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REGULATION OF HUMAN AROMATASE CYTOCHROME *P*450 GENE EXPRESSION

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Summary—In the human, estrogen biosynthesis occurs in several tissue sites, including ovary, placenta, adipose, and brain. Recent work from our laboratory has indicated that tissue-specific expression of aromatase cytochrome *P*450 (*P*450arom), the enzyme responsible for estrogen biosynthesis, is determined, in part, by the use of tissue-specific promoters. Thus the expression of *P*450arom in human ovary appears to utilize a promoter proximal to the translation start-site. This promoter is not utilized in placenta but instead, the promoter used to drive aromatase expression in placenta is at least 40 kb upstream from the translational start-site. In addition, there is a minor promoter used in the expression of a small proportion of placental transcripts which is 9 kb upstream from the start of translation. Transcripts from these promoters are also expressed in other fetal tissues including placenta-related cells such as JEG-3 choriocarcinoma cells, hydatidiform moles, and other fetal tissues such as fetal liver. On the other hand, in adipose tissue expression of *P*450arom may be achieved by yet another, adipose-specific promoter. The various 5′-untranslated exons unique for expression driven by each of these promoters are spliced into a common intron/exon boundary upstream from the translational start-site. This means that the protein expressed in each of the various tissue-specific sites of estrogen biosynthesis is identical.

OUTLINE

1. INTRODUCTION

In the human, the conversion of androgens to estrogens occurs in a number of cells and tissues including ovarian granulosa cells, testicular Sertoli and Leydig cells, placenta, adipose tissue of both males and females, and various sites of the brain including hypothalamus, amygdala and hippocampus. In a number of species, it is known also to occur in the preimplantation blastocyst [1–8]. In most other species, estrogen biosynthesis does not enjoy such a wide tissue specific distribution, but rather is limited to at most two sites, namely the gonads and the brain. While in the human, the physiological role of estrogen biosynthesis in other sites such as adipose is unclear, such extragonadal synthesis has considerable pathophysiological importance. For example, the ability of human adipose to convert circulating plasma androstenedione to estrone increases as a function of obesity and aging [10–12] and this increase bears a striking relationship to the incidence of endometrial cancer, which is a disease primarily of elderly, obese women. Evidence is also accumulating to suggest a role for estrogens produced by adipose tissue in the pathogenesis of certain forms of breast cancer [13].

The biosynthesis of estrogens is catalyzed by an enzyme complex known as aromatase, whose activity results in aromatization of the A ring of androgens, to form the phenolic A ring characteristic of estrogens with concomitant loss of the C19 angular methyl group. This enzyme complex is localized to the endoplasmic reticulum of cells in which it is expressed and consists of two components. The first is a cytochrome

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*P*450 known as aromatase cytochrome *P*450 (*P*450arom) [14–16], the product of the CYP19 gene [17]. This heme protein is responsible for binding the C19 steroid substrate and catalyzing the concerted series of reactions leading to formation of the phenolic A ring. The second component is a flavoprotein, NADPH cytochrome *P*450 reductase, which is a ubiquitous protein in the endoplasmic reticulum of most cell types and is responsible for transferring reducing equivalents from NADPH to any microsomal cytochrome *P*450 species with which it comes into contact. The aromatase reaction utilizes 3 mol of oxygen and 3 mol of NADPH for every mole of C19 steroid metabolized [18] (Fig. 1). There is general agreement that the first two oxygen molecules are utilized in the oxidation of the C19 angular methyl group. A growing consensus of opinion considers that the third oxygen attack is also on the C19 methyl group resulting in its loss as formic acid. Concomitant with this, aromatization of the A ring takes place with loss of the 2β-hydrogen and resulting rearrangement to form the phenolic A ring characteristic of estrogens [19]. That this complex series of reactions takes place at a single catalytic site was confirmed by expression of a full-length cDNA encoding human *P*450arom in COS-1 monkey kidney tumor cells [20]. Addition of androstenedione, testosterone or 16α-hydroxyandrostenedione led to formation of the corresponding estrogen thus leaving no doubt as to the catalytic activity of the single expressed protein.

