Pergamon

0960-0760(95)00032-1

J. Steroid Biochem. Molec. Biol. Vol. 53, No. 1–6, pp. 181–190, 1995

0960-0760/95 $9.50 + 0.00

Transcriptional Regulation of the Human Aromatase Cytochrome *P*450 Gene Expression in Human Placental Cells

Katsumi Toda, Li-Xia Yang and Yutaka Shizuta*

Department of Medical Chemistry, Kochi Medical School, Nankoku, Kochi 783, Japan

The human aromatase cytochrome *P*450 gene, CYP 19, spans more than 75 kb in the human genome. Recently, it is proposed that the expression of the CYP 19 gene is regulated in part by tissue-specific promoters through the use of mechanisms involving alternative splicing of a number of untranslated exons. In this study, we have characterized *cis*-acting elements involved in the transcriptional regulation of the gene in human placental cells, where the majority of the transcripts contain the 5′-untranslated sequence encoded by exon I.1. By transient expression analyses in human BeWo choriocarcinoma cells using the bacterial chloramphenicol acetyltransferase gene as a reporter gene, we localized an enhancer element in the region between −242 and −166 relative to the major cap site of the gene. Furthermore, we demonstrate that the element between −2141 and −2115 participates in the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-mediated enhancement of gene expression. By screening a human placental cDNA expression library, we have isolated a cDNA clone (λ1–2) encoding a peptide which binds specifically to the element between −2141 and −2115. Sequence analysis of the clone revealed that the insert of λ1–2 encodes a part of the amino acid sequence of NF-IL6 (also termed as LAP and C/EBPβ). Northern blot analysis reveals expression of the NF-IL6 gene in BeWo cells and human placenta. These results indicate that NF-IL6 is one of the nuclear factors which participate in TPA-mediated transcriptional enhancement of CYP 19 gene expression.

J. Steroid Biochem. Molec. Biol., Vol. 53, No. 1–6, pp. 181–190, 1995

INTRODUCTION

The human aromatase cytochrome *P*450 (*P*450arom) gene is a member of the cytochrome *P*450 gene superfamily, in which the gene is classified into a unique gene family and designated as CYP 19 [1]. *P*450arom forms an enzyme complex with a ubiquitous flavoprotein, NADPH-cytochrome *P*450 reductase in the endoplasmic reticulum and catalyzes the final step of the synthesis of various estrogens by three sequential hydroxylation reactions of corresponding androgens [2, 3]. Recently, much attention has been paid to the elucidation of the regulatory mechanisms of the CYP 19 gene expression, because not only is the expression responsible to various chemical stimuli such as cAMP, phorbol ester, glucocorticoids and several growth factors [4–11], but estrogens are also considered to play a major role in promoting the growth of hormone-dependent cancer [12–14] as well as in maintaining bone mass [for review, see 15].

Molecular genetic studies on the structure of the CYP 19 gene revealed that the gene is organized with at least five untranslated exons, designated I.1, 2a, I.4, I.2 and I.3, and nine coding exons, designated II–X, which span over more than 75 kb in the human genome [11, 16–21]. Studies on the regulation of the CYP 19 gene expression have showed that each untranslated exon is transcribed in a tissue-specific manner, indicating the involvement of the tissue-specific promoter activity for tissue-specific regulation of the CYP 19 gene expression [22, 23 and for review, see 24].

In order to elucidate the molecular mechanisms underlying the regulation of the CYP 19 gene

Proceedings of the IX International Congress on Hormonal Steroids, Dallas, Texas, U.S.A., 24–29 September 1994.
*Correspondence to Y. Shizuta.
The nucleotide sequence data reported in this paper appear in the GenBank, EMBL and DDBJ Nucleotide sequence databases with the accession numbers Y07508 for cDNA sequence, X55983 and D13391 for the 5′-flanking sequence of Exon I.1, and D14473 for the alternative 5′-untranslated exon.
Abbreviations: *P*450arom, aromatase cytochrome *P*450; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

expression involving exon I.1, whose expression predominates in the human placenta as well as in human choriocarcinoma cells, we characterized the 5′-flanking region of exon I.1 by transient expression analysis in human BeWo choriocarcinoma cells using chimeric *P*450arom/chloramphenicol acetyltransferase (CAT) gene recombinants [11, 25, 26]. In the present communication, we first provide evidence that a cell-type specific enhancer element is located between −242 to −166 relative to the major cap site of the gene. Secondly, we report localization of regulatory elements required for 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-mediated transcriptional enhancement of the gene between −2141 and −2115. Thirdly, we show that the ubiquitous transcription factor, termed NF-IL6 (also termed as LAP or C/EBPβ), is a component of the nuclear factors binding to the *cis*-acting element between −2141 and −2115.

EXPERIMENTAL AND RESULTS

Structural organization of the CYP 19 gene

Using various regions of cDNAs for CYP 19 as probes, we screened two types of human genomic DNA libraries, one constructed into phage vector, EMBL3, and the other into cosmid vector, Lorist 2 [11]. On the basis of restriction endonuclease mapping and Southern blot analysis probed with various regions of the cDNA, we determined the structural organization of the CYP 19 gene as shown in Fig. 1.

The CYP 19 gene is a single unique gene in humans. It spans more than 75 kb of genomic DNA and consists of 14 exons. Each exon is composed of 103 (exon I.1), 109 (exon 2a), 65 (exon I.4), 191 (exon I.2), 205 (exon I.3), 183 (exon II), 151 (exon III), 155 (exon IV), 177 (exon X) nucleotides, respectively, Exon II contains the translational start codon, ATG and exon X encodes carboxyl terminal 82-amino acid residues and the entire 3′-untranslated sequence. The five untranslated exons, I.1, 2a, I.4, I.2, and I.3 are transcribed in a tissue-specific manner. Nevertheless, sequences transcribed from these exons are spliced into the same splicing acceptor site located 5′ of the translational start codon, and thus the coding region and hence the protein product is identical in all cases. Recently, two more untranslated exons are reported. One termed as I.5 consists of 90 nucleotides and the transcript containing this exon occurs mainly in human fetal liver and intestine [27]. The other termed as 1f consists of 135 nucleotides and the transcript is detected exclusively in the human brain [28]. However, the location of neither exon with respect to the rest of the gene has been determined.

