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Regulation of estrogen receptor α gene mediated by promoter B responsible for its enhanced expression in human breast cancer

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ABSTRACT

We have previously reported that transcription from a distal promoter (promoter B) of the estrogen receptor α (ERα) gene is responsible for the increased expression of ERα in human breast carcinomas. This paper first characterized the promoter B region in terms of transient transfection experiments with luciferase using MCF-7 cells. Gradual deletions from the 5′-end of promoter B resulted in a decrease in promoter activity corresponding to the deleted lengths; a deletion of 39 bp in a non-coding exon 1a, drastically diminished the activity, indicating existence of an important *cis*-element. Furthermore, electrophoretic mobility shift assay and subsequent mutational analysis indicated that this element containing nucleotide sequence CTGGAAAG forms a specific DNA–protein complex. This element was capable of transactivating a heterogeneous SV40 promoter in MCF-7 cells, confirming that the element is a transcriptional enhancer; the *trans*-acting factor binding to the element was named ERBF-1 (estrogen receptor promoter B associated factor-1). The ERBF-1 was exclusively expressed in those cells expressing ERα mRNA transcribed from promoter B. Our findings indicate that ERBF-1 plays an important role in the expression of the ERα gene transcribed from promoter B, which is selectively utilized in breast cancer.

INTRODUCTION

Estrogen and its receptor play an important role in promotion and progression of steroid hormone-dependent cancers including breast cancer. In breast cancer, the expression status of the estrogen receptor α (ERα) is closely associated with cancer biology. For example, the presence of ERα is associated with less aggressive phenotypes (1,2) and the expression level of ERα in tumor tissues is a good predictor of prognosis in endocrine treatment (3), which aims to inhibit the mitogenic stimulus produced by the ERα bound to estrogen. Recently, a second ER subtype (ERβ) has been discovered in various tissues of rat, mouse and human (4–6), though the contribution of this gene to carcinogenesis is still unknown.

ERα belongs to a family of nuclear receptors that have steroid and thyroid hormones as known ligands and upon binding to estrogen response elements (ERE) in the regulatory region of the target genes, it regulates transcription of various genes as a transcription factor (7,8). The human ERα gene is located on chromosome 6q25.1 spanning >140 kb and it contains eight exons and seven introns (9,10). After isolation of human ERα cDNA and its partial genomic clone (11), several laboratories have attempted to identify and analyze the 5′ flanking region of this gene, revealing that transcription of this gene occurs from at least two different promoters (12–14). Transcription from these two promoters has been analyzed in various cell lines including mammary tumor cells (15–17). A proximal promoter (promoter A) present immediately upstream of the coding region is used both in normal human mammary epithelium and in ER-positive human mammary tumor cell lines, while the message from the distal promoter (promoter B) has been predominantly detected in mammary tumor cell lines with different levels of expression. Comparison of the 5′ flanks of the human, mouse and rat ERα gene has shown that the distal promoter B is well conserved among these species, while the sequence of the proximal promoter A region is more divergent (18). In fact, the determined 5′-ends of rat ERα mRNA revealed that this gene does not possess a functional promoter A, suggesting that the well-conserved promoter B is physiologically important.

To elucidate which promoter is responsible for the expression of ERα in human mammary tumor tissues, we have recently carried out a quantitative analysis of the transcription originating from the two different promoters on a tumor, the tumor's surrounding tissue and normal mammary tissue obtained from patients with breast cancer, who have different statuses of ERα expression and clinical characteristics (19). We found that the enhanced ERα protein expression in breast tumors correlates well with the increased mRNA expression from the distal promoter B,

