Efp as a primary estrogen-responsive gene in human breast cancer

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Received 3 December 1999; received in revised form 16 March 2000

Edited by Shozo Yamamoto

Abstract We have previously isolated the efp (estrogen-responsive finger protein) that is required for the normal estrogen-induced cell proliferation. Here, we show the genomic organization of the human efp gene which consists of nine exons. The efp mRNA was expressed in human breast tumors and the estrogen-induced expression of the efp was found in MCF-7 human breast cancer cells. Moreover, efp promoter activity was enhanced through the estrogen-responsive element dependent on estrogen and estrogen receptor. These results suggest that the efp can mediate estrogen actions such as cell growth in human breast cancer as a primary responsive gene.

Key words: Estrogen; Estrogen receptor; Breast cancer; Estrogen-responsive element; Transcriptional regulation

1. Introduction

Estrogen plays important roles in the development/maturation of the female reproductive organs and sexual behavior [1]. Estrogen is also involved in cell cycle progression and generation/promotion of tumors such as breast, uterus and prostate cancers [2,3]. Estrogen actions are assumed to be mediated by estrogen receptors (ERα and β) which regulate the transcription of target genes [1]. Therefore, uncovering the regulation and function of estrogen-responsive genes must lead to the clarification of the molecular mechanism of estrogen action in target tissues including breast cancer.

Estrogen-responsive finger protein (efp), a member of the RING finger family, has been isolated by genomic binding site cloning using a recombinant ER protein [4]. The efp gene has an estrogen-responsive element (ERE) at the 3′-untranslated region (UTR) and is predominantly expressed in female reproductive organs including uterus, ovary and mammary gland [4,5]. Estrogen-induced expression is found in the uterus, brain and mammary gland cells [4,5]. It is revealed that estrogen responses are markedly attenuated in uterus of efp knockout mice, suggesting that efp is essential for estrogen-induced cell growth [6]. Therefore, we have inferred that the efp mediates some estrogen actions in target organs [4–6].

We previously analyzed the 5′-flanking region of the human efp gene [7] and demonstrated that the region −173 to −1 relative to the translation initiation site had full promoter activity. In this region, an E-box element which bound the upstream stimulatory factor (USF) was indispensable for basic promoter activity. A GC-rich region with three putative GC-boxes adjacent to the E-box contained a positive regulatory region (−120 to −110) where an unknown factor(s) bound. In the mouse efp promoter, both the E-box and the GC-rich positive regulatory region were well conserved [8]. Besides, a negative regulatory region was revealed upstream of the promoter region of the human efp gene [7].

In the present study, we describe the structure of the human efp gene, and its expression and estrogen-induced promoter activity in human breast cancer cells.

2. Materials and methods

2.1. Breast tumor samples and cell culture

Fifteen samples of human breast tissues were collected from patients. Tumor tissues and adjacent normal tissues were obtained from the same resection specimen. Informed consent was obtained from all patients and permission was obtained from the local Ethical Committee. MCF-7 cells derived from a human breast cancer and 293T cells derived from a human embryonic kidney cell line transformed with SV40 large T antigen were maintained in Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal calf serum (FCS) at 37°C in 5% CO_2 and a humidified atmosphere. To investigate estrogen responsiveness of efp mRNA, MCF-7 cells were maintained in phenol red-free DMEM containing 0.5% dextran-coated charcoal-treated FCS for 48 h. Subsequently the medium was changed to one containing or not containing 10^{-8} M 17β-estradiol and the cells were harvested at indicated times to isolate total RNA.

2.2. RNA extraction and RNase protection assay

Total RNA was extracted from breast tumor tissues and adjacent normal tissues and MCF-7 cells using Isogen (Nippongene, Toyama, Japan) and the RNase protection assay was performed using an RPA II kit (Ambion, Austin, TX, USA) [7]. Total RNA (20 μg) was hybridized with efp and GAPDH RNA probes described elsewhere [5]. Hybridization signals were quantified using the BAS 2000 phosphoimaging system (Fuji, Inc.). The signal intensities of efp (330 bp) and GAPDH (114 bp) were measured using the same area, respectively, and the values of efp signals were divided by the corresponding one of GAPDH to normalize them. Statistical analysis was done using Scheffé's *F* test.