Because of the obvious importance of estrogen biosynthesis in both physiological and pathophysiological processes in the human, we have sought to characterize the gene encoding *P*450arom in order to understand the molecular basis of its regulation, both in terms of factors which in any given cell type alter the rate of expression of the enzyme, as well as the nature of the tissue-specific expression of cytochrome *P*450arom.

2. CHARACTERIZATION OF THE AROMATASE CYTOCHROME *P*450 GENE

Characterization of the cDNA encoding *P*450arom isolated from human placental libraries indicated that it is a typical member of the cytochrome *P*450 superfamily [20]. The gene encoding human cytochrome *P*450arom has been isolated from human genomic libraries by three different laboratories using nucleotide sequences of the human cDNA as probes [21–23]. The coding region comprises nine exons spanning a total distance of 32 kb (Fig. 2). The heme-binding region in common with that of most other cytochromes *P*450 is on the last coding exon, which also contains two polyadenylation signals. This is consistent with the presence of two messenger RNA species of 3.4 and 2.9 kb which are the consequence of usage of these alternative polyadenylation signals. Examination of the intron/exon boundaries reveals poor correspondence with those of other microsomal *P*450's, in particular 17α-hydroxylase

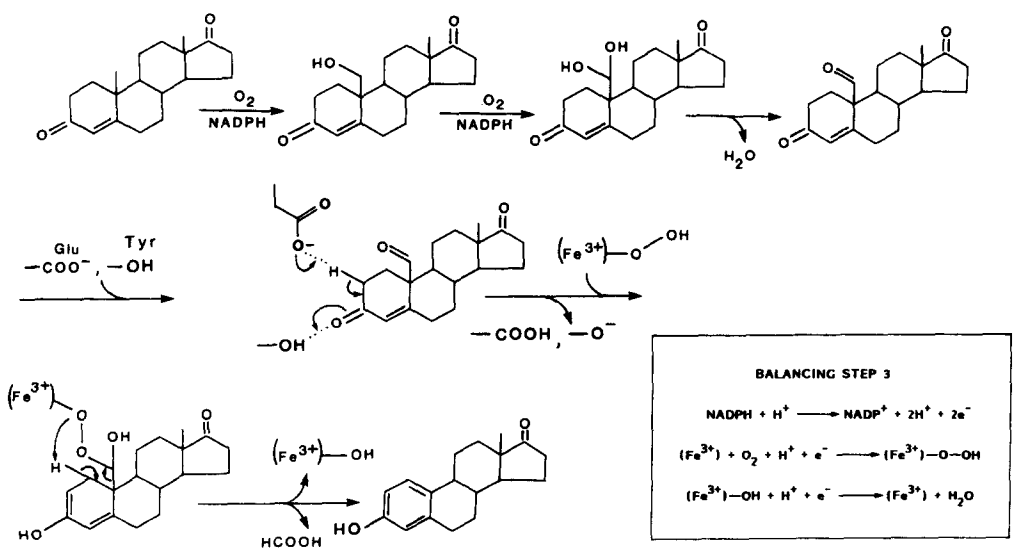

Fig. 1. Putative mechanism of the aromatization reaction. Of the three oxygen insertion reactions, only the third is shown in detail. Details of the third oxygen insertion and concomitant aromatization reaction are based on the proposed model of Cole and Robinson [19].

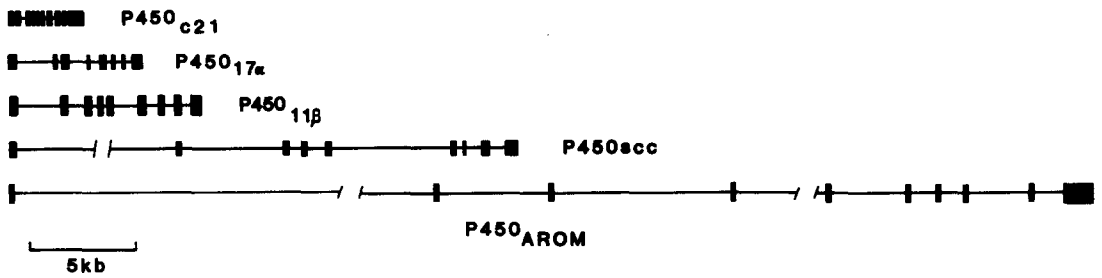

Fig. 2. Diagram of the structures of the five human steroidogenic P450s involved in the formation of corticosteroids, androgens, and estrogens. The exons are indicated by the black boxes and the relative sizes of the introns are shown.

