranslated exon. The 2nd and 3rd clones overlap, whereas the others do not. The nine exons in the coding region (II–X) are indicated, as in an exon in the 5′-untranslated region (exon I). The location of the heme-binding region (HBR) on exon I is indicated, as are the two polyadenylation signals.

steroidogenic cytochromes $P450$. The exon–intron boundaries of human $P450_{17\alpha}$ and $P450_{C21}$ agree rather well at least in the 3′ portion of the molecule, although there are several differences towards the 5′-end. On the other hand, the human $P450_{AROM}$ gene shows no boundary identities when compared to those of $P450_{17\alpha}$ and $P450_{C21}$, although there are several boundaries in the 3′ portion of the molecule which are close to those of the other two microsomal $P450$s. Towards the 5′-end of the molecule, however, attempts to align the boundaries with those of the other two microsomal $P450$s become impossible. Poor exon–intron boundary alignment between the various genes is a characteristic feature of the cytochrome $P450$ superfamily, leading to speculation that the ancestral gene had many more exons than the modern counterparts [12].

NUMBER OF AROMATASE CYTOCHROME *P450* GENES PRESENT IN THE HUMAN GENOME

The issue of whether there is more than one aromatase enzyme in the human is an important one for several reasons. In the first place, it has been suggested [17] that different aromatase enzymes exist in placenta, ovary, and adipose tissue since the major estrogen produced in each of these tissues is different, namely estriol in the placenta, estradiol in the ovary, and estron in the adipose. Equally, however, this could be due to the presentation to the same enzyme of different substrates, namely 16α-hydroxylated androgens in the case of the placenta, testosterone in the case of the ovary, and androstenedione in the case of adipose tissue. The issue is also of importance clinically. At present there is much interest in the development of more effective inhibitors of aromatase for use clinically in the management of patients with breast cancer [18]. However, the only source of aromatase available for the testing of such inhibitors is that derived from human placenta. Moreover, there is evidence to suggest that the estrogen which may be of consequence in the development of breast tumors is that produced in the breast adipose tissue surrounding the developing tumor. If the enzyme responsible for such estrogen in the adipose tissue were different from that in the placenta, then clearly inhibitors developed against the placental enzyme might be less efficacious toward the enzyme present in adipose.

Studies on the expression of the cDNA encoding $P450_{AROM}$ [19] together with previous work on the purified enzyme [20, 21] suggest that a single enzyme is capable of metabolizing all three categories of C_{19} substrate and that it is not necessary to postulate the presence of different enzymes in the different tissues which synthesize estrogens. Our present work on the characterization of the gene encoding $P450_{AROM}$ is consistent with this view. In all of our restriction mapping and Southern analysis, we have obtained no evidence to suggest that there is more than one cytochrome $P450_{AROM}$ gene within the human genome.

CHARACTERIZATION IS A 5′-UNTRANSLATED EXON

DNA sequence upstream from the bases encoding the start of translation contains putative CAAT and TATA boxes, however, primer extension using human placental poly A^+ RNA failed to reveal any start of transcription associated with these. Moreover, an oligonucleotide prepared against a region commencing 39 bp downstream from this putative TATA box failed to hybridize in Northern analysis of poly A^+ RNA from human placenta. We conclude, therefore, that an intron is present in the DNA 5′ of the exon encoding the start site of translation and that the intron–exon boundary occurs at the point where the genomic sequence and that of the cDNAs all become identical. A sequence similar to the splice junction consensus sequence is also present at this site [22]. By contrast, the region of the gene which we have called exon I fulfills the criteria of a 5′-untranslated exon (Figs 3 and 4), although the clone that contains it does not overlap with that encoding the translated gene product.

PUTATIVE REGULATORY SEQUENCES WITHIN THE HUMAN AROMATASE CYTOCHROME *P450* GENE

5′-Flanking sequence comprising 918 bp upstream of exon I has been sequenced (Fig. 4). Analysis of the 5′-flanking region of exon I indicates the presence of a putative TATA box (ATAAA). Downstream from this (23 bp) is the site of transcription initiation as revealed by primer extension. Putative CAAT binding sequences are present at −54, −63, and −82 bp. In addition, sequences with partial homology to glucocorticoid response elements (GRE), cAMP