2.3. Chloramphenicol acetyltransferase (CAT) assay

Oligonucleotides 5′-CCGCTCGAGTTCAGGGTCATGGTGACCCTGATCTCGAGCGG-3′ and 5′-CCGCTCGAGATCAGGGTCACCATGACCCTGAACTCGAGCGG-3′ containing the ERE of the 3′-UTR of the human efp gene [4] were annealed and inserted into the *Xho*I site of pMSG-CAT (Pharmacia, Piscataway, NJ, USA) self-ligated with *Bam*HI to delete the NEO coding sequence (pMSG-CATERE). The human efp promoter regions of CAT reporter plasmids (pCAThe-235, pCAThe-173, pCAThe-120, pCAThe-114) described previously [7] were transferred to pMSG-CATERE. They were designated pCAThe-235ERE, pCAThe-173ERE, pCAThe-120ERE and pCAThe-114ERE, respectively. pCAThe-173mutERE1 and pCAThe-173mutERE2 which contained 2 bp mutations: TTTCATGGTGACC in a half-site and 4 bp mutations: TTTCATGGTGATT in both half-sites of the ERE of the 3′-UTR of the human efp gene, respectively, were constructed like pCAThe-173ERE except for using the corresponding mutated oligonucleotides.

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PII: S0014-5793(00)01421-6

Oligonucleotides 5′-CCGCTCGAGCCCGAGGGCAGGGTGACC-TCCTGCTCGAGCGG-3′ and 5′-CCGCTCGAGACGGAGGTCA-CCCTGCCCTAGGGCTCGAGCGG-3′ containing the ERE of the 3′-UTR of the mouse efp gene were used to construct pCAThe-173mERE. The CAT assay was performed as described [9].

3. Results

3.1. Structural organization of the efp gene

During a database search, we noted that a sequence of chromosome 17 (clone hRPC1107 A 17, accession number AC004584) deposited in GenBank contained the full length of the efp cDNA. Comparison between the cDNA and the chromosome 17 sequence revealed the genomic organization of the efp gene (Fig. 1). The human efp gene expands approximately 25 kb of genomic DNA and consists of nine exons and eight introns. The first methionine codon of the open reading frame is located in exon 1, whereas the stop codon and poly(A) addition signal are located in the last exon 9 which contained the whole 3′-UTR. Fig. 1B schematically shows the efp cDNA with nine exons and coding regions for the conserved domains below. RING finger (R) and B1 box (B1) and B2 box (B2) domains were encoded in exon 1, the coiled-coil domain (C-C) ranged from the end of exon 1 to exon 4 and the C-terminal domain (C) located in the N-terminal portion of exon 9. The sequences of exon/intron junctions are shown in Fig. 1C. All exon/intron boundaries conform to the consensus donor/acceptor sequence (gt/ag) for RNA splicing.

3.2. Expression of efp in breast cancer

To investigate the expression of efp mRNA in human breast cancer, an RNase protection assay was performed using total RNAs isolated from breast tumor tissues and adjacent normal tissues. RNA probes for efp and GAPDH were degraded with RNase digestion and gave specific protected signals of efp (330 bp) and GAPDH (114 bp), respectively. Some smaller background bands which may be caused by degradation of the probe or the protected fragment were seen [10,11]. Efp mRNA was expressed in all 15 samples tested. Although the amount of mRNA in a tumor relative to adjacent normal tissue varied among the specimens, efp mRNA expression of the tumor tissue was greater than that of the adjacent normal tissue in nine of 15 samples. In particular, there were two instances where the expression of the efp in tumor tissue was elevated more than twice (Fig. 2).

3.3. Estrogen responsiveness of efp mRNA and promoter activity

Since MCF-7 cells derived from human breast cancer mainly express ERα and are known to respond to estrogen [12], we investigated the estrogen responsiveness of efp

