Regulation of Placenta-specific Expression of the Aromatase Cytochrome P-450 Gene

INVolVEMENT OF THE TROPHOBlast-SPECIFIC ELEMENT BINDING PROTEIN*

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The aromatase (cytochrome P-450,AROM) gene contains multiple untranslated exons I that are differentially transcribed in a tissue-specific manner. DNA sequences within the initial –301 upstream of placenta-specific exon I (exon la) are sufficient for placenta-specific expression of aromatase. In gel mobility shift assay, three separate domains in this region form specific binding complexes with proteins extracted from choriocarcinoma JEG-3 nuclei. A fragment containing these domains activates transcription driven by a heterologous promoter in a cell type-specific manner. Two of the binding domains that form major complexes in gel shift assay compete with each other and with a DNA fragment containing the trophoblast-specific element (TSE), which is derived from the enhancer region of the human chorionic gonadotropin α-subunit gene and is believed to confer placenta-specific expression of the gene. The core sequence RCNTNNRG is sufficient for recognition of the TSE-binding protein, which is detected only in nuclear extracts prepared from placenta and choriocarcinoma. A mutation introduced in the distal TSE core in aromatase promoter resulted in marked reduction of transcriptional activity, although TSE region by itself did not show enhancer activity as that in human chorionic gonadotropin α-subunit gene.

Aromatase (cytochrome P-450,AROM) is a unique member of the cytochrome P-450 superfamily. It catalyzes the conversion of androgen to estrogen, which is a rate-limiting step in estrogen biosynthesis (1). Aromatase is expressed in various cells and tissues including ovarian (1–4), testicular Sertoli and Leydig cells (5, 6), placenta (7, 8), adipose tissue (9), skin fibroblasts (10), and various parts in the brain including the amygdala and hypothalamus (12, 13). Recent investigations revealed that aromatase mRNAs expressed in adipose tissue, ovary, fetal liver, and brain have differences in the untranslated exon I from that reported previously for the placenta (14–18). The use of alternative transcription start sites that occur as a consequence of employing tissue specific promoters is a unique feature of the aromatase gene expression and seems to be the underlying molecular basis for the complexity of the expression of this gene. However, how individual promoters exert their tissue specificities is not known.

The placenta is the primary site of estrogen synthesis in pregnant women and 16α-hydroxydehydroandrosterone sulfate derived from the fetal adrenal and liver is the major precursor for placental estrogen production. Human aromatase mRNA expressed in placenta has a unique untranslated exon I, which is designated as exon la in this laboratory (16, 17) or I-1 by Mahendroo and others (14, 15). This type of aromatase mRNA is detected only in placenta and in cultured cells of trophoblast origin. Although cDNAs containing other types of untranslated 5′-region have been isolated from placental cDNA libraries (19, 20), the 5′-end of exon la is the major initiation site in placenta. A choriocarcinoma cell line JEG-3 expresses aromatase mRNA of placental type (14, 21). This cell line expresses aromatase in unstimulated condition as well as in response to phorbol ester and various reagents that raise intracellular cAMP levels (21, 22). The aromatase activity in the cells was closely related to the content of aromatase mRNA that was very labile, suggesting transcriptional control is the main regulatory point for the enzyme activity. Thus, this cell line seems to provide a good system to investigate transcriptional regulation of placental type aromatase.