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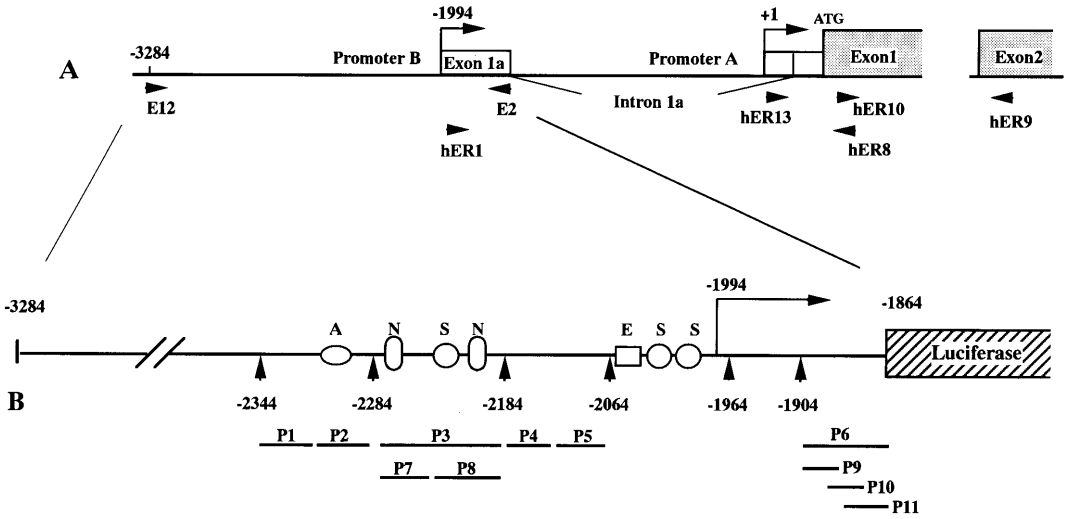

Figure 1. Schematic representation of the 5′ flanking region of the human ERα gene. (**A**) Location of the two promoters in the ERα gene. Arrows with numbers indicate the positions of the two major transcription start sites. The position of the proximal transcription start site is designated as +1. Arrowheads with names indicate the positions of primers used for isolation of the promoter B region of the ER gene and detection of ER mRNA expression by RT–PCR as described in Materials and Methods. (**B**) Schematic representation of promoter B constructs with the reporter gene, luciferase. Vertical arrows with numbers indicate positions of either 5′- or 3′-ends of deletion constructs. Putative binding sites for known transcription factors are also shown. Open circle with S indicates the site for Sp-1; open square with E, E-box; horizontal oval with A, AP-1; vertical oval with N, NF-κB. Lines with P1–P11 indicate oligonucleotides used for EMSA.

not that from promoter A, indicating that the transcription from promoter B plays an essential role in the overexpression of ERα protein in human breast carcinogenesis.

Thus, functional analysis of the regulatory region of the ERα gene, especially that of the distal promoter B, may give us new insight into understanding breast carcinogenesis as well as the physiological function of the ER gene. So far, two *trans*-acting factors, ERF-1 and AP-1, have been shown to be involved in the regulation of human ERα gene expression (15,20): ERF-1, a member of the AP2 transcription factor family, binds to elements in the 5′-non-coding region of the ERα gene and was found to be important for the transcriptional regulation of promoter A in ER-positive T-47-D cells (15,21); AP-1 binds ~3.7 kb upstream from the proximal transcription start site, which was critical for the high level of ERα expression in ER-positive MCF-7 cells (20). However, neither ERF-1 nor AP-1 has been identified as an essential factor in the promoter B-specific transcription of the ERα gene.

In this report, we performed functional analysis of promoter B in MCF-7 cells, where endogenous expression from this promoter was observed, and identified a novel *cis*-element within the upstream non-coding exon 1a that was critical for the transcriptional activity of promoter B in ER-positive cell lines.

MATERIALS AND METHODS

Cells and culture

Human breast cancer cells, MCF-7, T-47-D, ZR-75–1, MDA-MB-231 and BT-20, and all other tumor cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 2 μg/ml of gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reporter plasmid constructions