and 21-hydroxylase P450s [21], which share many intron/exon boundaries in common. This poor homology speaks to the antiquity of the CYP19 gene lineage, and is consistent with sequence homology comparisons [17].

3. REGULATION OF AROMATASE CYTOCHROME P450 GENE EXPRESSION IN HUMAN PLACENTA

Examination of the sequence upstream of the translational start site reveals a putative promoter sequence (Fig. 3). In order to determine if this TATAA sequence was serving as a promoter of human cytochrome P450arom, polyA$^+$ RNA derived from human placenta was used in primer extension analysis to determine the transcriptional start site. The resulting fragment did not extend to the expected position, some 25 to 20 bp downstream from the TATAA box, but rather extended to a position almost completely on top of this sequence, indicative that the P450arom RNA isolated from human placenta is too long to be promoted from this region. This observation, however, is consistent with previous studies in which the 5′ ends of clones contained in a primer-extended cDNA library prepared from human placental polyA$^+$ RNA were sequenced [21]. This library was found to contain two different 5′ ends which also differed from the genomic sequence upstream of a common boundary. Downstream from this boundary the sequence of the cDNAs as well as that of the gene became identical. It was realized that this boundary upstream of the start of translation is in fact an intron/exon boundary and that the two different 5′ ends represent 5′-untranslated exons which are spliced into this junction during processing of the initial transcript to the mature forms. When sequences corresponding to these 5′ ends were used as probes in Northern analysis of P450arom transcripts in human placental polyA$^+$ RNA, it was found that by far the majority of the transcripts contained sequences corresponding to one of these 5′ ends, whereas, the other was present in less than 1% of the transcripts. These sequences were also used as probes to rescreen the human genomic libraries and isolate and characterize the corresponding genomic clones.

Based on these considerations, the exon containing the start of translation has been renumbered exon II and the remaining coding exons, exons III through X. The exon which comprises the most abundant 5′ terminal found in placental transcripts is now named I.1 [24], whereas the exon comprising the 5′ end of lesser abundance is numbered I.2 [25]. Southern blotting and restriction analysis of the genomic clones containing these exons revealed that whereas the genomic sequence corresponding to exon I.2 is located some 9 kb upstream from the translation start site, the genomic clone containing exon I.1 could not be overlapped with any of the other genomic clones, indicating that it is at least 40 kb upstream from the start of translation (Fig. 4). Primer extension analysis utilizing human placental polyA$^+$ RNA indicated a transcription start site some 23 bp downstream from a putative TATAA sequence located within the exon I.1 genomic clone (ATAAA). Upstream from this sequence there are at least three potential CCAT box sequences.

4. REGULATION OF AROMATASE CYTOCHROME P450 GENE EXPRESSION IN HUMAN OVARY

In order to investigate which 5′-ends might be present in ovarian transcripts, and in particular to determine if transcripts containing exon I.1 or I.2 specific 5′ sequences were present in cytochrome P450arom messenger RNA in human ovary, Northern analysis was performed using human ovarian corpus luteum polyA$^+$ RNA and exon I.1- and I.2-specific sequences as probes [24]. No hybridization was detectable under these conditions. In order to increase the

sensitivity of this analysis, an attempt was made to amplify exon I.1- and I.2-specific sequences from corpus luteum polyA$^+$ RNA utilizing PCR technology. Even with the vastly increased sensitivity of this technique, no transcripts containing these sequences were apparent although transcripts containing sequences corresponding to coding exon II were readily apparent. It must be concluded therefore that ovarian transcripts of *P*450arom do not contain the 5′ ends found in human placental transcripts, suggesting that the placental promoters I.1 and I.2 are not responsible for *P*450arom expression in the human ovary. The question then arises as to

