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Unique regulation of expression of human aromatase in the placenta☆

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Abstract

The expression of human placental aromatase is transcriptionally regulated through the promoter region of exon 1a (I.1) of the gene. We examined the transcriptional regulation by using human choriocarcinoma-derived JEG-3 cells which also express aromatase mRNA transcribed under the control of the placenta-specific promoter of the exon 1a. Aromatase in the cells was induced by forskolin (cAMP) and phorbol ester (TPA) in both levels of the activity and the mRNA. However, any elements responsible for the cAMP-responsiveness have not yet identified. To identify and characterize the specific elements, CAT assay of the placenta-specific promoter was performed. We reconstructed an 11.5 kb gene structure consisting of exons 1a (I.1), 1b (I.4), 1c (I.3), and 1d (PII) and their proximal promoter regions to mimic the native structure of human aromatase gene and performed a promoter assay by the transient expression of a CAT reporter carrying the mini-gene structure. The construct was transcribed from exon 1a in JEG-3 cells and exon 1b in HepG2 cells to produce tissue-specific mRNAs from the exons 1–CAT hybrid gene, indicating that the mini-gene structure contained promoter regions essential for the tissue-specific expression. However, unexpectedly exons 1–CAT hybrid mRNA in JEG-3 cells was not induced by forskolin. Then, we prepared JEG-3 cells transformed by incorporation of the exons 1–CAT hybrid gene into the chromosomal DNA. The cells stably expressed the hybrid reporter gene which was transcribed from exon 1a and induced by forskolin and TPA. These results suggest that enhancers on the promoter regions of exons 1b, 1c, and 1d might interact with a transcriptional machinery of exon 1a in the induction by forskolin and TPA. Finally, a β-galactosidase gene connected with the 11.5 kb gene structure was introduced into mouse eggs to produce transgenic mice. The hybrid gene was transcribed from exon 1c in the gonadal tissues of all lines of the transgenic mice in accordance with the tissue-specificity of human aromatase gene, whereas it was not transcribed from exon 1a, but from exons 1b and 1c in the all placentae. The results suggest that the mouse placenta might lack in the transcriptional elements or factors essential for the placenta-specific expression of human aromatase gene.

Keywords: Aromatase; Placenta; JEG-3 cell; cAMP-responsive element; Transgenic mouse; Tissue-specific expression

1. Introduction

Aromatase (estrogen synthase) is a key enzyme catalyzing the conversion of androgen to estrogen in estrogen biosynthesis. In humans, it is expressed not only in gonadal tissues such as ovary [1], testis [2], placenta [3], and prostate [4], but also in extra-gonadal tissues such as brain [5], skin [6], liver [7], and bone [8], blood vessel [9], and adipose tissues [10] and participates in the various physiological functions of those tissues through the tissue-specific regulation of estrogen production by various kinds of cytokines and lipids.

The presence of multiple exons 1 in human aromatase gene were first shown by Means et al. [11]. Furthermore, we reported that the aromatase gene was tissue-specifically regulated through the alternative utilization of the multiple exons 1 consisting of exons 1a (I.1), 1b (I.4), 1c (I.3), 1d (PII), 1e (I.2), and 1f specific for expression in the placenta, skin/fetal liver/bone/blood vessel/adipose tissues, ovary, ovary/prostate/testis, placenta, and brain, respectively [12]. Each of the tissue-specific exons 1 is flanked by a unique promoter region containing basic and regulatory promoter or enhancer elements. These results strongly indicate that the expression of aromatase in various human tissues is regulated by tissue-specific utilization of the multiple exons 1 and promoters. Because it was fairly difficult to obtain suitable cell lines for transcriptional analyses of each of the multiple promoter regions of human aromatase gene, except for availability of human choriocarcinoma-derived JEG-3 or BeWo cells in which aromatase was transcribed from the placenta-specific exon 1a, the early study about the transcriptional regulation of human aromatase gene was thoroughly done on the placenta-specific promoter region. The choriocarcinoma-derived cells express placenta-specific aromatase transcripts inducible by phorbol ester (TPA) and various reagents that raise intracellular cAMP levels [13]. Thus, these cells seem to provide a good model system

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to investigate the transcriptional regulation of placental aromatase expression.