The 1.4 kb DNA fragment (–3284 to –1864) including the 5′ flanks as well as non-coding exon 1a of the ERα gene was amplified by LA (long and accurate) PCR using LA Taq polymerase (Takara). The following primers were used for the amplification: forward primer E12, 5′-TAG AGC ATG GGT GGC CAT TG-3′; backward primer E2, 5′-CTG CTG GAT CAA GAA CGT CT-3′. The amplified DNA fragment was subcloned into a cloning vector, pGEM7Zf(+) (Promega) to give a plasmid pGEMProB1.4 and confirmed by sequencing with an ALF Auto Read Sequencing Kit (Pharmacia) and found to have no amplification error. Various fragments of the ER promoter B region were prepared from pGEMProB1.4 and subcloned into a reporter plasmid pGL2 Basic (Promega), which encodes firefly luciferase as a reporter gene. Fragments used for the construction were as follows: a 1.4 kb *Sal*I–*Sal*I fragment (ProB1.4 kb); a 1.35kb *Sal*I–*Nco*I fragment (ProB1.35 kb); a 0.47 kb *Eco*RV–*Acc*I fragment (ProB0.47 kb). To generate other fragments of promoter B, PCR was performed using specific primers and pGEMProB1.4 as a template. The following primers were used for amplification of fragments: E12 and REV, 5′-TTC ACA CAC TGA GCC ACT CG-3′, for the ProB 1.3 kb fragment; PB1, 5′-CAG CAG AAA GCA TAG GGT AC-3′, and E2 for the ProB 0.39 kb fragment; PB2, 5′-TTC TCT GGC TGT GCC ACA CT-3′, and E2 for the ProB 0.31 kb fragment; PB3, 5′-AAG TGC CCT GCC TAC TGG CT-3′, and E2 for the ProB 0.19 kb fragment. All the amplified fragments were also subcloned into pGL2 Basic vector. Schematic diagrams of these constructs are shown in Figures 1B and 2.

Transfection and luciferase assay

Transient transfection was performed essentially as described previously with a slight modification (22). Briefly, MCF-7 cells were grown on plastic culture dishes in RPMI medium with 5% FCS to 30–50% confluency and then the medium was replaced

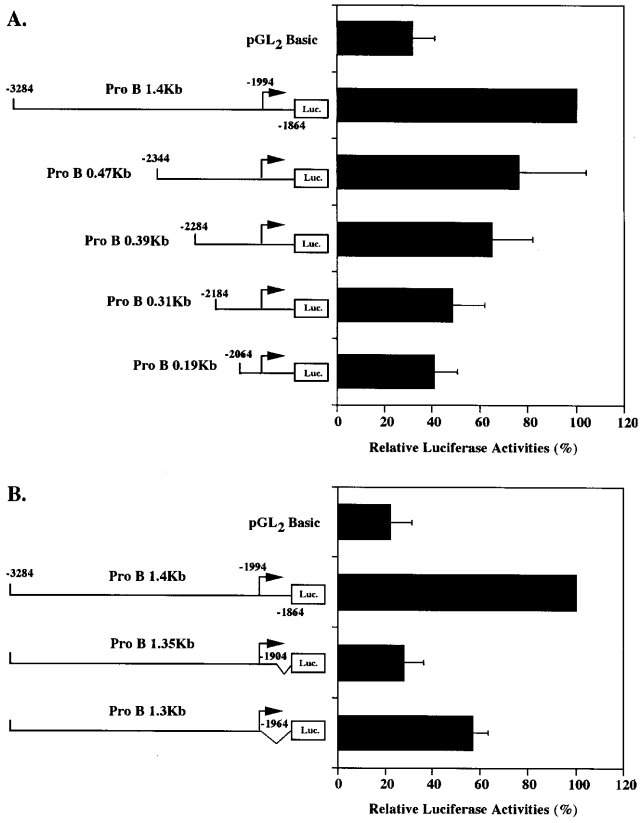

Figure 2. Promoter activities of 5′ and 3′ deletion constructs of the promoter B region of the human ERα gene. (**A**) Schematic diagrams of 5′ deletion constructs of the human ER promoter B region fused to a reporter gene, luciferase. All the constructs were transiently transfected into MCF-7 cells as described in Materials and Methods. After 48 h incubation, cells were lysed and the luciferase activity was measured. All the measured luciferase activities were normalized against protein concentration. Normalized luciferase activities were shown as relative luciferase activities, where that of the longest construct was taken as 100%. The results are averages of three transfection experiments for each plasmid. The error bars show standard deviations. (**B**) Schematic diagrams of 3′ deletion constructs of the human ER promoter B region fused to a reporter gene, luciferase. Results of the transfection were shown as in the above.