In the present paper, we describe analysis of the 5′-flanking region of aromatase exon Ia for the placenta-specific expression of this gene. We show that the initial –301 upstream of the exon Ia is sufficient for the basal expression of the exon. This region, conferring cell specificity, contains at least three binding domains. Two of them are recognized by the same transacting factor that binds to the trophoblast-specific element previously located in the enhancer region of human glycoprotein hormone α-subunit (23). A common transcription factor appears to be involved in the expression of two hormone-related genes that are characteristic of human placenta.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer or were obtained commercially from Toa Gosei Co. (Tsukuba, Japan). The sequences of the double-stranded oligonucleotides used in this paper are as follows (only leading sequences are shown): C2, 5′-AGGTGCTTTAGGCCTCAGGAAACAGAA-3′; C2m, 5′-AGGTGCTTTAGGAATCAGGAAACAGAA-3′; C2l, 5′-AGGTGCTTTAGGCCTCTGGAAACAGAA-3′; C2l2, 5′-AGGTGCTTTAGGAATCAGGAAACAGAA-3′; C2p, 5′-AGGTGCTTTAGGCCTCAGGAAACAGAA-3′; C2p, 5′-AGGTGCTTTAGGCCTCAGGAAACAGAA-3′; C3, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C3m, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C3l, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C3l2, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C3p, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C3p, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C4, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C4m, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C4l, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C4l2, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C4p, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′; C4p, 5′-GACCCTTCCAGGAGGCCATTAGTG-3′. The complementary oligonucleotides were annealed to form double-stranded oligonucleotides. Their concentrations were determined by densitometry after electrophoresis through 2% SeaPlaque GTG-agarse (FMC) gels and staining with ethidium bromide. The following oligonucleotides were used as
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FIG. 1. Schematic illustration of the aromatase exon 1a 5′-flanking region. Bold arrows indicate the 5′-end of the designated CAT constructs. Numbers are relative positions from the initiation start site of aromatase exon 1a. Thin arrows represent PCR primers used in the amplification of promoter fragments. The fragments used for construction of CAT cassettes and gel mobility shift assays are shown below with their identities indicated on the left. Recognition sequences of potential cis-acting elements Sp1, PEA3, and Ad4 are boxed.

Luciferase and Chloramphenicol Acetyltransferase Assay—The reference plasmid RSV/Luci in CAT assay expresses the luciferase protein under control of the Rous sarcoma virus promoter. Luciferase assays were performed as described by Brasier et al. (28). 10 µl of lysate from each plate was added to 100 µl of 25 mM glycylglycine buffer (pH 7.5), 5 mM MgCl2, and 5 mM ATP, and the reaction was started by injection of 100 µl of 25 mM glycylglycine buffer (pH 7.5) containing 0.1 µmol of luciferin and 1 mM dithiothreitol into the sample. Light output was measured for 30 s at 25°C using a Luminescence Reader BLR-301 (Aloka). A CAT assay was conducted as described (29) with samples containing equal amounts of luciferase units. The amounts of acetylated chloramphenicol on TLC plates were quantitated with a Bioimage Analyzer BA100 (Fujifilm Co.).

Preparation of Nuclear Extracts—Nuclear extracts from JEG-3, HeLa, and HepG2 cells were prepared as described by Urbiumi et al. (30) and from human placenta according to the procedure described by Gorski et al. (31). Protein concentrations were determined with a BCA protein assay kit using bovine serum albumin as a standard.

Gel Mobility Shift Assays—DNA fragments FB, FD, and HB were created by polymerase chain reaction and size selected by electrophoresis through 2% SeaPlaque GTG agarose gels. They were end labeled with [α-32P]dCTP by the Klenow fragment of Escherichia coli DNA polymerase I. The nuclear extract was incubated with radioactive probe (approximately 10 000 cpn) at room temperature for 30 min in a final volume of 22 µl of 20 mM HEPES KOH (pH 7.9) buffer containing 2 µg of poly(dI-dC) (Pharmacia Biotech Inc.), 50 mM KCl, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol. For the competition analysis, nuclear extract was incubated for 10 min with the indicated molar excess of cold oligonucleotide prior to addition of the radiolabeled probe. The samples were then loaded on a 5% non-denaturing acrylamide gel (acrylamide:bisacrylamide ratio, 29:1) and run for 2.5 h at 4°C at 175 V. Gels were dried and exposed to Kodak-Omat AR film at −70°C in the presence of intensifying screens for 16–48 h.