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EXON I

aagctttgtc catttcctcc caactcaaac gttctgtgac ctacttgaag aactgtcatg tatccttatc tcttttcatc caggtatcag  -832
gtttagggcc aatggggagg agccactgag aagggagggt tgacactcag catcattcag atcttcattt aattcagtaa acacttaact  -742
gagcacttcc tgtgcctggt atgataatgg gcattggaga tacggaagta aaaaagacaa gcagattacc ctgtgagcag ctggaacacc  -652
gtggagcctt ctgggcttct ctttatccta acattcattg cagttactgt tgactgatca tctctcagca atacccacca ttaaccatgg  -562
aatagaagcc tcctgaggct agggccatgt cacccccaac acatagcaca gttcctgatt tggaattcac tattacccgt tgaataaatg  -472
agtgaatgaa cgaatgaata aatgaaaagc tgtatagtga aggcacttct atgtagaaca atgtggtgtg tggtgggggg tgtgatctta  -382
                                 GRE
tgattacatg tatatagatt tttgtccatg gttcttgtcc ataactccca tgacacttgc tgaggtcttt tgctataatg tagaggtgct  -292
                                                                                          CRE
ttaggcctca ggaaacagaa tatttctctg gccttctttg ccctcctttc atccacccaa ggcggactct ataatctgat tgtggtcata  -202
agaccctcat tccagaggag gtcatgcccc ataccctgga ggaaggaatg ctgcacaaag agaggaagaa gaatctggac agacagacct  -112
                                                                AP1
tgctgagatt agatcatacc cttttgtcc aatcacattt tgttcaatca catgcttcag tcatggacaa caaatgaaat ctccataaaa  -22
                      +1
ggcccaaagg acagggttca gGGAGTTTCT GGAGGGCTGA ACACGTGGAG GCAAACAGGA AGGTGAAGAA GAACTTAATC CTATCAGGAC  69
GGAAGGTCCT GTGCTCGGGA TCTTCCAGAC GTCGCgtatg tatctcttaa tctgactgag ccctcaacct gtgggatcag acactctttc  159
caggtagata gtgttggaac tgagttggag gacacccagc tggtgcccgc tgcttggtgt gtgggggaaa atcccctaca tttggtcgca  249
gaagtcttct gtgttgatga ctgttgtcgt ggtgtgagag cagagggaaa acactgcttg agtgtttttc tgaaatagga gggcctaaga  339
actatactgt tctatgccac agaactggcc ccctac.....>19 kb......
        GRE
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Fig. 4. Sequence of exon I of the gene encoding $P450_{AROM}$. Exonic sequence is indicated by bold caps, upstream sequence and intronic sequence by lower case letters. The exon–intron boundary between exon I and intron I is indicated by underlining. The putative transcriptional start site is indicated by +1, and upstream from this, enclosed by boxes, are a putative TATA box and three putative CAAT boxes. Other sequences of potential regulatory significance are indicated in bold with underlining, and their possible roles so designated. GRE—glucocorticoid regulatory element; CRE—cAMP regulatory element.

response elements (CRE) and API response elements, are indicated.

ARE THERE ALTERNATIVE PROMOTER SEQUENCES?

Sequencing of inserts from the primer-extended placental cDNA library revealed the presence of yet another divergent 5′-region upstream from the splice junction, suggestive of a second untranslated exon within the human cytochrome $P450_{AROM}$ gene which may be subject to differential splicing. This, of course, raises the exciting possibility that these exons may be differentially spliced in different tissues that express aromatase, and that such differential splicing might account for some of the complexities of the regulation of expression of this gene that have been observed in these tissues.

More recently, we have found that this second promoter is responsible for the expression of a minor fraction of the $P450_{AROM}$ mRNA species in human placenta, whereas the promoter present in exon I is the major promoter driving expression in placenta. However, neither of these exons is present in RNA derived from human ovary or adipose, indicative that neither of these placental promoters is responsible for $P450_{AROM}$ expression in ovary or adipose. Conceivably therefore, the putative promoter sequence upstream from exon II may be responsible for expression in either or both of these tissues, although further studies will be required to resolve this issue.

NATURE OF THE TRANSCRIBED RNA MESSAGE

Although there appears to be only one cytochrome $P450_{AROM}$ gene within the human genome, nonetheless, Northern analysis of RNA from tissues in which the gene is expressed reveals the presence of two hybridizable mRNA species, one at 3.4 kb and one at 2.9 kb. We believe that this is due to the use of alternative polyadenylation signals for the following reasons. In the first place, there are two putative polyadenylation signals within the 3′-untranslated region of the human cytochrome $P450_{AROM}$ gene (Fig. 4) and we have isolated cDNAs that have polyadenylated tails corresponding to the use of each of these polyadenylation signals [19, 23]. In all other aspects the sequences of the cDNAs are identical, including the sequence of the coding region. Secondly, when an oligonucleotide complementary to the area of the 3′-untranslated region between these polyadenylation signals is used as a hybridization probe in Northern analysis of human placental poly A^+ RNA, only one of these mRNA species hybridizes, namely the 3.4 kb band. This proves convincingly that the RNA species of 2.9 kb does not contain the 3′-untranslated region between the polyadenylation signals. Thirdly, an estimate of the size of the

mRNA that would be expected based on the lengths of these cDNAs is in good agreement, since sizes of the corresponding cDNAs are 3.0 and 2.7 kb, and most polyadenylated tails are 0.2–0.4 kb long.

CONCLUSIONS

These studies represent initial efforts to understand the regulation of expression of the genes encoding steroidogenic enzymes. This is of interest, not only because of the importance of steroids in such diverse phenomena as the physiological response to stress, control of salt balance, and maintenance reproductive capacity, but also because of the multifactorial nature of the regulation of these genes. The observation that classical cAMP-responsive elements appear not to be present in the upstream regions of many of these genes, and that the genes do not share a common cAMP-responsive element among themselves, suggests the possibility that each of the steroidogenic *P*450 genes may interact with different members of the CREB family of transcription regulating factors. This situation will be clarified by further definition of the *cis*-acting elements which mediate cAMP responsiveness present on these genes, as well as isolation and characterization of the corresponding *trans*-acting factors. The ovarian model provides a particularly useful system to study this regulation because of the dramatic changes in expression of the steroidogenic genes which occur during the ovarian cycle. In the case of the aromatase enzyme, the situation is further complicated by the apparent utilization of tissue-specific promoters, which can provide a basis for understanding the diversity of regulation of estrogen biosynthesis in the various tissues in which this occurs.

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