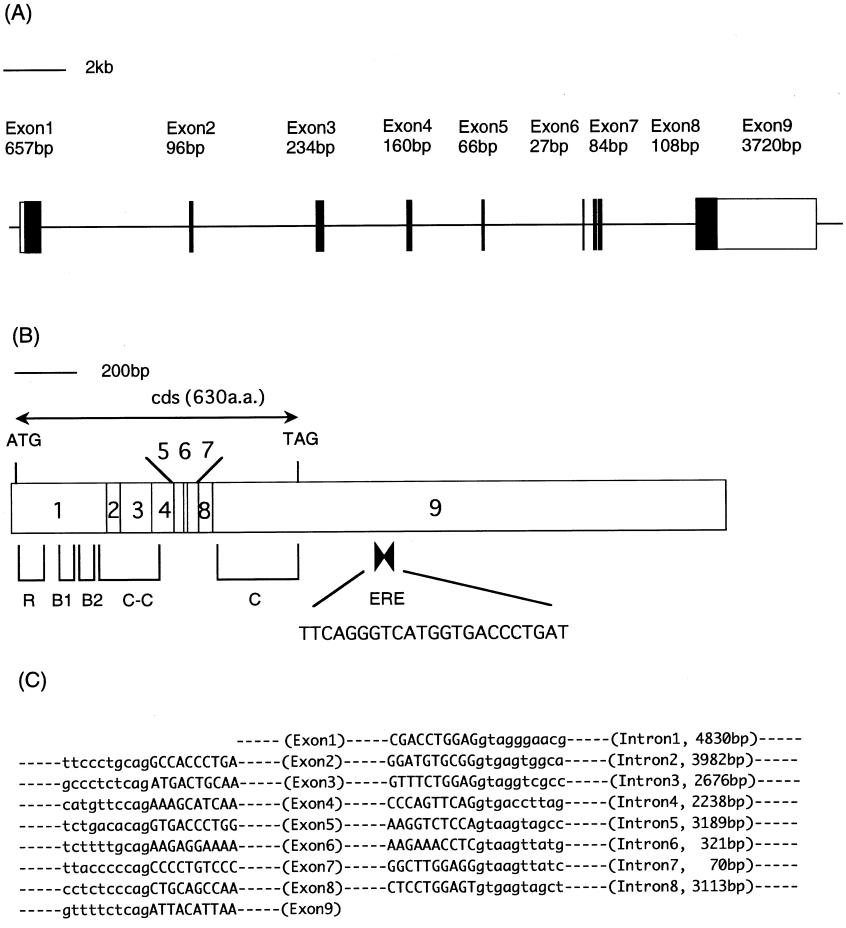

Fig. 1. Gene structure of efp. A: Genomic organization of the human efp gene. Exons are shown as boxes. The filled and open boxes indicate the coding and non-coding sequences, respectively. Exon sizes are also shown. B: cDNA structure of human efp. The boxes correspond to the exons shown in A in order. C: Exon/intron splice junction sequences.

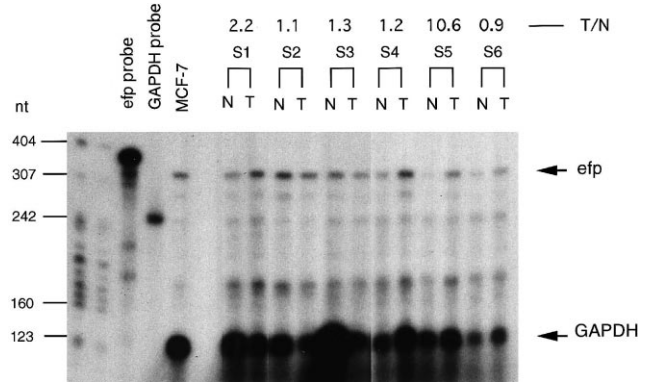

Fig. 2. Expression of efp mRNA in human breast cancer. RNase protection assay was performed using 20 μg of total RNAs from breast tumor tissues (T) and adjacent normal tissues (N) with efp and GAPDH RNA probes. The ratio of the amount of efp in a tumor to adjacent normal tissue (T/N) is shown above.

mRNA using this cell line. Total RNAs were extracted from MCF-7 cells treated with or without estrogen and an RNase protection assay was performed. The induction rates of the efp mRNA levels were plotted (Fig. 3A). The expression of the efp mRNA was elevated by estrogen treatment within 0.5 h and reached a peak (about 1.7 times) at 1 h. The expression level of efp mRNA returned to the level before treatment at 8 h. Thus, the estrogen induction of efp mRNA was confirmed in MCF-7 cells.