The characterization of the placenta-specific promoter region was performed by transient expression of a CAT reporter in BeWo cells, and the region spanning from −242 to −166 bp upstream of the exon 1a was identified as a cell-type specific enhancer element [14]. Furthermore, a NF-IL6 binding site localized between −2141 and −2115 bp was identified as a *cis*-acting element important for the transcriptional enhancement in response to TPA [15]. However, no typical cAMP-responsive elements (CRE) have found in the placenta-specific promoter region, and any *cis*-acting elements responsible for the transcriptional induction of the gene by cAMP have not yet unidentified. On the other hand, the essential promoter region for the placenta-specific expression of human aromatase gene was studied using the transient promoter assay in cultured cells [16] and the stable promoter assay in the transgenic mouse by truncating the proximal promoter region to the placenta-specific exon 1a [17]. Both assays indicated that the 5′-flanking region within 500 bp of the exon 1a (I.1) was essential for the placenta-specific expression of the gene. However, it has remained unclear whether the 500 bp promoter region contained necessary and sufficient elements for the placenta-specific expression of the gene.

In the present study, we first characterize the regulatory mechanism of the transcriptional induction from the placenta-specific promoter of human aromatase gene in response to cAMP by expression of a newly developed CAT reporter gene in the stable transformant cells. Furthermore, we also reconstruct an artificial gene consisting of the multiple exons 1 and promoters of human aromatase gene, and verify the essential promoter region for the placenta-specific expression of human aromatase gene by analyzing tissue-specific utilization of the multiple exons 1 of the artificial gene in the in vitro culture cell system and in vivo transgenic mouse system.

2. Transcriptional regulation of human aromatase gene in JEG-3 cells

As human choriocarcinoma-derived JEG-3 cells showed high levels of aromatase mRNA transcribed from placenta-specific exon 1a, we adopted JEG-3 cells as a model for a study of the placenta-specific expression of human aromatase gene. Regulation of aromatase gene expression in JEG-3 cells was examined in the levels of the activity and the mRNA by TPA and forskolin raising intracellular cAMP levels as shown in Fig. 1. Both the activity and the mRNA of aromatase in the cells were almost parallel increased by TPA and forskolin. Consequently, aromatase was increased 1.5–3-fold by TPA and 3–5-fold by forskolin. Simultaneous treatment of the cells with TPA and forskolin caused synergistical increase (6–12-fold) of both levels of aromatase activity and mRNA.

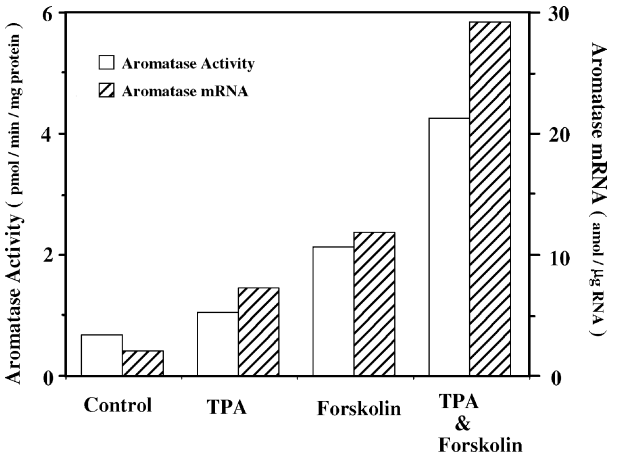

Fig. 1. Induction of aromatase activity and mRNA in JEG-3 cells by forskolin and TPA. Aromatase activity in JEG-3 cells was measured 24 h after drug treatment using 1β-[^{3}H] androstenedione as the substrate [9]. The levels of aromatase mRNA in JEG-3 cells were fluorometrically measured 12 h (TPA) or 24 h (forskolin) after drug treatment by quantitative RT-PCR in the presence of standard RNA [34].