RESULTS

Specificity of the Promoter/Regulator Region of Exon 1a—When various lengths of truncated 5′-flanking region of aromatase exon 1a up to 12 kilobases were transiently expressed in JEG-3 cells, a choriocarcinoma cell line, the shortest construct that showed the full transcription activity was a CAT construct containing −301 to +22 of aromatase exon 1a (p-301ACAT). A

The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); CAT, chloramphenicol acetyltransferase; tk, thimidine kinase; TSE, trophoblast-specific element; TSEB, TSE-binding protein.

[Image 49x500 to 552x742]
shorter construct, p-212ACAT, exhibited 14% of full activity, and p-115ACAT had minimal transcription activity (Fig. 2). Thus, the region downstream of -301 is likely to contain the regulatory domain for basal expression of exon Ia, namely, placental type aromatase mRNA.

These three constructs and a CAT construct with the tk promoter were transfected into two other cell lines: a hepatic cell line, HepG2, and HeLa cells. In HepG2, exon Ib-type aromatase mRNA, a major form expressed in fibroblasts and fetal liver, was found. HeLa cells did not express detectable aromatase mRNA, a major form expressed in fibroblasts and fetal cells. In HepG2, exon Ib-type aromatase mRNA, a major form expressed in fibroblasts and fetal liver, was found. HeLa cells did not express detectable aromatase mRNA, a major form expressed in fibroblasts and fetal liver, was found.

### Determination of Binding Domains

For further analysis of this regulator/promoter region, labeled DNA fragments encompassing this region and nuclear extracts from JEG-3 cells were prepared for gel mobility shift assay. In Fig. 1, a schematic diagram of the promoter region of aromatase Ia is illustrated with the size and location of tested fragments in the gel mobility shift assay (start points of the CAT constructs listed in Fig. 2 are shown with arrows).

As shown in Fig. 3, a fragment spanning -307 to -142 (designated as FB) gave two specific bands (panel A, lane 1), which are indicated with arrows. These bands were diminished with a 400× molar excess of unlabeled FB as a competitor (lane 2). As illustrated in Fig. 1, this region contains several potential cis-elements such as Ad4 (32), PEA3 (33), and Sp1 (34). Ad4 sequences exist in regulatory regions of various steroidogenic P-450 genes, and this element dictates expression specific to steroidogenic tissues. Oligonucleotides containing consensus sequences for Ad4 (5′-GGACATACCCAAAGTCCCTTTT-3′) (32) or PEA 3 (5′-TCGAACTTCTTGCTCGA-3′) (33) did not compete for binding (Fig. 3A, lanes 6 and 7). The major binding complex formed with labeled FB was also competed by some segments of FB, namely, FD (-307 to -210), FC1 (-307 to -240), and HB (-217 to -142) (lanes 3-5). The smallest fragment that competed for the binding was the 27-bp fragment, C2 (-300 to -274), included in the FD fragment (Fig. 3A, lane 8). The formation of the minor binding complex was inhibited by the HB and HM (-217 to -166) fragments (lanes 5 and 9). When the FD fragment was used as a probe (Fig. 3B), only the major band appeared as a specific binding complex (Fig. 3B, lane 6). Unlabeled FD, FC1, HB, and C2 (Fig. 3B, lanes 1-4) competed for this binding.

In experiments where the HB fragment was used as a radiolabeled probe (Fig. 3A, panel C), two specific bands appeared as shown by the arrow and arrow head. The major band was inhibited by excess amounts of unlabeled HB, FC1, C2, and C4.
C, (data not shown). Both of the resulting fragments of digested HB probe completely lost the ability to form the major band with excess of unlabeled C2 fragment as a competitor in lane 1. MvaI cuts the HB fragment at least one base in each of the four pairs was necessary for the recognition. These mutated C2 fragments also showed similar competitive effects on formation of the major binding complex with HB probe, which contains proximal binding domain C4. This result further shows that these two binding domains are recognized by a same trans-factor.