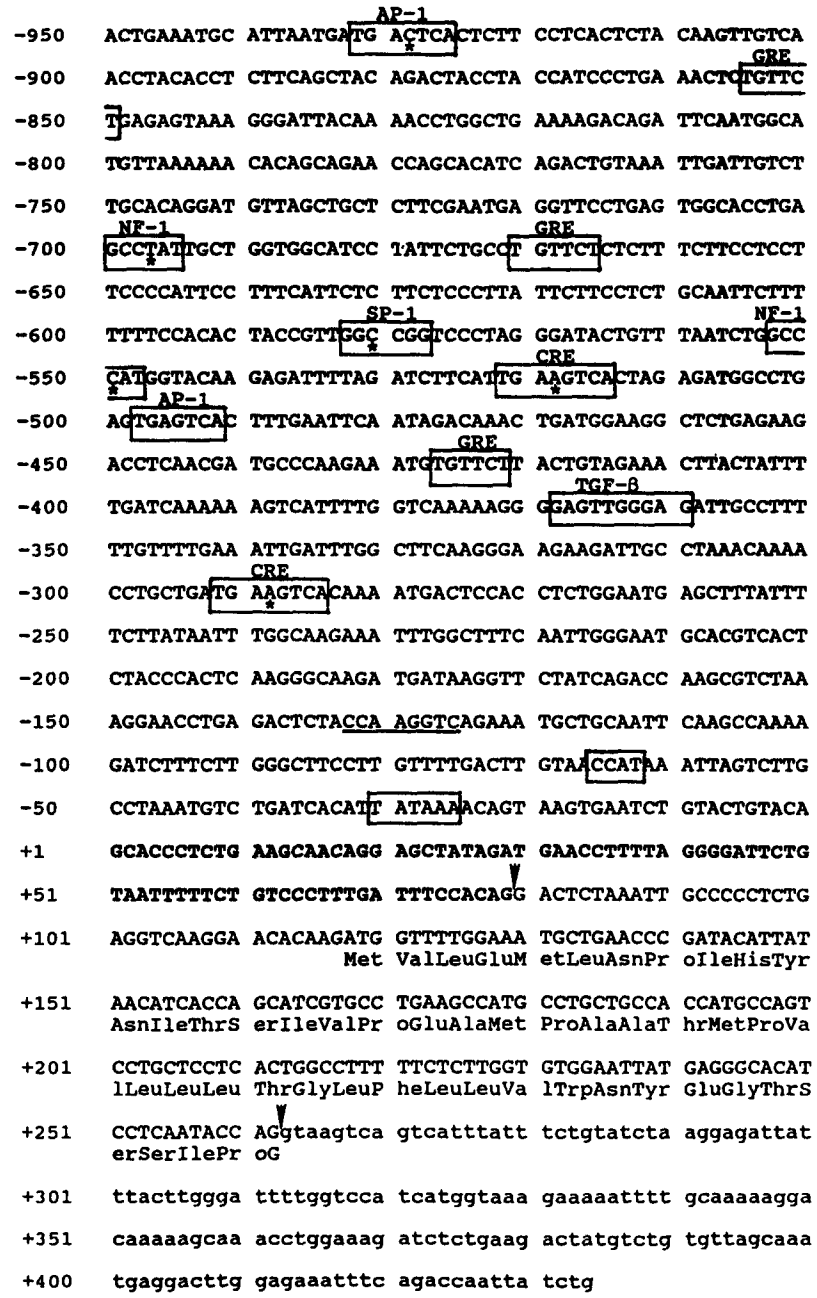

Fig. 3. Sequence of the 5′ flanking DNA upstream of exon II corresponding to promoter II. Sequences with similarity to the consensus sequences for binding of known transcriptional activators are boxed, as are the putative TATA and CAAT box. Nucleotides that diverge from the consensus sequences are indicated with an asterisk. The underlined sequence has been found to be important in the regulation of several steroidogenic genes, notably 11β-hydroxylase. The arrows indicate the position of the placental intron/exon II boundary, and the boundary between exon II and the first intron in the coding region. The bases are numbered such that +1 represents the start of transcription using promoter II as defined by the primer extension and S1-nuclease protection experiments described in this paper.