To identify DNA elements responsible for the transcriptional induction of aromatase gene in JEG-3cells by TPA and forskolin, a conventional CAT assay was carried out for the 5′-promoter region proximal to the placenta-specific exon 1a. As shown in Fig. 2, the CAT reporter carrying the 5′-flanking region within 810 bp upstream of the exon 1a was

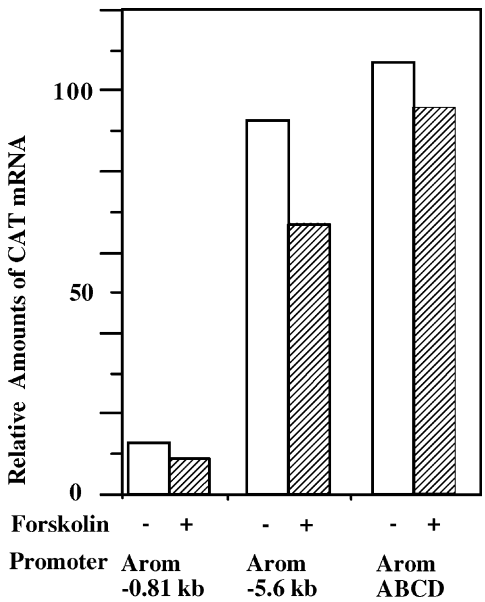

Fig. 2. Transient promoter activities of the 5′-flanking regions of exon 1a of human aromatase gene in JEG-3 cells in the presence or absence of forskolin. JEG-3 cells were transiently transfected with CAT reporter genes carrying 0.81 kb (Arom −0.81 kb) and 5.6 kb (Arom −5.6 kb) 5′-flanking regions upstream of exon 1a of human aromatase gene, and a compact construct of the multiple exons 1/promoters of the gene (Arom ABCD) [34,35], and cultured for 36 h. The promoter activity was analyzed 24 h after forskolin-treatment by measuring the CAT reporter mRNA in the transfected JEG-3 cells by quantitative RT-PCR [34].

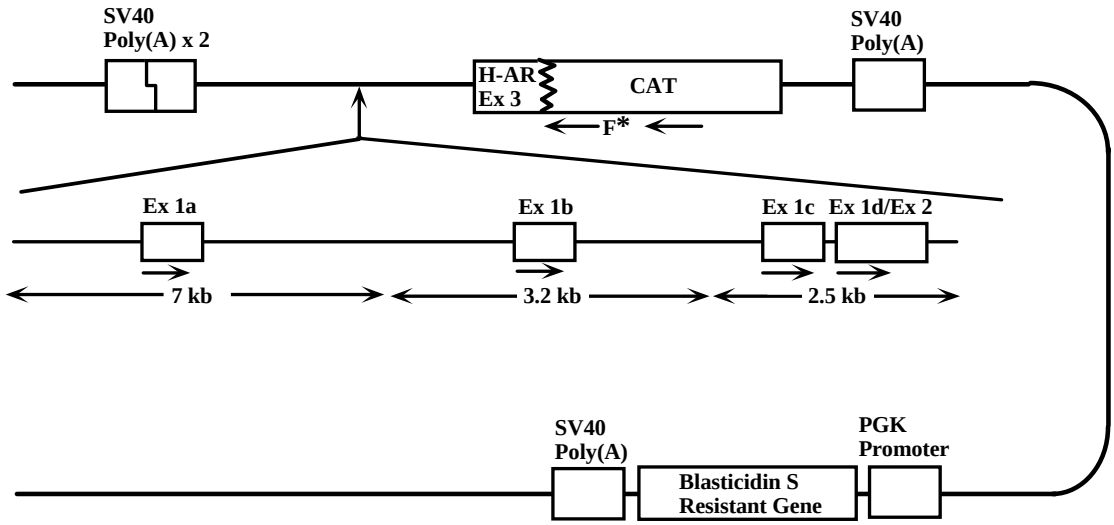

Fig. 3. The construct of a CAT reporter gene for a promoter assay in the stable transformant JEG-3 cells. JEG-3 cells were transfected with the CAT reporter gene, and selected by blasticidin *S*-resistance to establish the stable transformant cells. The promoter activity was measured by determination of the CAT mRNA by RT-PCR. The positions of each of exons 1-specific sense primers and the CAT gene-specific antisense primers were indicated in the figure by arrows. F* shows a fluorescent dye, 2-FAM.

transcribed in JEG-3 cells after liposome (lipofectamine; Gibco-BRL)-mediated transfection. The transcriptional activity of the CAT reporter was further increased when the 5′-promoter region within 5.6 kb upstream of the exon 1a was used in the reporter. However, in both cases, addition of forskolin to JEG-3 cells did not give any increase of the transcription from the reporter gene, rather suppressed the transcriptional activities. The promoter elements responsible for the forskolin responsiveness were further searched for the promoter region up to −9.1 kb upstream from the exon 1a, but any elements could not be found (data not shown).