Identification of an enhancer element in the 5′-flanking sequence of exon I.1

To demonstrate the promoter activity of the upstream sequence of exon I.1, the 5′-flanking region was ligated to the CAT gene and tested by transient expression analysis in human BeWo choriocarcinoma cells. As shown in Fig. 2, construct 1, the 576 bp DNA fragment which contains 500 bp of the 5′-flanking sequence and 76 bp of exon I.1 showed promoter activity in the cells. On the other hand, the 5′-flanking region ligated in the reverse orientation relative to the CAT gene did not exhibit any promoter activity (data not shown). To further characterize the promoter region, deletion mutants of the 5′-flanking region were ligated to the 5′ end of the CAT gene and transfected into BeWo cells (Fig. 2, constructs 2–4). When the 5′-flanking region between −500 and −362 was deleted, the promoter activity was enhanced 7.6-fold. Moreover, deletion extending to nucleotide position −243 resulted in an additional 2.6-fold enhancement

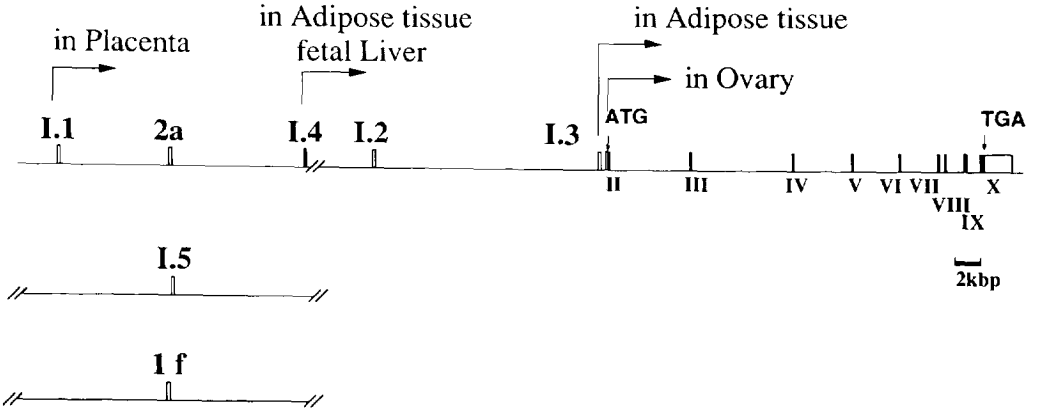

Fig. 1. Schematic representation of the exon–intron organization of the human CYP 19 gene. The closed boxes represent translated sequences which are encoded by nine exons, exon II–X. The translational initiation codon indicated by ATG is located 38 bp downstream of the 5′ end of exon II. The translational termination codon indicated by TGA is located 247 bp downstream of the 5′ end of exon X. The distance between exon I.4 and I.2 is not determined due to a lack of a genomic clone which bridges two exons. The location of exon I.5 and exon 1f is also not determined. The major transcriptional start sites in placenta, adipose tissue, fetal liver and ovary in humans are indicated by arrows. Scale is shown in length of 2 kbp.

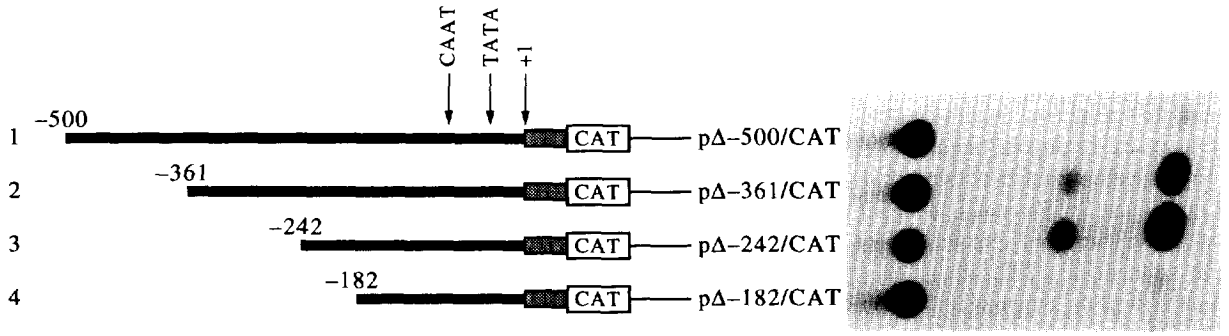

Fig. 2. Promoter activity of various 5′-deletion mutants of the CYP 19 gene in BeWo cells. The diagram shows structures of the 5′-deletion mutants used in these experiments. Relative positions of the transcriptional initiation site (+1), a TATA box (TATA), a CAAT box (CAAT) are indicated with vertical arrows on top of the panel. The autoradiogram shows the results of CAT assays using the 5′-deletion mutants as indicated on the left of the panel. Note that each CAT activity was measured using a suitable amount of each cell lysate containing the same level of β-galactosidase activity simultaneously expressed in the host cells as an internal standard.

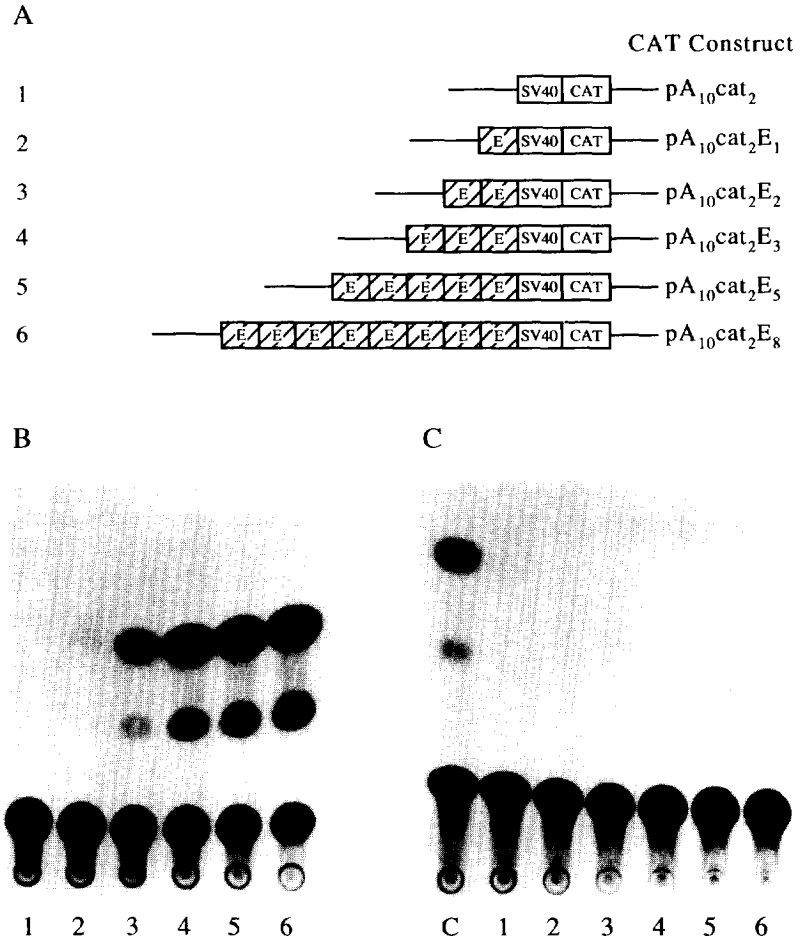

Fig. 3. Enhancement of SV40-basal promoter activity by the 77 bp fragment spanning −242 to −166. (A) The diagram shows the structures of the chimeric constructs used in these experiments. In the vector, $pA_{10}cat_2$, the bacterial CAT gene as indicated by a box with CAT is controlled by the 132 bp basal core promoter of the SV40 early gene as indicated by a box with SV40, (construct 1). The 77 bp fragment indicated by a striped box with E was inserted into the 5′ end of the SV40 promoter sequence in various copy-numbers (construct 2–6). After transfection of these constructs into BeWo cells (B) or HeLa cells (C), CAT activities expressed were measured. Lane 1, $pA_{10}cat_2$; lane 2, $pA_{10}cat_2E_1$; lane 3, $pA_{10}cat_2E_2$; lane 4, $pA_{10}cat_2E_3$; lane 5, $pA_{10}cat_2E_5$; lane 6, $pA_{10}cat_2E_8$. In experiment with HeLa cells, a CAT construct controlled by the human β-actin promoter was used as a positive control as shown by lane C.