As we found that this sequence requirement for C2 and C4 binding was not conflicting with that of TSE, which resides in the upstream region of human chorionic gonadotropin α-subunit gene (23, 35, 36), the 24-bp segment (−182/−159), designated as TSE in the promoter of the α-subunit gene (37), was synthesized and examined for competition with the binding of C2, C4, and HB. As shown in Fig. 6A, TSE effectively inhibited formation of the binding complex of C2, C4 (lanes 2 and 5), and the major complex with HB (lane 9). The binding factor(s) for TSE is referred to as the TSE-binding protein (TSEB) (23). Little is known about TSEB except its cell line-specific occurrence and that it cannot bind to a TSE homologue that has a C to T mutation at −172 (TSEm−172) (37). The failure of this mutated TSE to compete for the binding of C2, C4, and HB (lanes 1, 6, and 10) further confirms that the binding activity to the C2 and C4 regions arises from the same factor with TSEB. As C2 and C4 are recognized by the same factor(s) TSEB, their core binding domains are designated as TSE-Cprom 1 and 2, respectively. Interestingly, the minor complex formed with labeled HB was competed by both TSE and TSEm−172 (indicated by an arrow) (lanes 9 and 10). In panel B, three sequences are aligned to give a consensus sequence for competitive effects on formation of the major binding complex with HB probe, which contains proximal binding domain C4. This result further shows that these two binding domains are recognized by a same trans-factor.

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TSEB recognition. The consensus sequence RNCCTNNRG also suffices requirement for the binding shown in Fig. 5. The observation that the C4 fragment with a mutation at −166 and −167 (CC to GG) failed to compete for the binding of C4 (data not shown) also supports this alignment.

In the last experiment transcriptional activity of this region was further examined (Fig. 7). The HB fragment that contains the TSE-C exon 2 and C3 enhanced transcription when linked to the tk promoter in both directions and placed downstream of tk. The full activity of pFBCAT was not retained when the fragment was reversely placed upstream or downstream of tk promoter. The C2 fragment only marginally activated transcription when single, double, or four copies of the 26-bp fragment were linked to the tk promoter. As the distal TSE core sequence did not enhance transcription by itself, transcriptional activity of a pFBCAT that has two G to T mutations at −283 and −282 (corresponding C2n-type mutation in Fig. 5) was examined. This mutation resulted in marked reduction of the transcription activity.

DISCUSSION

In the present study, we have shown that DNA sequences within the initial −301 upstream of placenta-specific exon 1 (exon Ia) are sufficient for basal transcription of placental type aromatase mRNA. Transcription activity directed by this promoter/regulator is cell type specific. Further deletion resulted in gradual loss of the transcriptional activity, suggesting involvement of multiple regulatory elements as shown in other systems (38). The fragment encompassing the distal half of the region has an activity to enhance transcription driven by a heterologous tk promoter, which was also cell type specific.

Gel mobility shift assay demonstrated three distinct binding domains in this region. Two separate binding domains at −300 to −274 (C2) and −177 to −153 (C4) form a major complex. The mutual trans-acting factor for C2 and C4 was further found to be the same factor that recognizes the TSE, which resides in the upstream region of the human glycoprotein hormone α-subunit gene (Fig. 6) (23, 35–37). In human, this gene is expressed in the pituitary as subunits of glycoprotein hormones as well as α-subunit of chorionic gonadotropin in placenta. The placental expression of this gene has been extensively studied; it requires a multi-component enhancer composed of tandem cAMP-responsive elements and an adjacent upstream regulatory element. TSE is described as a subdomain of upstream regulatory element and is considered to be a regulatory element that confers tissue-specific expression. Although little is known about its binding protein, TSEB (23, 37), the cytosine at −172 in TSE is critical for recognition of TSE sequence by TSEB. The observation that TSE with a C to T substitution at −172 also failed to compete for binding to C2 or C4 gives support to the conclusion that TSEB is the factor. The two new TSE-like sequences in the aromatase promoter and the competition analysis of their homologues with sequentially introduced mutations now reveal the sequence required for TSEB recognition, namely, the core sequence of TSE (TSE-C) as RNCCTNNRG.