We supposed that the structure of human aromatase gene consisting of multiple exons 1 and promoters is necessary for the forskolin-responsive expression. So we isolated all regions of the multiple exons 1 and promoters of the gene, reconstructed to be a compact reporter gene structure containing minimum exons 1 and promoters by truncating the redundant DNA regions, and analyzed forskolin-responsive expression of the compact reporter gene in transfected JEG-3 cells. Even the compact reporter gene consisting of exons 1a, 1b, 1c, and 1d and their proximal promoter regions did not show any increase by addition of forskolin to the cells.

All conventional CAT assays using the promoter region of exon 1a and multiple exons 1/promoters did not give any positive results concerning forskolin-inducible promoter activity in JEG-3 cells. So, the promoter activity of the compact reporter gene was analyzed after it had been stably incorporated into the chromosomes of JEG-3 cells to mimic the chromosomal structure of aromatase gene in the cells. As shown in Fig. 3, a compact reporter gene carrying multiple exons 1/promoters and a blasticidin S-resistant gene as a selection marker was constructed and then introduced into JEG-3 cells by liposome-mediated transfection. The transfected cells were selected by the blasticidin *S*-resistance and 30–40 clonal cells of the stable transformants were isolated for the promoter assay. The transformant cells (ABCD in Fig. 4) showed 7-fold increase of CAT mRNA by forskolin and 14-fold increase of the mRNA by forskolin plus TPA, in good accordance with inductive responses of

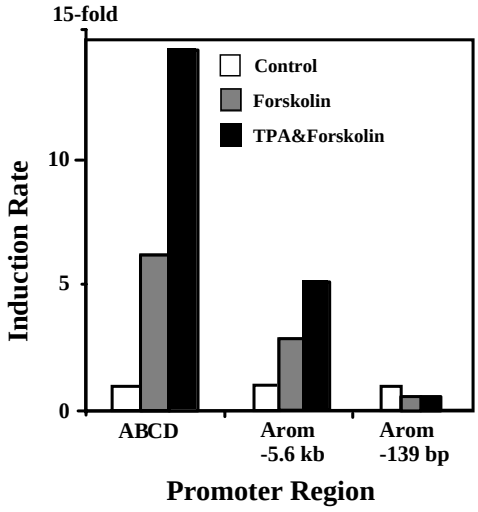

Fig. 4. Stable promoter activities of the 5′-flanking regions of exon 1a of human aromatase gene in JEG-3 cells in the presence or absence of forskolin and TPA. The CAT reporters carrying 139 bp (Arom −139 bp) and 5.6 kb (Arom −5.6 kb) 5′-flanking regions upstream of exon 1a of human aromatase gene, and a compact construct of the multiple exons 1/promoters of the gene (ABCD) were used to prepare stable transformant JEG-3 cells (Fig. 3). JEG-3 cells transformed with the CAT reporter gene were treated with forskolin and TPA, and measured expression levels of the reporter CAT mRNA by quantitative RT-PCR 24 h after the treatment [34].

aromatase gene by the drugs in intact JEG-3 cells. Furthermore, all of the CAT transcripts were transcribed from the placenta-specific exon 1a (data not shown). The removal of exons 1b, 1c, and 1d and their promoter regions decreased inducibility of CAT mRNA in the cells by the drugs. The promoter region within 5.6 kb upstream from the exon 1a (Arom −5.6 kb in Fig. 4) caused significant but less increases of CAT mRNA by the drugs, whereas that within 139 bp upstream of the exon 1a (Arom −139 bp in Fig. 4) did not cause any increases by the drugs. These results suggested that the transcription from placenta-specific exon 1a was regulated by both the proximal and distal promoter regions, including the other promoter regions of the multiple exons 1 of human aromatase gene, and the proper chromosomal structure of the gene was necessary for the accurate transcriptional regulation from exon 1a by the inducers.