of the activity. Deletion to position −183 caused a substantial reduction in the activity. These results suggest that the region between −500 and −243 might modulate transcription of the gene in a negative fashion and that the region between −242 and −183 contains important elements for transcriptional enhancement of the gene in BeWo cells. Actually, we have demonstrated by transient expression analyses that the fragment spanning −242 to −166 contains an enhancer element which augments its own promoter activity [25]. Furthermore, as shown in Fig. 3(B) the same fragment can enhance heterologous promoter activity in a copy-number dependent manner and the effect seems not to be additive but cooperative. Importantly, the fragment functions as an enhancer only in BeWo cells but not in other cell lines tested including HeLa cells [Fig. 3(C)], human embryonic brain fibroblast (Flow3000), human ovarian carcinoma (TYK-nu) cells, human mixed glioma (KG-1-c) cells and mouse adrenal tumor (Y-1) cells [25]. These results show that the enhancer element has a strict cell-type specificity.

Identification of TPA-responsible cis-acting elements in the 5′-flanking sequence of exon I.1

As shown in Fig. 4, levels of CYP 19 mRNA in BeWo cells are increased by TPA in a time-dependent manner. An initial increase in levels of the mRNA was observed at 8–12 h after addition of TPA followed by a remarkable increase up to 10-fold relative to the basal levels. This observation is well compatible with the recent report of Ritvos and Voutilainen [9]. In order to identify the *cis*-acting transcriptional elements responsible for TPA mediated enhancement of CYP 19 gene expression, we examined whether or not the genomic fragment derived from the upstream sequence of exon I.1 has any effect on the expression of the linked CAT reporter gene. We found that the region of 362 bp between −2408 and −2027 relative to the cap site enhanced the expression of the CAT gene 5.7-fold over control levels in response to TPA. By DNase I protection assays and electrophoretic mobility shift assays, we found that the region spanning −2238 to −2214 (5′-CTTTCTGGCCTAAGGGTTGGAGAGC-3′) (termed hATRE-1) and the region spanning −2141 to −2098 (5′-CTTTTATGTTGCCCAATACCT-GCTCTGCCTCGAGGGTCACTGTC-3′) (termed hATRE-2) are the sites to which nuclear factors bind. Electrophoretic mobility shift assays demonstrate that hATRE-2 effectively competes for binding of the nuclear factors to hATRE-1, whereas hATRE-1 does not compete well for binding of the nuclear factors to hATRE-2, suggesting that hATRE-2 contains at least two different sites for binding of nuclear factors, namely one which is specific to hATRE-2 and another which is homologous to hATRE-1 [26]. By a series of electrophoretic mobility shift assays with various synthetic oligonucleotides, we localized the binding site of the nuclear factors specific to hATRE-2 in the region between −2141 and −2115 (5′-CTTTTATGTTGC-CCAATACCTGCTCTG-3′) (Fig. 5). Transient expression analyses in BeWo cells showed that five copies of oligo-e which spans −2141 to −2115 significantly enhance the basal expression of the reporter gene as well as the expression in response to TPA (Fig. 6). These results indicate that the nuclear binding factors specific to hATRE-2 participate in TPA-mediated transcriptional activation of the gene.

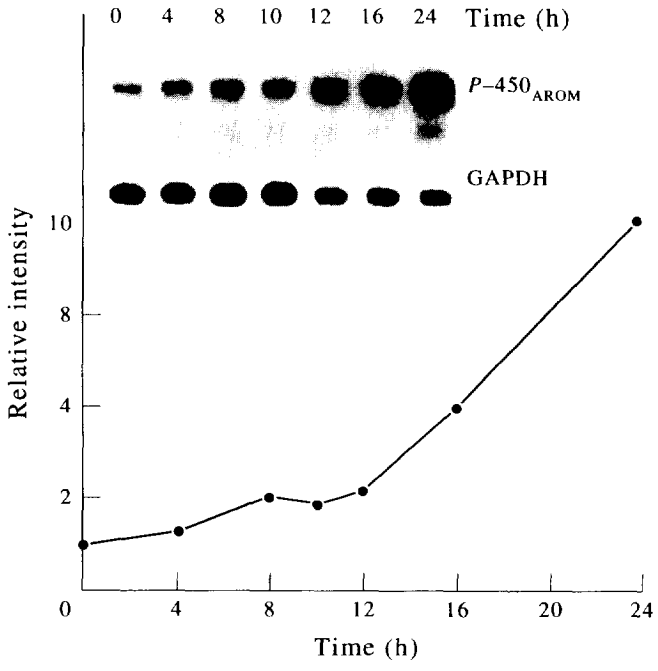

Fig. 4. Accumulation of the CYP 19 transcript in BeWo cells by the TPA treatment. BeWo cells were incubated with 100 ng/ml of TPA for indicated time intervals. Total cellular RNA (20 μg) prepared from the cells was electrophoresed, blotted to a nylon membrane filter and hybridized with the ^{32}P-labeled cDNA probe for *P*450arom (*P*450arom). The same filter was hybridized again with the ^{32}P-labeled cDNA probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to serve as a loading control. Quantification of the intensity of each band was performed with Bioimage Analyzer BAS2000 (Fuji).