In the α-subunit promoter, TSE is associated with another element, αACT, adjacent to two tandemly repeated cAMP-responsive elements to compose a tissue-specific enhancer. TSE in the α-subunit promoter has little independent transcriptional activity when linked to homologous or heterologous promoters, and only when linked to the cAMP-responsive elements does it stimulate basal and cAMP-dependent expression. Likewise, the trophoblast-specific enhancer in the aromatase gene may be composed of multiple cis-elements including two
TSE-Cs, although neither of them is associated with cAMP-responsive element or other known cis-elements. The 27-bp DNA fragment containing TSE-C<sub>2ram</sub> was inactive to stimulate tk-driven transcription by itself even when two or four tandem copies were introduced. The binding to TSE-C<sub>2ram</sub>1, however, seems to make an indispensable part of the aromatase enhancer. A pFBCAT2 analogue that has a mutation which eliminates TSE-C<sub>2ram</sub>1 binding loses most of the transcriptional activity that the parent pFBCAT2 shows. At present we do not have the direct evidence of the contribution of the proximal TSE-C or the C3 region to the enhancer activity. The two TSE-Cs seem to be equivalent in terms of recognition by TSEB, but their function may be different because of their position. Indeed, as shown in Fig. 7, the transcriptional activities of FB and HB fragments seem to be affected by their orientation and position in the CAT constructs.

Importance of the region between –242 and –183, which contains the C3 domain, was shown by Toda et al. (39, 40) in transient transfection assays in BeWo cells. They reported that the 89-bp fragment had an enhancer activity when multiple copies were placed with a heterologous promoter. Although the binding to the C3 domain was not competed by the two TSE-C<sub>2ram</sub> sequences, the TSE derived from the α-subunit gene competed for binding to the C3 domain, and the TSE<sub>μ</sub>–172 that failed to compete for TSE and TSE-C<sub>2ram</sub>5 is competitive for binding to the C3 region. Pittmann et al. (37) recently described a new binding protein that recognizes the region partly overlapping TSE in the α-subunit promoter. This transacting element binds to a distinct recognition sequence from that of TSEB and seems to be involved in placenta-specific expression of the α-subunit gene as well. It is therefore of particular interest whether this second upstream regulatory element binding protein is also involved in the expression of placental aromatase.

In this study, we demonstrated that TSE-like elements are located in the promoter region of placenta-specific exon I of aromatase and that at least the distal TSE-like element is transcriptionally functional. TSE-like elements in aromatase gene seem to compose a placenta-specific enhancer with other factor(s), as shown in the α-subunit gene, though their compositions seem to be quite different. The observation that the element recognized by TSEB, originally found in the α-subunit gene, also functions in the promoter regions of unrelated genes to confer placenta specificity emphasizes its functional importance as a placenta-specific transcription element. Although the α-subunit and aromatase genes are not closely linked in evolution, both are involved in producing hormones characteristic of the human placenta. Interestingly, the placental expressions of these genes are observed only in some mammals. Nevertheless, the fact that the two hormones are secreted from an early stage of pregnancy and play important roles in development and maintenance of the human placenta highlights the significance of a common trans-acting factor governing their expression. A potential TSE-C sequence is seen in the immediate upstream of human chorionic gonadotropin β-subunit genes (41), though their functional significance has not been tested.

Although various types of transcription factor have been found in trophoblasts so far, only a few are specific to this cell type (42). Recently, transcription factors of the basic helix-loop-helix family that are important cell lineage determinants in many cell types have been identified with limited occurrence in extra-embryonic structures (43, 44). At present, however, their immediate target genes have not been shown. TSEB is likely to be a immediate regulator of the genes in the later stage of placental differentiation. Purification of TSEB and cDNA-don-
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