3. The promoter region responsible for placenta-specific expression of human aromatase gene

We analyzed the promoter region responsible for tissue-specific or cell-specific expression of human aromatase gene. The CAT reporter gene carrying the compact multiple exons 1/promoters of human aromatase gene was transiently introduced into JEG-3, HepG2, and HeLa cells by liposome-mediated transfection. Both of choriocarcinoma-derived JEG-3 cells and hepatocarcinoma-derived HepG2 cells significantly express aromatase mRNAs which are predominantly transcribed from exons 1a and 1b, respectively, whereas endocervical carcinoma-derived HeLa cells shows no expression of aromatase mRNA. As shown in Fig. 5, the CAT reporter gene was transcribed specifically from exon 1a in JEG-3 cells and from exon 1b in HepG2 cells in accordance with the exon 1 preference of aromatase transcripts in each cells. On the other hand, we could not observe any CAT transcripts in HeLa cells which was used as a negative control, although a thymidine kinase (tk) promoter-driven CAT control reporter, a positive control reporter, was greatly expressed in all of JEG-3, HepG2, and HeLa cells. These results strongly suggested that the compact multiple exons 1/promoters contained necessary elements for tissue-specific or cell-specific transcription from the exons 1a and 1b.

The analysis using in vivo expression system of a transgenic mouse was designed to confirm the conclusion obtained from the analysis using the in vitro culture cell system. The β-galactosidase structural gene controlled by the compact multiple exons 1/promoters of human aromatase gene was constructed as a reporter transgene. The in vivo tissue-specificity or cell-specificity of the compact reporter gene was evaluated by analyzing expression of β-galactosidase in various tissues of the transgenic mice. Fig. 6 shows expression levels of the β-galactosidase reporter mRNA in various tissues of the transgenic mice. In this experimental condition, the transcription of the reporter gene in the transgenic mice was expected to be regulated

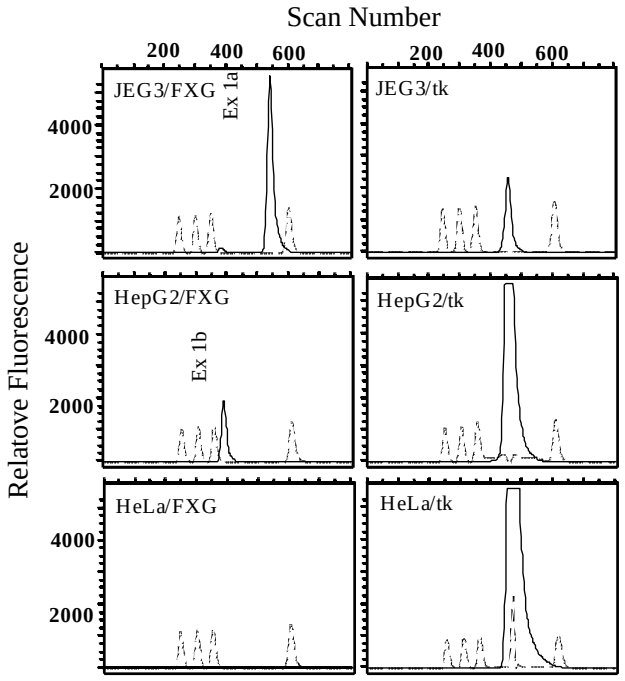

Fig. 5. Cell (tissue)-specific utilization of the multiple exons 1 of human aromatase gene in various cells. The CAT reporter gene carrying a compact construct of the multiple exons 1/promoters of human aromatase gene was transiently introduced into the cells, and both the promoter activity and the preference in utilization of the multiple exons 1 of the gene were analyzed in JEG-3, HepG2, and HeLa cells [34]. As controls, the CAT reporter gene carrying the 5′-promoter region of thymidine kinase (tk) gene was also introduced into the cells and analyzed.

by promoter regions of exons 1a, 1b, 1c, and 1d of human aromatase gene. The expression levels were quite high in gonadal tissues such as testis, ovary, and placenta, low but significant in the skin, brain, bone, adipose, and arterial tissues, and traceable in the liver and kidney. The expression

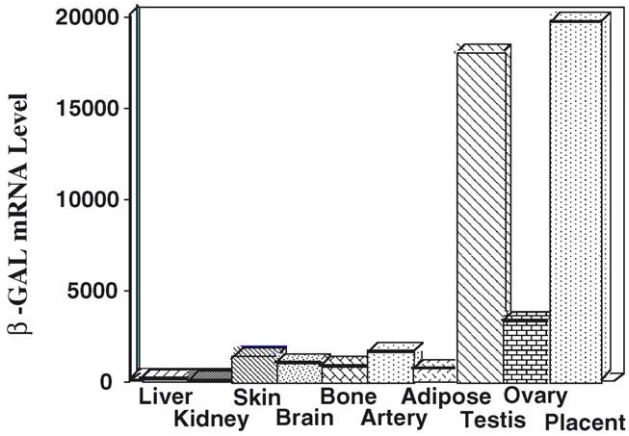

Fig. 6. Expression levels of the mRNA of a β-galactosidase reporter gene controlled under a compact construct of the multiple exons 1/promoters of human aromatase gene in various tissues of transgenic mice carrying the reporter gene. The expression levels of the reporter gene in various tissues was measured by quantitative RT-PCR [34].