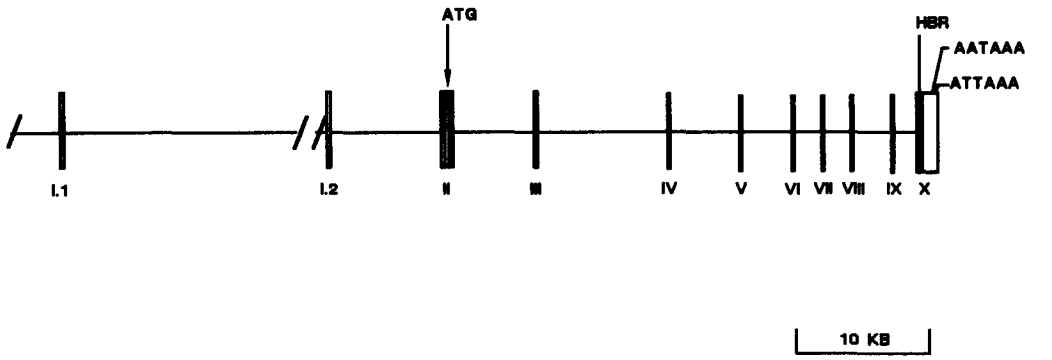

Fig. 4. Schematic representation of the human *P*450arom gene, based on sequences transcribed in ovary and placenta. The closed bars represent translated sequences. The septum in the open bar in exon II represents the splice junction for exons I.1 and I.2, sequences to the left of the septum would be present in mature RNA only when a putative TATA box 149 bp 5′ of the ATG is utilized to promote transcription.

what promoter is responsible for expression in the ovary. Recalling that upstream from the start of translation there was a TATAA sequence and a CCAT box sequence that appeared to be likely candidates for a promoter, primer extension and S1 nuclease protection analysis were performed utilizing polyA$^+$ RNA from human corpus luteum. Both of these techniques indicated a transcriptional start site 23 bp downstream from the proximal TATAA box, indicating the likelihood that this sequence was serving as a promoter of *P*450arom in human ovarian corpus luteum. By contrast, S1 nuclease protection analysis utilizing placental RNA revealed that the product extended to a site corresponding to the placental intron/exon splice boundary. It appears therefore, that in contrast to the situation in the placenta, *P*450arom expression in the human ovary is regulated by a proximal promoter upstream from the start of translation. This promoter sequence is now referred to as promoter II. When Northern analysis was performed utilizing as a probe sequences specific for expression supported by this promoter, that is sequence between the ovarian transcriptional start site and the intron/exon II splice boundary, it was found that this sequence readily hybridized to transcripts from ovarian corpus luteum. By contrast, there was no detectable hybridization to transcripts from human placenta, indicating once again that this proximal promoter was not utilized in human placenta, which instead uses the distal promoter upstream from exons I.1, and to a lesser extent, that upstream of exon I.2.

5. REGULATION OF AROMATASE CYTOCHROME *P450* GENE EXPRESSION IN HUMAN ADIPOSE

Although in many species, estrogen biosynthesis appears limited to the gonads and the brain, in humans, as well as in some primates, subcutaneous adipose tissue is another site of estrogen biosynthesis. The principal product is estrone which is synthesized from circulating androstenedione derived primarily from the adrenal cortex. In postmenopausal women as well as in men, adipose becomes a major site of estrogen biosynthesis. Estrogen biosynthesis by adipose tissue increases not only with obesity but also as a function of aging and is believed to be a principle causative agent in the incidence of endometrial cancer [9–12]. Local production of estrogens in breast fat has also been implicated in at least some forms of breast cancer [13]. For these reasons, it becomes of considerable interest to study the regulation of aromatase activity in adipose tissue. Utilizing adipose stromal cells as a model system, we have found that *P*450arom expression is induced in such cells by dexamethasone as well as dibutyryl cyclic AMP [26, 27]. The effect of cyclic AMP analogs is markedly potentiated by phorbol esters and is inhibited by a number of growth factors including EGF, TGFα, TGFβ, FGF, TNF, as well as IL-I [28].