Identification and characterization of a nuclear binding factor specific to hATRE-2

To isolate a cDNA encoding a nuclear binding factor specific to hATRE-2, we screened a human placental λgt11 cDNA expression library with hexameric oligo-e ([oligo-e]$_6$) that encompasses the sequence between −2141 and −2115 as described above. We isolated one positive clone from 5×10^5 recombinant phages by initial screening. The clone, termed as λ1–2, produced strong positive signals when probed with ^{32}P-labeled [oligo-e]$_6$ but not with the other oligonucleotides including pentameric oligo-g which spans −2121 to −2098 ([oligo-g]$_5$) [Fig. 7(A, B)]. Neither [oligo-e]$_6$ nor [oligo-g]$_5$ generates positive signals with the control filters which are blotted proteins expressed from λgt11 phages without inserts [Fig. 7(C, D)]. To further

investigate the DNA-binding specificity of the protein coded by λ1–2, we prepared lysogens of λ1–2 and control λgt11, and induced expression of their respective β-galactosidase-fusion genes to use in an electrophoretic mobility shift assay. Incubation of the extracts from λ1–2 lysogen which ^{32}P-labeled monomeric oligo-e generates a specific retarded band as shown in Fig. 8(A). Unlabeled monomeric oligo-e and [oligo-e]$_6$ compete for binding of the probe to the β-galactosidase-fusion protein in a concentration-dependent manner [Fig. 8(A, lanes 2–7)], although the latter seems to be a much stronger competitor. A 100-fold molar excess of the unlabeled oligonucleotides completely abolishes retardation of the probe [Fig. 8(A, lanes 4 and 7)]. Neither [oligo-g]$_5$ nor a nonspecific competitor DNA from pUC118 plasmid competes for binding of the ^{32}P-labeled probe to the fusion protein [Fig. 8(A, lanes 8–12)]. When we used ^{32}P-labeled [oligo-e]$_6$ as a probe in the assay, we obtained essentially the same results as those with monomeric oligo-e except that several retarded bands are formed [Fig. 8(B)]. However, neither ^{32}P-labeled oligo-e nor ^{32}P-labeled [oligo-e]$_6$ forms any specific retarded bands with the extracts from the lysogen of control λgt11 phage (data not shown). These results indicate that the β-galactosidase-fusion protein from λ1–2 lysogen forms sequence-specific DNA–protein complexes.

The nucleotide sequence of the insert of λ1–2 was determined and the open reading frame deduced from the sequence is presented in Fig. 9. By

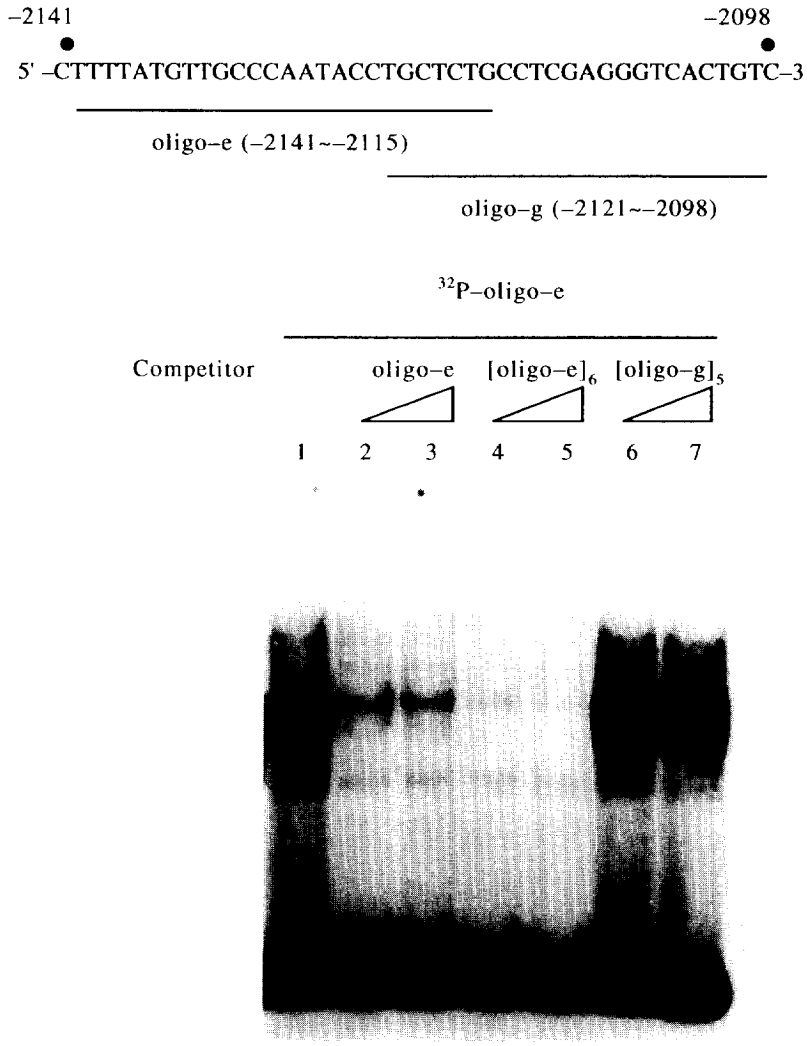

Fig. 5. Specific binding of nuclear factors to the region between −2141 and −2115. Two different synthetic double-stranded oligonucleotides, oligo-e spanning −2141 to −2115 and oligo-g spanning −2121 to −2098, were employed for these experiments. Oligo-e was radiolabeled with [^{32}P-γ]ATP and T4 polynucleotide kinase and used as a probe. The labeled probe was mixed with the nuclear extracts from BeWo cells in the absence (lane 1) or presence (lanes 2–7) of unlabeled competitor oligonucleotides. Lane 1, an electrophoretic mobility shift pattern after incubation of the probe in the absence of a competitor DNA; lanes 2 and 3, after incubation in the presence of 50-fold (lane 2) and 100-fold (lane 3) molar excesses of unlabeled oligo-e, lanes 4 and 5, after incubation in the presence of 50-fold (lane 4) and 100-fold (lane 5) molar excesses of unlabeled hexameric oligo-e, lanes 6 and 7, after incubation in the presence of 50-fold (lane 6) and 100-fold (lane 7) molar excesses of unlabeled pentameric oligo-g.