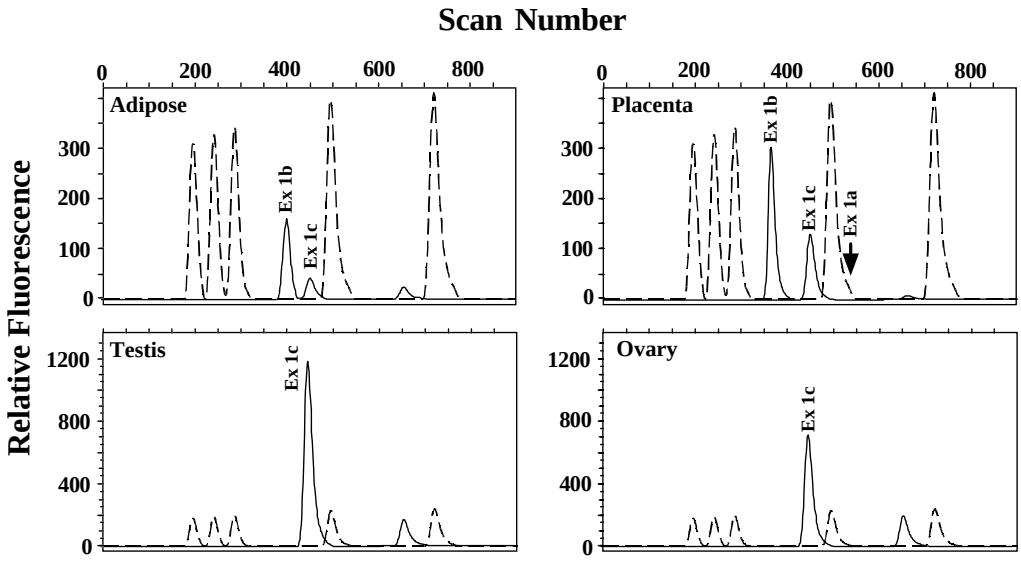

Fig. 7. Preference in the utilization of the multiple exons 1 of the reporter gene in various tissues of transgenic mice. The preference in the utilization of the multiple exons 1 of the reporter gene was analyzed by RT-PCR using sense primers specific for exons 1a, 1b, 1c, and 1d, and an antisense fluorescent dye (2-FAM)-labeled primer [34]. The transcripts from exons 1a, 1b, 1c, and 1d gave 456, 381, 422, and 409 bp, respectively, of PCR products (solid lines). The size standards (dashed lines) showed five peaks, corresponding to 262, 293, 317, 439, and 561 bp.

pattern was quite similar to that of aromatase gene in the human, not in the mouse. Then, preferential utilization of the multiple exons 1 of the reporter gene in various tissues of the transgenic mice was examined by RT-PCR analysis using primer pairs of a β-galactosidase-specific antisense primer and each one of specific sense primers for the multiple exons 1 (Fig. 7). As expected from tissue-specific utilization of the multiple exons 1of aromatase gene in the human, the reporter β-galactosidase mRNA was transcribed from exon 1b in the adipose tissue and from exon 1c in the testis and ovary. However, β-galactosidase mRNA in the placenta was transcribed from exons 1b and 1c, but not from placenta-specific exon 1a. The tissue-specific utilization of the multiple exons 1 of the β-galactosidase reporter gene in the transgenic mice reflected well that of aromatase gene in the human, except for that in the placenta. There was a discrepancy in placenta-specific expression of the compact reporter gene between the results from the in vitro cultured cells and from the in vivo transgenic mice.

4. Discussion

In the present study, we have shown that the chromosomal structure might play an important role of the transcriptional regulation of human aromatase gene by cAMP signaling pathway in the placenta, and that the 500 bp promoter region upstream of the placenta-specific exon 1a might be necessary but not sufficient for the placenta-specific expression of human aromatase gene.