For these reasons then, it was of great interest to determine the regulatory domains responsible for *P*450arom gene expression in human adipose tissue. In the first instance, we sought to determine whether or not promoters I.1 or I.2 might be utilized for expression in adipose stromal cells in culture [29]. In order to address this issue, we attempted to amplify transcripts containing these sequences by means of PCR from polyA$^+$ RNA extracted from adipose stromal cells as well as adipose tissue. Such transcripts were undetectable, indicating that neither exon I.1 nor I.2-containing transcripts were present in adipose tissue or adipose stromal cells in culture. In this sense then, *P*450arom transcripts in

adipose are similar to those in human corpus luteum and differ from those in human placenta. On the other hand, primer extension analysis of polyA$^+$ RNA from adipose stromal cells maximally stimulated with dibutyryl cyclic AMP plus phorbol ester indicated a transcriptional start site identical to that found in human corpus luteum, namely, 23 bp downstream from the proximal TATAA sequence, i.e. promoter II. However, S1 nuclease protection assay led to a somewhat different result. Although this analysis indicated that a population of the transcripts extended to the same position as the primer-extended product, namely 23 bp downstream from promoter II, at least 50% of the transcripts extended only to the placental intron/exon splice boundary, in other words, to the identical position found with transcripts derived from placenta. This is the expected result if promoter II was not being utilized. These results would indicate that at least 50% of the transcripts in these adipose stromal cells contained a 5′ end which was spliced into this junction, indicative of a 5′ untranslated exon. Since PCR analysis had ruled out the presence of exon I.1- or I.2-containing 5′ ends, this result is indicative that a third as yet unidentified 5′ untranslated exon is spliced into a number of the transcripts present in these adipose stromal cells [29].

6. THE MECHANISM OF TISSUE-SPECIFIC REGULATION OF HUMAN *P*450arom EXPRESSION

The results of this ongoing work can be summarized as follows (Fig. 5). The expression of *P*450arom in human ovary appears to utilize a promoter which is proximal to the translation start site, that is to say in the "normal" location. This promoter, promoter II, is not utilized in placenta but instead, the promoter utilized to drive aromatase expression in placenta is at least 40 kb upstream from the translational start site. In addition, there is a minor promoter used in the expression of a small proportion of placental transcripts which is 9 kb upstream from the start of translation. Transcripts from these promoters are also expressed in other fetal tissues including placenta-related cells such as JEG-3 choriocarcinoma cells, hydatidiform moles, and other fetal tissues such as fetal liver. We speculate that the ovarian promoter i.e. proximal promoter II, is the primordial promoter regulating *P*450arom expression. This is consistent with results from the laboratory of JoAnne Richards [30] indicating that in rat ovary, *P*450arom expression is regulated by a promoter proximal to the translation start site. Similarly, work from the laboratory of Michael McPhaul [31] indicates that in the chicken ovary, a promoter proximal to the start of translation is also utilized to regulate *P*450arom expression. Since rat placenta does not synthesize estrogens, and chickens do not have a placenta, one would not expect utilization of tissue-specific alternative promoters in these species. We speculate that when the human placenta acquired the ability to synthesize estrogens, since this capacity is very great, and since estrogen production by the placenta tends to be a function of placental size, instead of utilizing the ovarian promoter, a powerful distal