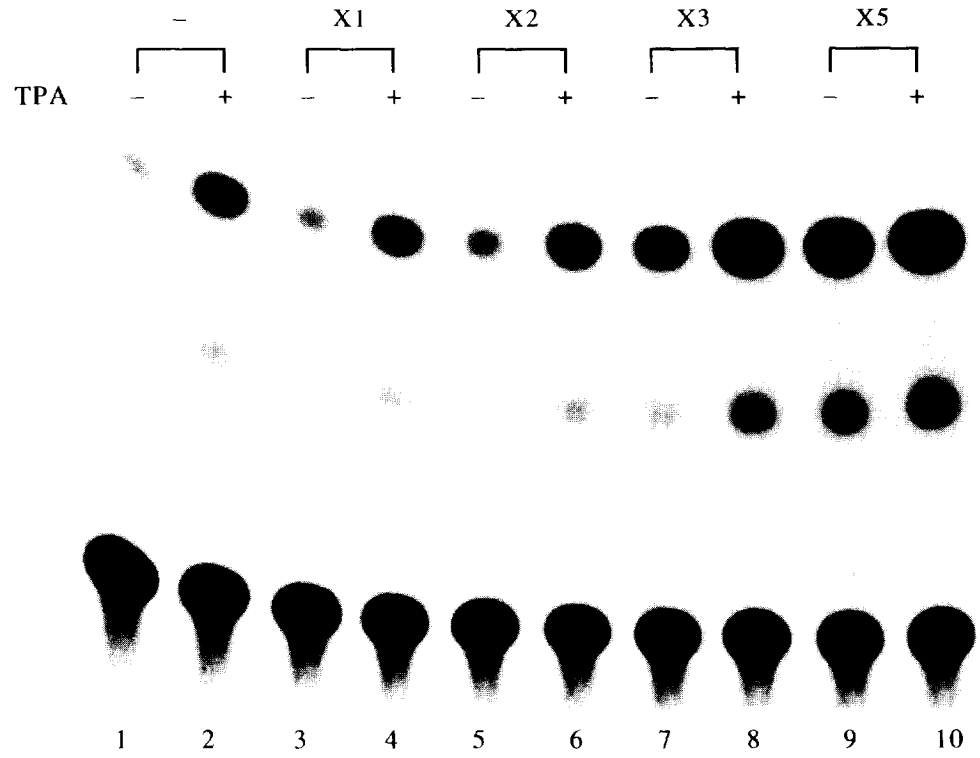

Fig. 6. Enhancement of the reporter gene expression by oligo-e. CAT constructs containing oligo-e in various copy numbers in pΔ-500/CAT were prepared as described previously [26]. After transfection of each CAT construct into BeWo cells, cells were cultured in the absence (−) or presence (+) of TPA for 44 h. Copy-numbers of the oligo-e in each construct is indicated on the top of the figure. A thick bar indicates no insertion of the oligonucleotide in the construct.

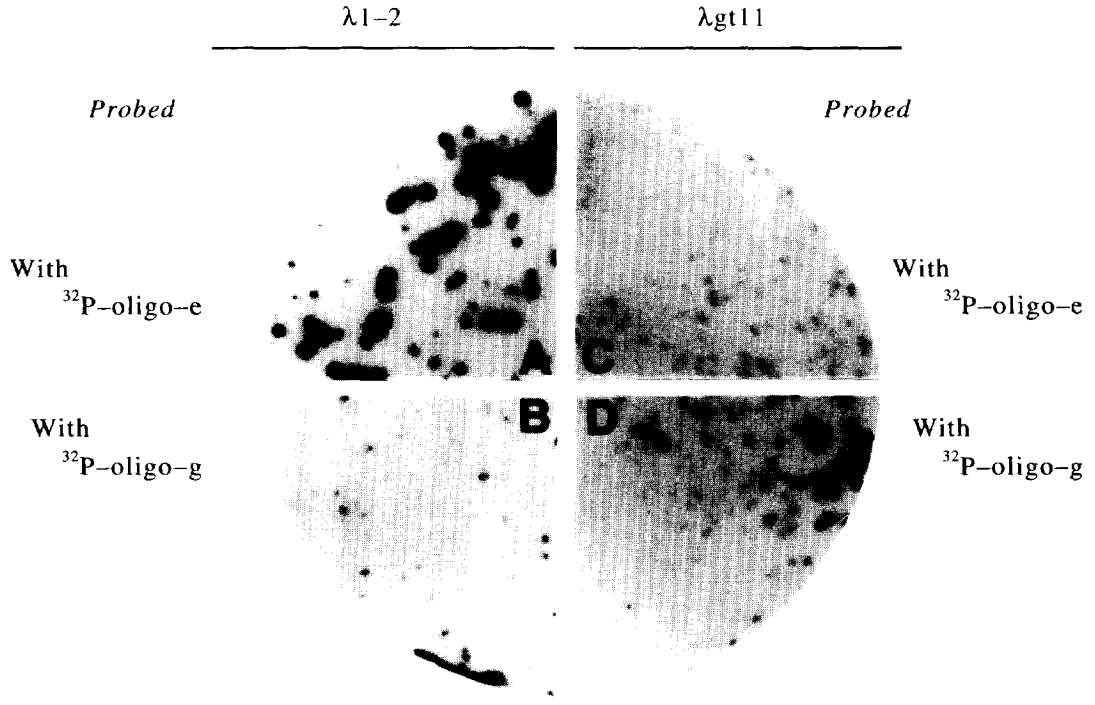

Fig. 7. Isolation of a λgt recombinant clone encoding a peptide which specifically binds to the region between −2141 and −2115. *E. coli*, strain Y1090, was infected with the recombinant clone λ1–2 (A and B) or λgt11 control phages (C and D). The cells were mixed with top agar and spread onto an agar plate. Each plate was overlaid with an IPTG-impregnated nitrocellulose filter and incubated for 6 hr at 37°C. The filter lifted plaques was cut into four pieces and each piece was probed either with ^{32}P-labeled hexameric oligo-e spanning −2141 to −2115 (A and C) or ^{32}P-labeled pentameric oligo-g spanning −2121 to −2098 (B and D). After washing, the filters were autoradiogramed.