The induction of aromatase in human placenta by cAMP signaling pathway is quite unique in the regard of cycloheximide-sensitive slow induction and no typical CRE motifs in the placenta-specific promoter region proximal to exon 1a [12]. These results may suggest that aromatase in the human placenta is induced through a *trans*-acting mechanism by cAMP. Cycloheximide-sensitive de novo synthesis of cAMP-inducible protein factors may be essential for the transcriptional induction of aromatase in the human placenta. It is noteworthy that similar observations were reported in the regulation of the placenta-specific expression of the β-subunit of human chorionic gonadotropin (hCG) [18–20]. It was well known that hCG is a member of the glycoprotein hormone superfamily which is a heterodimeric protein consisting of a common α-subunit and distinct β-subunits, and a cAMP-inducible protein expressed only in the human and horse placenta [21–23]. The α-subunit has been thoroughly studied about the transcriptional regulation for the placenta-specific expression, and consequently the gene regulation was reported to be directed by three distinct *cis*-acting elements, two copies of typical CRE, a trophoblast-specific element (TSE), and a GATA element, in the 5′-promoter region [22,24,25]. However, the transcriptional regulation of the human CG β-subunit gene was more complicated, and remains to be fully elucidated. The TSE motifs were found in the β-subunit genes as well as the α-subunit gene, and it was showed that they were coordinately regulated by a common TSE-binding protein [26]. However, no typical CRE motifs were found in the 5′-promoter region of the β-subunit gene, although cAMP-responsive and cycloheximide-sensitive slow induction of the gene was observed [19].

To elucidate the transcriptional response of aromatase gene in the human placenta to cAMP, we first analyzed the 5′-promoter region proximal to the exon 1a by a conventional CAT assay using choriocarcinoma-derived JEG-3 cells, but could not identify any positive elements responsible for aromatase induction by cAMP even when we used CAT gene reporters carrying the promoter region up to −9.1 kb upstream from the exon 1a or the compact promoter region truncated from the multiple exons 1/promoters of human aromatase gene for the transient promoter assay. So, we prepared stable transformant JEG-3 cells by transfection with the CAT reporter gene to make the reporter approached to intact aromatase gene in the human placental genome. Surprisingly, the transformed cells exhibited the transcriptional induction of the reporter gene by cAMP. As the gene incorporated into the chromosomes is susceptible to surrounding regulatory elements such as silencers or enhancers on the genome, the result was confirmed by repeated stable CAT assays using independent clones of the transformant cells. These results indicate that incorporation of the CAT reporter gene into chromosomes makes it possible to induce the reporter gene by cAMP, suggesting that the chromosomal structure may be essential for cAMP-responsiveness of aromatase gene in the human placenta. As histone acetyltransferase is known to participate in the transcriptional activation of many regulatory genes by unwinding specific gene loci on the chromosomes which are constructed with histone octamers to form the rigid super-double helix structure, induction of aromatase gene in the human placenta by cAMP might be mediated by activation of histone acetyltransferase through a *trans*-acting mechanism (Fig. 8).

We have previously identified and characterized three nuclear factor-binding sites necessary for transcriptional regulation within the 500 bp promoter region upstream of the exon 1a of human aromatase gene [27]. It was revealed that one of these sites is a trophoblast-specific element and glial cells missing (GCMa), a kind of transcription factor, specifically binds to the sits. As GCMa is a essential factor for placenta-specific expression [28], it was reported that its deficiency (GCM knockout mice) is embryonic lethal by placental failure [29]. In our study to characterize placenta-specific expression of human aromatase gene, the compact reporter gene constructed from the multiple exons 1/promoters of human aromatase gene was tissue-specifically transcribed in human choriocarcinoma-derived JEG-3 cells and in human hepatocarcinoma-derived HepG2 cells. A reporter further truncated from the compact gene, in which the 7 kb promoter region of the exon 1a was replaced with the 500 bp promoter region, was also tissue-specifically transcribed in the both cells (data not shown). This fact is well consistent with our previous data concerning GCMa [27,28].