Subsequently, we analyzed the estrogen responsiveness of the efp promoter using the CAT assay in this cell line (Fig. 3B). Since we have found that the negative and positive regulatory regions and an E-box are important for transcription regulation [7], the sequence −235 to −1 relative to the translation initiation site containing all these elements, the sequence −173 to −1 not containing the negative regulatory region, the sequence −120 to −1 containing the positive regulatory region together with the E-box, and the sequence −114 to −1 containing only the E-box were constructed with the ERE of the efp in the 3′-UTR. The results are shown in Fig. 3B. pCAThe-173ERE, which had about five times higher activity than pCAThe-114ERE without estrogen, was enhanced three times by estrogen treatment. These results show the ERE in the 3′-UTR functions as an enhancer in response to estrogen treatment. However, pCAThe-120ERE and pCAThe-114ERE did not show significant estrogen-induced activities, indicating that the promoter region −173 to −1 is needed to respond to estrogen. The activity of pCAThe-235ERE was not enhanced by estrogen, suggesting that the negative regulatory region −235 to −174 has strong silencer activity.

3.4. ERE of efp responds to both ERα and ERβ and the mutations thereof attenuate the enhancing activity

Next, we examined whether the efp promoter/enhancer could respond to ERα or ERβ. 293T cells that do not express either ER were transiently cotransfected with ERα or ERβ expression plasmids and assayed for CAT activities (Fig. 4). When no ER expression vector was cotransfected, estrogen-induced promoter activities were not observed and the patterns were similar to that of MCF-7 without estrogen. On the other hand, estrogen-induced activities of pCAThe-173ERE, pCAThe-120ERE and pCAThe-114ERE were indicated if not only the ERα but also the ERβ expression vector was cotransfected. Since estrogen-induced activities of pCAThe-120ERE and pCAThe-114ERE were obviously lower than that of pCAThe-173ERE and were observed only after the ER expression vectors were cotransfected, the sequence −173 to −1 of the efp promoter is necessary for the entire activity through the ERE in the 3′-UTR. To confirm the function of the ERE in the 3′-UTR, mutated ERE sequences were introduced into pCAThe-173ERE (see Section 2) (Fig. 5). pCAThe-173mutERE1 containing a 2 bp mutation in a half-site of the ERE possessed about half of the activity to respond to estrogen compared to pCAThe-173ERE; on the other hand, pCAThe-173mutERE2 containing a 4 bp mutation in both half-sites of the ERE could hardly respond to estrogen either in MCF-7 cells or 293T cells cotransfected with ERα or ERβ. These results indicate that the ERE in the 3′-UTR is

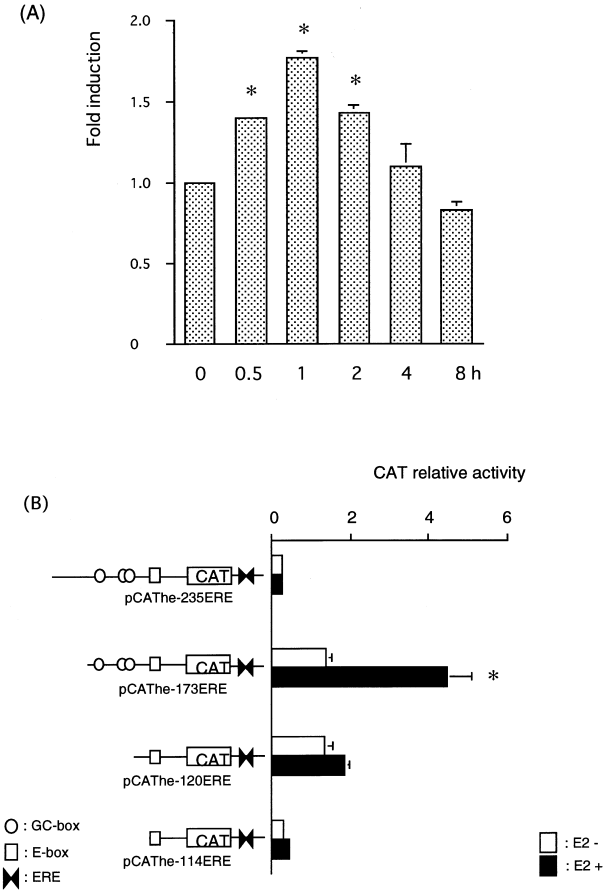

Fig. 3. Estrogen responsiveness of human efp in MCF-7 breast cancer cells. A: Estrogen-induced expression of efp mRNA. MCF-7 cells were treated for the indicated hours with or without 10^{-8} M 17β-estradiol and total RNAs were extracted. The RNase protection assay was carried out using 20 μg of total RNAs with efp and GAPDH RNA probes. The intensity of each signal of efp and GAPDH was measured by phosphoimager analysis and efp mRNA levels were normalized using GAPDH mRNA levels as reference. Each experiment was carried out in triplicate and the results are shown as mean+S.D. $*P < 0.01$. B: Estrogen-induced promoter activity of efp in MCF-7 cells. The structures of the CAT reporter plasmids are shown schematically (left) and their relative CAT activities are shown on the right of each construct. GC-box, E-box and ERE are shown by the open circle, open box and filled triangles, respectively. Each experiment was carried out in triplicate and the results are shown as mean+S.D. $*P < 0.01$ compared to transfections without estrogen.