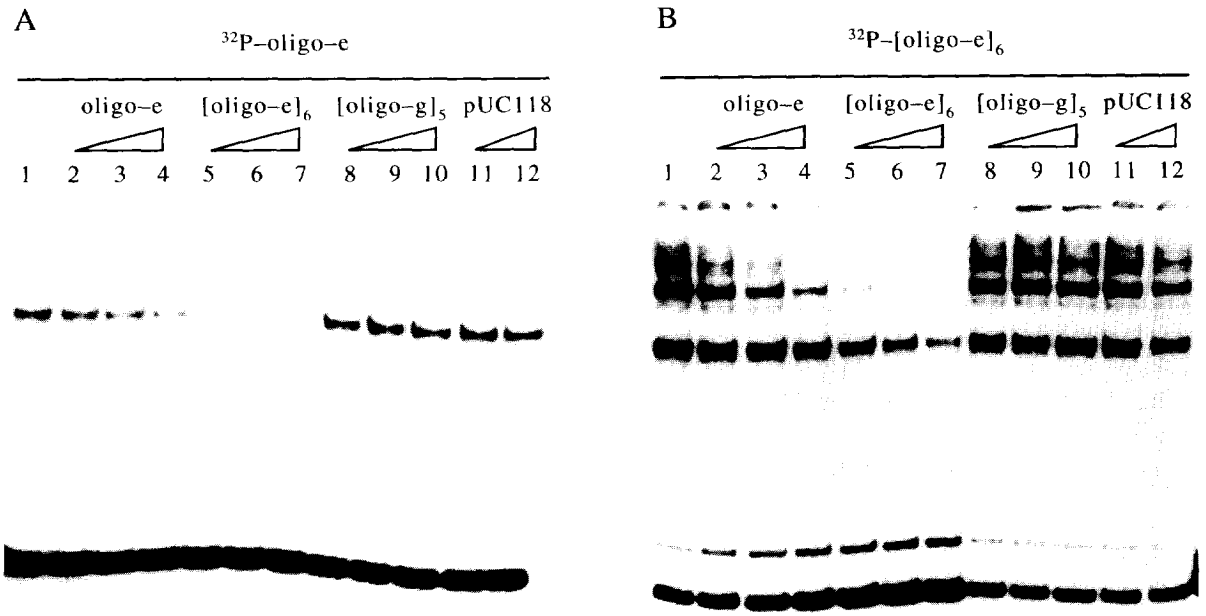

Fig. 8. Specific binding of the β-galactosidase fusion protein from λ1–2 to the region between −2141 and −2115. The synthetic double-stranded oligonucleotides, oligo-e and oligo-g, were employed for these experiments. ^{32}P-labeled monomeric oligo-e (A) or ^{32}P-labeled hexameric oligo-e (B) were used as probes. The probe was mixed with the extracts from λ1–2 lysogen in the absence (lane 1) or presence (lanes 2–12) of unlabeled competitor oligonucleotides. Lane 1, electrophoretic mobility shift patterns after incubation of the probe in the absence of a competitor DNA; lanes 2–4, after incubation in the presence of 10-fold (lane 2), 50-fold (lane 3), and 100-fold (lane 4) molar excesses of unlabeled oligo-e; lanes 5–7, after incubation in the presence of 10-fold (lane 5;, 50-fold (lane 6), and 100-fold (lane 7) molar excesses of unlabeled hexameric oligo-3; lanes 8–10, after incubation in the presence of 10-fold (lane 8), 50-fold (lane 9), and 100-fold (lane 10) molar excesses of unlabeled pentameric oligo-g; lanes 11 and 12, after incubation in the presence of 50-fold (lane 11) and 100-fold (lane 12) molar excesses of an unlabeled non-specific DNA fragment prepared by digestion of pUC118 with *Pvu*II-HindIII located between positions 782 and 872.

computer-searching in a Protein Sequence Database, we found that the deduced amino acid sequence of the insert is exactly identical to amino acid number 242 to 323 of NF-IL6 (also known as C/EBPβ) [29]. Consequently, the insert encodes the amino acid sequence for the entire DNA binding domain and a N-terminal half portion of the leucine zipper domain of NF-IL6 as shown in this figure. Northern blot analysis detected

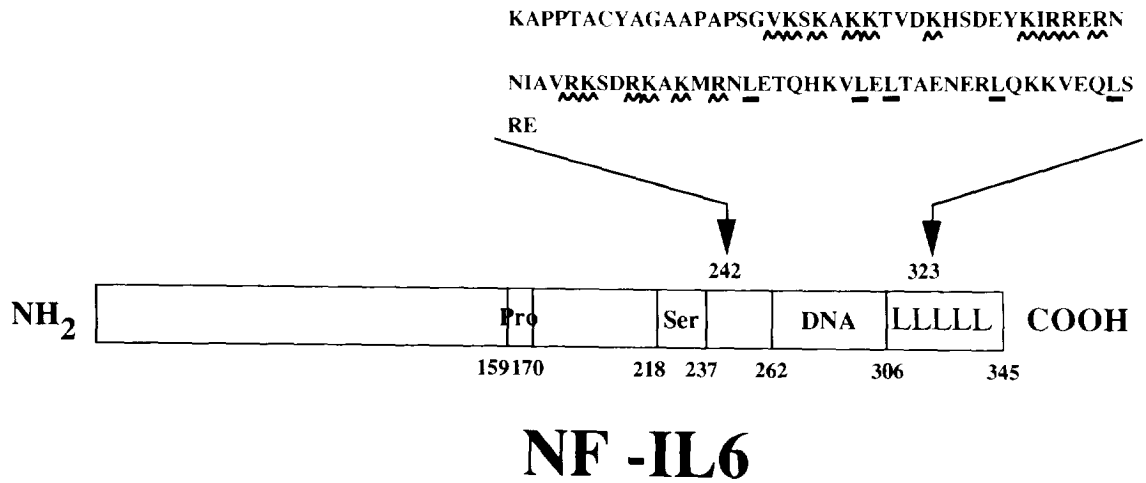

Fig. 9. Amino acid sequence deduced from the nucleotide sequence encoded by the insert of λ1–2. The deduced amino acid sequence is presented by the single letter symbol for amino acid. Wavy line and under line respectively indicate basic amino acid residue and leucine residues which may participate in the leucine zipper structure. The domain structure of NF-IL6 is schematically illustrated at the bottom of the figure. Pro and Ser indicate domains rich in proline residue and in serine residue, respectively. DNA indicates a domain rich in basic amino acid residue and that required for binding to DNA. LLLLL indicates a domain generating the leucine zipper structure. Number represents the position of amino acid residue [29].

expression of the NF IL-6 gene in BeWo cells as well as in human placenta (Fig. 10). These results led us to the conclusion that one of the components of the nuclear factors which bind specifically to hATRE-2 is NF-IL6.

DISCUSSION

By screening genomic libraries and cDNA libraries prepared by the RACE (Rapid Amplifcation of cDNA Ends) procedure, it has been established that the heterogeneity observed in the 5′-untranslated sequences in the transcripts of the CYP 19 gene is generated by an alternative splicing event or alternative initiation of the transcription [16, 18–23, 27, 28]. Up to date, seven untranslated exons, I.1, 2a, I.4, I.2, I.3, I.5, and 1f, have been reported. Consequently, the CYP 19 gene is conceived to be organized at least with 16 exons, of which seven are untranslated exons and nine are protein-coding exons. It is noteworthy that each untranslated exon is transcribed in a tissue-specific manner. Thus, these observations indicate that alternative promoters participate in regulation of the tissue-specific expression of the CYP 19 gene, although all of the region upstream of each untranslated exon has not been proved to direct the transcription of the gene in a tissue-specific manner [18–23]. In the present study, we have attempted to elucidate the regulatory mechanisms for the CYP 19 gene expression in human placenta, where the majority of the transcripts contains the 5′-untranslated sequence encoded by exon I.1.

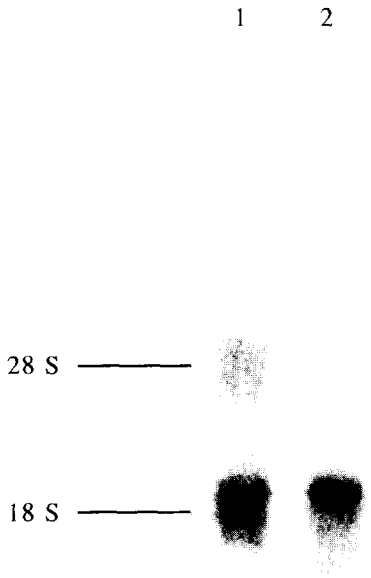

Fig. 10. Expression of the NG-IL6 gene in BeWo cells and human placenta. Total cellular RNA (20 μg) prepared from BeWo cells (lane 1) and human placenta (lane 2) was electrophoresed, blotted to a nylon membrane filter and hybridized with the ^{32}P-labeled cDNA fragment prepared from λ1–2.