On the other hand, when placenta-specific expression of human aromatase gene was examined in vivo by transgenic mice carrying β-galactosidase reporter gene conrolled by the multiple exons 1/promoters of the gene, inconsistent results with the data of the in vitro analysis using JEG-3 cells were obtained. The transgenic mice showed tissue-specific expression of the compact reporter gene in various tissues in consistent with that of aromatase gene in the human, except for the expression in the placenta. The transgenic mice showed high level expression of the reporter transcripts from the exon 1c in the ovary and testis and low level expression of the transcripts from the exon 1b in the adipose tissue, whereas little expression in the liver and kidney [30]. The expression of the reporter gene in the placenta was greatly high, as compared with the other tissues, in consistent with that of human aromatase gene, but was transcribed from exons 1b and 1c. The switching in the utilization of the multiple exons 1 of the reporter gene occurred in the placenta of the transgenic mice as often observed in human breast cancer tissues [12,31,32]. Recently, regulatory regions for the placenta-specific expression of human aromatase gene were examined in the primary cultures of human placental syncytiotrophoblast cells by a reporter gene, P450arom–hGH fusion gene of human GH gene linked to the placenta-specific promoter region proximal to the exon I.1 (1a) [16]. The reporter gene carrying the 501 bp promoter region upstream of the exon I.1 (1a) gave significant expression only in the placental cells, but not in non-placental cells. This analysis indicated that elements responsible for trophoblast-specific expression were present within 501 bp promoter region of the exon I.1 (1a) and elements necessary for high-level expression were present between −501 and −246 bp of exon I.1 (1a). Furthermore, the 501 bp promoter region of human

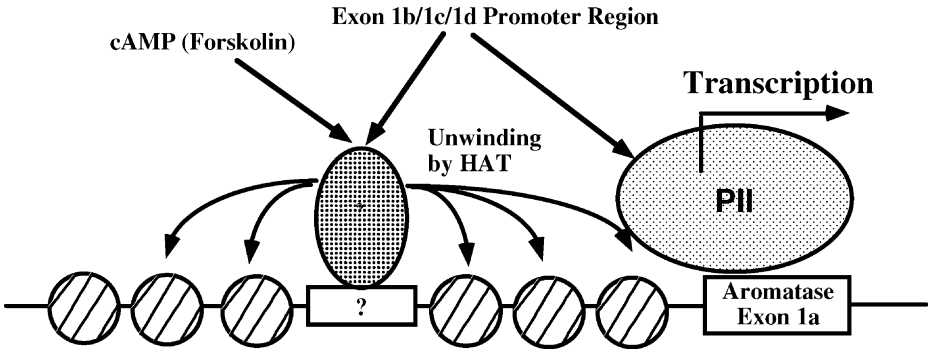

Fig. 8. Schematic illustration of transcriptional regulation of aromatase gene in the human placenta. HAT: histone acetyltransferase.

aromatase gene was showed to mediate placenta-specific expression in the transgenic mice which were introduced the P450arom–hGH fusion gene [17]. These results are consistent with that of our in vitro reporter assay using JEG-3 cells, but inconsistent with that of our transgenic mice which showed significant expression of the reporter gene but switching from the placenta-specific exon 1a to the exons 1b/1c. The significant difference between both experimental systems was constructs of the reporter genes. The promoter region of the reporter gene in our study consisted of the multiple exons 1/promoters of human aromatase gene to analyze tissue-specific utilization of multiple exons 1/promoters of the gene and to observe switching of the exons 1 when transcriptional enhancers and silencers in the tissue-specific promoters dysfunction, whereas the promoter region of the gene in the report consisted of only the 5′-flanking region proximal to the exon I.1 (1a) to analyze the reporter transcripts only from the exon I.1 (1a). Because aromatase is not expressed in the mouse placenta [33], mouse placenta might be deficient in transcription factors such as tissue-specific enhancers or silencers, essential for the tissue-specific expression of human aromatase gene. The discrepancy might be also explained by the presence of factors strictly suppressing transcription from the exon 1a of human aromatase gene in non-placental mouse tissues and by the absence of factors strictly enhancing transcription from the exon 1a and the presence of factors loosely enhancing transcription from all the exons 1 in mouse placenta. The transgenic mice carrying the reporter construct containing 2400 bp promoter region proximal to exon I.1 (1a) showed placenta-specific expression in three of the seven mice and no expression in four of the seven [17]. This result may suggest the presence of silencer elements between −2400 and −501 bp of the exon 1a. As our reporter gene construct contained the 7 kb promoter region proximal to the exon 1a, there might be several silencer elements on the promoter region and their binding factors might make it difficult to express tissue–specifically in the placenta. Further investigation should hopefully give more information on the tissue-specific and cAMP-responsive transcriptional regulation of aromatase gene in the human placenta.

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