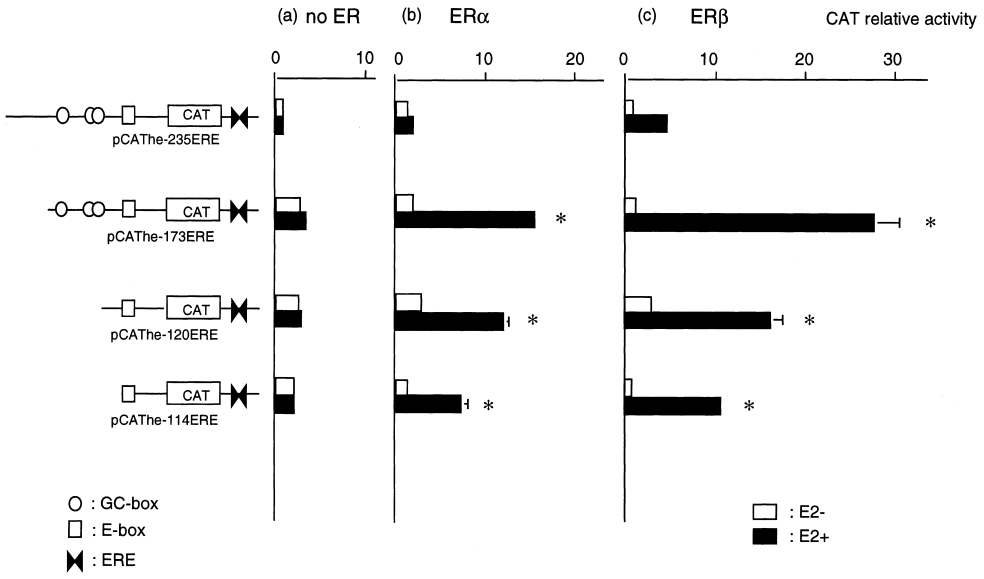

Fig. 4. Efp promoter with ERE responds to both ERα and ERβ. CAT reporter plasmids containing human efp promoter regions were transfected together with no ER expression vector (A), 1 μg of pCXN2-hERα (B) or 1 μg of pCXN2-hERβ (C) into 293T cells. Each experiment was carried out in triplicate and the results are shown as mean+S.D. $*P < 0.01$ compared to transfections without estrogen.

indispensable for the estrogen-induced activity of the human efp promoter. In addition, pCAThe-173mERE containing the ERE in the 3′-UTR of the mouse efp gene, which contains a 1 bp mismatch to the consensus ERE (GGTCANNNTGACC), could also respond to estrogen, albeit to a lower degree.

4. Discussion

The human efp gene expands approximately 25 kb of genomic DNA and has nine exons. Genetic alteration is a common event in breast cancer. Particularly, gene amplifications and allelic losses are frequently found on the long arm of chromosome 17 on which the efp gene is mapped [13]. For example, the BRCA1 gene, mapped at chromosome 17q21, is a well-known breast cancer susceptibility gene involved in DNA repair, transcription regulation and apoptosis [14,15]. The c-ErbB-2 gene, also mapped at chromosome 17q21, is a proto-oncogene encoding a tyrosine kinase receptor and is amplified in breast cancer [16]. The information of the genomic organization or exon/intron junction of the efp gene, which was mapped to the 17q23.1 region by FISH [17], will be useful for genetic analysis of breast cancer.