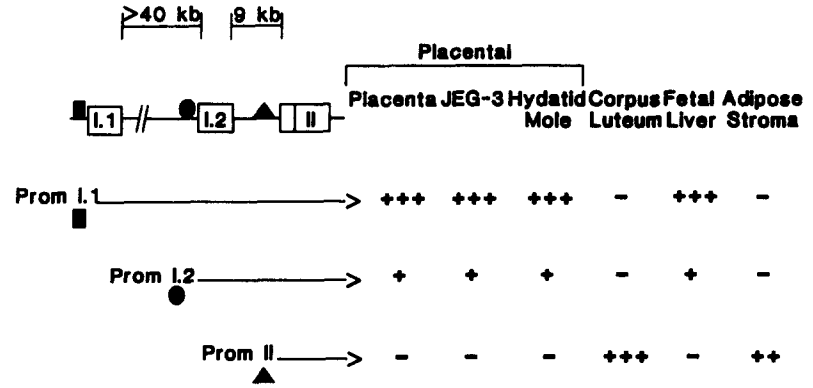

Fig. 5. Schematic representation of the relative utilization of the promoters driving *P*450arom expression. Promoter I.1 (closed box) and Promoter I.2 (closed circle) are expressed in tissues of fetal origin, while promoter II (closed triangle) is expressed in adult tissues. The relatively low expression of exon I.2 containing transcripts in fetal tissues is indicated by a single plus (+). The more abundant form of transcript in each tissue is indicated by three plusses (+ + +). Data from adipose stromal cells in culture (120) suggest the existence of another promoter in this tissue in addition to promoter II; therefore the expression of promoter II has been designated (+ +) to indicate this possibility.

promoter was utilized instead, namely, that upstream from exon I.1. In this context, it is also interesting to speculate on the origins of the secondary placental promoter 1.2. Whether this has any specific functions during particular stages of development or whether it is perhaps a residual promoter sequence whose function has become irrelevant with the passage of evolutionary time, remains to be determined. The situation in adipose again appears to be quite different. Although sequences consistent with the use of the proximal promoter II are present in *P*450arom transcripts in adipose stromal cells, at least one other alternative 5′ end is also present in many such transcripts. The nature of this 5′ untranslated exon and its location within the genome remains to be determined. It is worth noting that the 5′ untranslated exons which have so far been characterized are spliced into a common intron/exon boundary upstream from the translational start site. This means that the protein which is expressed in each of the various tissue-specific sites of estrogen biosynthesis is identical, in contrast to a number of other genes in which alternative splicing results in differences in the amino-terminus of the protein itself.

It appears that human *P*450arom is the first cytochrome *P*450 to be shown to utilize alternative promoters in the regulation of tissue-specific expression. Although no other cytochrome *P*450 has been shown to utilize this form of regulation of expression, a growing number of other proteins do. These include the genes for IGF-II and -I, glucokinase, c-myc, c-fms, and aldolase A [32–36]. Interestingly, at least two other genes have been shown to utilize a different promoter to regulate expression in placenta and related tissues, as compared to their classical tissue of expression. Thus, human prolactin expression in decidua appears to be regulated by a distal promoter [37], as does growth-hormone releasing hormone expression in placenta [38]. Thus, at least 3 genes utilize a distal promoter to regulate their expression in placenta or uterine tissue as compared to other tissues. This, however, does not appear to be a universal truth. For example, α-glycoprotein gene expression in placenta appears to utilize a unique regulatory region upstream from a single promoter rather than an alternative promoter.

It is pertinent to ask why the *P*450arom gene resorts to this mechanism for regulation of tissue-specific expression, whereas this is not so for any of the other *P*450 genes so far characterized. The answer probably relates to the unique tissue-specific distribution of estrogen biosynthesis in the human. In contrast to most other species where estrogen biosynthesis is confined to the gonads and the brain, in the human it is also present in adipose and in placenta. In both these tissues *in vivo*, estrogen biosynthesis appears to be relatively unregulated, although dramatic regulation can be achieved utilizing adipose stromal cells in culture. By contrast, the expression of estrogen biosynthesis in the placenta is very great, whereas that in adipose is rather low. It is apparent that the estrogen produced in each of these tissues in the human subserves a different function and consequently, the regulation of this biosynthesis must be different in each tissue, and this is apparent from *in vitro* studies. Consequently, it can be surmised that a single promoter with a number of upstream regulatory sequences would be inadequate to permit such sophisticated complexity of tissue-specific regulation, and it is for this reason that this particular gene has resorted to the use of alternative promoters to allow for greater versatility in determining tissue-specific regulation of expression.

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