By means of transient expression analysis in human BeWo choriocarcinoma cells using the CAT gene as a reporter gene, we localized an enhancer element between −242 and −166 relative to the transcriptional initiation site of the CYP 19 gene in placenta. We demonstrated that the element enhances its own promoter activity as well as a heterologous promoter activity in a manner independent of position and orientation but dependent on the multiplicity of the element [25]. Importantly, the enhancer activity is detected only in human BeWo choriocarcinoma cells, but not in other types of cells tested (Fig. 3). These results indicate that the enhancer element has a strict cell-type specificity. In addition, we observed that nuclear extracts from BeWo cells form protein–DNA complexes with the enhancer element in a sequence specific manner [25]. These observations suggest that nuclear binding factors to the enhancer element may play a key role in regulation of CYP 19 gene expression in human choriocarcinoma cells and hence in human placenta.

Levels of *P*450arom mRNA in BeWo cells is increased by treatment with TPA (Fig. 4). A series of studies including transient expression analysis, electrophoretic mobility shift assay and DNase I footprinting analysis have allowed the identification of *cis*-acting elements required for TPA-mediated transcriptional enhancement of CYP 19 gene expression. The elements are localized in the region between −2238 to −2214 (hATRE-1) and between −2141 to −2098 (hATRE-2). hATRE-2 is further dissected into two subelements, namely, one spanning −2141 to −2115

Table 1. *Distribution of possible binding sites for NF-IL6 in the upstream region of exon I.3 and I.4*

Exon	Location	Sequence
I.3	− 388 to − 379	CCTCTGCAAT
	− 382 to − 373	CAATTCTTTT
I.4	− 374 to − 365	TTTGGCCAAT
	− 291 to − 282	GTTGCGCAAT
	− 208 to − 199	GTTCCAGAAG
Consensus		TTNNGNAAT/G

The nucleotide sequence is numbered beginning with the nucleotide at the 5′ ends of exon I.3 and I.4 as +1 according to the reports [18 and 20, respectively] and the upstream sequences from those are indicated by negative numbers.

which is specific to hATRE-2 and another spanning −2121 to −2098 which shares specificity with hATRE-1. The element specific to hATRE-2 is shown to activate transcription of the gene in BeWo cells. Nevertheless, we assumed that the binding factors to hATRE-2 correspond to the basic building blocks over which other nuclear factors assemble to form a functional regulatory complex for TPA-mediated transcriptional enhancement of expression, based on the following observations. First, the nuclear binding factors specific to hATRE-2 are present in BeWo cells regardless of the TPA-treatment. Secondly, DNase I footprinting patterns with nuclear extracts prepared from TPA-treated cells showed the same protection patterns as those observed with nuclear extracts from TPA-untreated cells. Thirdly, nuclear binding factors specific to hATRE-2 are present in HeLa cells and in TYK-nu cells but they appear not to participate in TPA-mediated transcriptional enhancement in those cells.

In order to characterize the nature of the nuclear binding factor specific to hATRE-2, we attempted to isolate cDNA clone by screening the λgt11 expression library according to the method described by Vinson *et al.* [30]. Characterization of the clone obtained demonstrates that the insert encodes a part of the amino acid sequence of NF-IL6. This nuclear factor is originally identified as a transcriptional regulatory factor responsible for induction of the IL-6 gene by IL-1 and turned out to be a member of the CEBP gene family [29]. Recent studies have indicated that NF-IL6 is a pleiotropic transcriptional regulator for many genes involved in acute-phase reaction, inflammation, hematopoiesis as well as in adipocyte differentiation [29, 31–33]. The expression of NF-IL6 in adipocyte seems to be particularly interesting because, in humans, adipose tissue is one of the major tissue sites for estrogen biosynthesis [4–7]. The transcripts detected in adipose tissue do not possess the nucleotide sequence encoded by exon I.1 in the 5′-untranslated sequence, but they have sequences encoded by exon I.4 or I.3 as demonstrated by Mahendroo *et al.* [18, 20]. This finding suggests that transcription in adipose tissue is regulated in a different fashion from that in placenta, possibly by using alternative promoters. Consequently, the upstream sequences of exon I.3 and I.4 are assumed to be promoter sequences responsible for expression in adipose tissue because they contained potential binding sites for various transcriptional regulatory factors including Sp1, CREB, NF-1 and so on [18, 20]. In addition to these *cis*-acting elements, a nucleotide sequence possibly recognized by NF-IL6 (consensus: 5′-TT/GNNGNAAT/G-3′) also appears in the 5′-flanking sequences of those exons as shown in Table 1. This observation thus suggests that NF-IL6 may participate in the transcriptional regulation of the CYP 19 gene not only in placenta but also in other tissue sites where the gene is expressed.