We have shown here a rather robust expression of the efp mRNA in human breast tumors and in MCF-7 breast cancer cells. Moreover, the expression of the efp mRNA in MCF-7 cells was elevated as early as 30 min after estrogen treatment, in line with the notion that the efp is directly regulated by estrogen. Efp is a member of the RING finger protein family [6] which appears to be involved in transcription, differentiation as well as oncogenesis. For example, PML [18] is fused to the retinoic acid receptor α in acute promyelocytic leukemia and BRCA1 [14] is mutated in some early-onset breast cancers and ovarian cancer. Thus, the efp may function as an ER primary responsive gene and mediate an estrogen action leading to growth in cancer. Indeed, the estrogen-responsive pro-

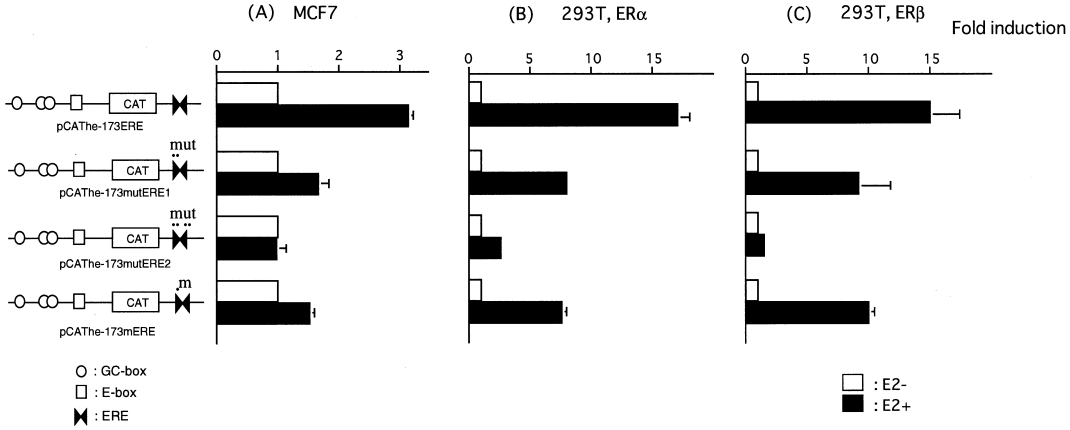

Fig. 5. Function of ERE in the 3′-UTR of the human and mouse efp genes for estrogen-induced transactivation. pCAThe-173ERE, pCAThe-173mutERE1, pCAThe-173mutERE2 and pCAThe-173mERE (see Section 2) were transfected together with pCXN2-hERα into MCF-7 cells (A) and 293T cells (B) or together with pCXN2-hERβ into 293T cells (C). Each experiment was carried out in triplicate and the results are presented as fold induction compared to transfections without estrogen.

liferation of uterine cells which express abundant ERα was impaired in efp knockout mice, suggesting that the efp is a mediator of cell growth [6]. We also found that the efp was expressed in some ovarian cancers by RNase protection assay (data not shown) and, recently, it was shown that the efp was upregulated by bile acids in gastric cancer cells in which bile acids appeared to be involved in carcinogenesis [19]. Further studies will provide useful information about the contribution of the efp gene in various cancers.

CAT analysis of the estrogen responsiveness of the efp promoter demonstrated that the ERE in the 3′-UTR efficiently functions as an enhancer. Recently, a second estrogen receptor, ERβ, has been isolated and its tissue distribution and ligand specificity have been shown to be different from those of ERα [20,21], suggesting differential biological roles of ERα and ERβ. It is reported that some human breast tumors express ERα and/or ERβ [22,23]. The efp promoter could be enhanced by both ERα and ERβ under estrogen treatment, whereas it was not observed when neither ER expression vector was transfected. Thus, the efp could respond to estrogen as a common downstream gene of ERα and ERβ and may mediate estrogen actions in both ERα- and ERβ-positive breast tumors.

Taking together the results presented here and described previously [7], we consider the mechanism of transcription regulation of the human efp gene to be as follows. The E-box-bound USF is indispensable for the basal activity in the TATA-less human efp promoter [7]. USF is also known to recognize an initiator sequence around a transcription initiation site and to interact with basal transcription factors [24]. Moreover, both ERα and ERβ could further regulate the promoter activity through the ERE in the 3′-UTR. We assume that the efp acts as one of the primary estrogen responsive genes in ERα- and/or ERβ-positive breast tumors and would mediate estrogen functions such as cell proliferation. Some transcription cofactors of ER as well as negative and positive regulatory factors described above may be involved in the regulation although their mechanisms remain to be determined. Studies of transcriptional regulation of estrogen-responsive genes such as efp will help clarify the mechanism of estrogen actions in cancer.

Acknowledgements: K.I. is supported by the Sankyo Foundation of Life Science. This work was supported in part by a grant from Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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