Acknowledgements—Human choriocarcinoma (BeWo) cells, human embryonic brain fibroblast (Flow3000), human ovarian carcinoma (TYK-nu) cells, human mixed glioma (KG-1-c) cells and mouse adrenal tumor (Y-1) cells were obtained from Japanese Cancer Research Resources Bank. This work was supported in part by research grants from The Mochida Memorial Foundation for Medical and Pharmaceutical Research, and by Grants-in-Aid for Science from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Nelson D. R., Kamataki T., Waxman D. J., Guengerich F. P., Estabrook R. W., Feyereisen R., Gonzalez F. J., Coon M. J., Gunsalus I. C., Gotoh O., Okuda K. and Nebert D. W.: The *P*450 superfamily: update on new sequence, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12** (1993) 1–51.
2. Fishman J. and Goto J.: Mechanism of estrogen biosynthesis, participation of multiple enzyme sites in placental aromatase hydroxylations. *J. Biol. Chem.* **256** (1981) 4466–4471.
3. Akhtar M., Calder M. R., Corina D. L. and Wright J. N.: Mechanistic studies on C19 demethylation in oestrogen biosynthesis. *Biochem. J.* **201** (1982) 569–580.
4. Simpson E. R., Ackerman G. E., Smith M. E. and Mendelson C. R.: Estrogen formation in stromal cells of adipose tissue of women: induction by glucocorticosteroids. *Proc. Natn. Acad. Sci. U.S.A.* **78** (1981) 5690–5694.
5. Lanoux M. J., Cleland W. H., Mendelson C. R., Carr B. R. and Simpson E. R.: Factors affecting the conversion of androstenedione to estrogens by human fetal hepatocytes in monolayer culture. *Endocrinology* **117** (1985) 361–368.
6. Mendelson C. R., Corbin C. J., Smith M. E., Smith J. and Simpson E. R.: Growth factors suppress and phorbol esters potentiate the action of dibutyryl adenosine 3′, 5′-monophosphate to stimulate aromatase activity of human adipose stromal cells. *Endocrinology* **118** (1986) 968–973.
7. Evans C. T., Corbin C. J., Saunders C. T., Merrill J. C., Simpson E. R. and Mendelson C. R.: Regulation of estrogen biosynthesis in human adipose stromal cells: effects of dibutyryl cyclic AMP, epidermal growth factor, and phorbol esters on the synthesis of aromatase cytochrome *P*-450. *J. Biol. Chem.* **262** (1987) 6914–6920.
8. Lobo J. O. and Bellino F. L.: Estrogen synthetase (aromatase) activity in primary culture of human term placental cells: effects of cell preparation, growth medium, and serum on adenosine 3′,5′-monophosphate response. *J. Clin. Endocr. Metab.* **69** (1989) 868–874.
9. Rivos O. and Voutilainen R.: Regulation of aromatase cytochrome *P*-450 and 17β-hydroxysteroid dehydrogenase messenger ribonucleic acid levels in choriocarcinoma cells. *Endocrinology* **130** (1992) 61–67.
10. Berkovitz G. D., Chen S., Migeon C. J. and Levine M. A.: Induction and superinduction of messenger ribonucleic acid specific for aromatase cytochrome *P*-450 in cultured human skin fibroblasts. *J. Clin. Endocr. Metab.* **74**, (1992) 629–634.
11. Toda K., Terashima M., Kawamoto T., Sumimoto H., Yokoyama Y., Kuribayashi I., Mitsuuchi Y., Maeda T., Yamamoto Y., Sagara Y., Ikeda H. and Shizuta Y.: Structural and functional characterization of human aromatase *P*450 gene. *Eur. J. Biochem.* **193** (1990) 559–565.
12. Miller W. R., Anderson T. J. and Jack W. J. L.: Relationship between tumour aromatase activity, tumour characteristics and response to therapy. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 1055–1059.
13. Esteban J. M., Warsi Z., Haniu M., Hall P., Shively J. E. and Chen S.: Detection of intratumoral aromatase in breast carcinomas. *Am. J. Path.* **140** (1992) 337–343.
14. Bulun S. E., Price T. M., Aitken J., Mahendroo M. S. and Simpson E. R.: A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome *P*450 transcripts using competitive polymerase chain reaction after reverse transcription. *J. Clin. Endocr. Metab.* **77** (1993) 1622–1628.
15. Turner R. T., Riggs B. L. and Spelsberg T. C.: Skeletal effects of estrogen. *Endocrine Rev.* **15** (1994) 275–300.

16. Means G. D., Mahendroo M. S., Corbin C. J., Mathis J. M., Powell F. E., Mendelson C. R. and Simpson E. R.: Structural analysis of the gene encoding human aromatase cytochrome *P*-450, the enzyme responsible for estrogen biosynthesis. *J. Biol. Chem.* **264** (1989) 19,385–19,391.
17. Harada N., Yamada K., Saito K., Kibe N., Dohmae S. and Takagi Y.: Structural characterization of the human estrogen synthetase (aromatase) gene. *Biochem. Biophys. Res. Commun.* **166** (1990) 365–372.
18. Mahendroo M. S., Means G. D., Mendelson C. R. and Simpson E. R.: Tissue-specific expression of human *P*450arom: the promoter responsible for expression in adipose is different from that utilized in placenta. *J. Biol. Chem.* **266** (1991) 11,276–11,281.
19. Kilgore M. W., Means G. D., Mendelson C. R. and Simpson E. R.: Alternative promotion of aromatase cytochrome *P*450 expression in human placenta. *Molec. Cell. Endocr.* **83** (1992) R9–R16.
20. Mahendroo M. S., Mendelson C. R. and Simpson E. V.: Tissue-specific and hormonally-controlled alternative promoters regulate aromatase cytochrome *P*450 gene expression in human adipose tissue. *J. Biol. Chem.* **268** (1993) 19,463–19,470.
21. Toda K. and Shizuta Y.: Molecular cloning of a cDNA showing alternative splicing of the 5′-untranslated sequence of mRNA for human aromatase *P*450. *Eur. J. Biochem.* **213** (1993) 383–389.
22. Means G. D., Kilgore M. W., Mahendroo M. S., Mendelson C. R. and Simpson E. R.: Tissue-specific promoters regulate aromatase cytochrome *P*450 gene expression in human ovary and fetal tissues. *Molec. Endocr.* **5** (1991) 2005–2013.
23. Harada N., Utsumi T. and Takagi Y.: Tissue-specific expression of the human aromatase cytochrome *P*450 gene by alternative use of multiple exon 1 and promoters, and switching of tissue-specific exon 1 in carcinogenesis. *Proc. Natn. Acad. Sci. U.S.A.* **90** (1993) 11,321–11,316.
24. Simpson E. R., Mahendroo M. S., Means G. D., Kilgore M. W., Hinshelwood M. M., Graham-Lorence S., Amarneh B., Ito Y., Fisher C. R., Michael M. D., Mendelson C. R. and Bulun S. E.: Aromatase cytochrome *P*450, the enzyme responsible for estrogen biosynthesis. *Endocrine Rev.* **15** (1994) 342–355.
25. Toda K., Miyahara K., Kawamoto K., Ikeda H., Sagara Y. and Shizuta Y.: Characterization of a *cis*-acting regulatory element involved in human aromatase *P*450 gene expression. *Eur. J. Biochem.* **205** (1992) 303–309.
26. Toda K. and Shizuta Y.: Identification and characterization of *cis*-acting regulatory elements for the expression of the human aromatase cytochrome *P*-450 gene. *J. Biol. Chem.* **269** (1994) 8099–8107.
27. Toda K., Simpson E. R., Mendelson C. R., Shizuta Y. and Kilgore M. W.: Expression of the gene encoding aromatase cytochrome *P*450 (CYP 19) in fetal tissues. *Molec. Endocr.* **8** (1994) 210–217.
28. Honda S-I., Harada N., and Takagi Y.: Novel exon 1 of the aromatase gene specific for aromatase transcripts in human brain. *Biochem. Biophys. Res. Commun.* **198** (1994) 1153–1160.
29. Akira S., Isshiki H., Sugita T., Tanabe O., Kinoshita S., Nishino Y., Nakajima T., Hirano T. and Kishimoto T.: A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* **9** (1990) 1897–1906.
30. Vinson C. R., LaMarco K. L., Johnson P. F., Landschulz W. H. and McKnight S. L.: *In situ* detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev.* **2** (1988) 801–806.
31. Poli V., Mancini F. P. and Cortese R.: IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* **63** (1990) 643–653.
32. Descombes P., Chojkier M., Lichtsteiner S., Falvey E. and Schibler U.: LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4** (1990) 1541–1551.
33. Cao Z., Umek R. M. and McKnight S. L.: Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5** (1991) 1538–1552.