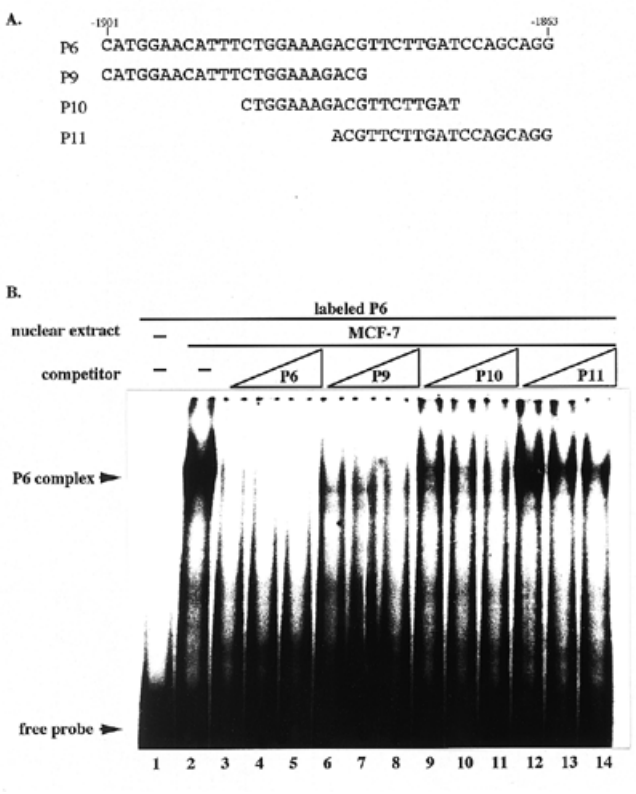

Figure 3. EMSA using the P6 oligonucleotide probe with nuclear extracts from MCF-7 cells. (**A**) Sequences of the sense strand of the oligonucleotides used in EMSA. (**B**) Annealed P6 oligonucleotides were radiolabeled by fill-in reaction using [α-^{32}P]dCTP. The probe was incubated with 7 µg of nuclear extract prepared from MCF-7 cells on ice for 30 min and electrophoresed in a 5% polyacrylamide gel. Lane 1, no nuclear extract; lane 2, no competitor; lanes 3–5, P6 oligonucleotides; lanes 6–8, P9 oligonucleotides; lanes 9–11, P10 oligonucleotides; lanes 12–14, P11 oligonucleotides as competitor. From lane 3, each three lanes contained 50, 100 and 200 times molar excesses of cold oligonucleotide, respectively. Arrows indicate the positions of a major DNA–protein complex and free probes.

with serum-free RPMI 1640 medium. Plasmid cocktail was mixed with Trans IT™ LT-1 reagent (Takara) and added to the culture. After 4 h of incubation, the medium was replaced with fresh RPMI 1640 medium with 5% FCS and the cells were further cultured for 40 h. Then the cells were lysed in a lysis buffer and luciferase activity was measured using the Luciferase Assay System (Promega). Protein concentration of the cell extracts was determined using the BCA Protein Assay kit (Pierce). All the measured luciferase activity was normalized against the determined protein concentration. In some experiments, transfection was also performed with an internal control vector pRL-TK (Promega), which encodes a *Renilla* luciferase reporter gene driven by a TK (thymidine kinase) promoter, and the luciferase activity of the constructs was also normalized to the *Renilla* luciferase activity using the Dual Luciferase Assay Kit (Promega).

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described previously (23). Briefly, the double-stranded oligonucleotide probes were end-labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I (Takara). Sequences of the oligonucleotides encompassed are shown in Figures 3A and 4A. Nuclear extracts (7 µg protein/reaction) prepared from cancer cells as described previously (23) were incubated with 1.2 ng of labeled oligonucleotide probes in a 10 µl reaction mixture containing 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT, 4% glycerol and 500 ng poly(dI–dC) (Pharmacia LKB) for 30 min on ice. Radioinert competitor DNA was added when indicated. The reaction mixture was then loaded onto a 5% non-denaturing polyacrylamide gel containing 0.5× TBE (40 mM Tris, 40 mM borate, 1 mM EDTA). Gels were run at 130 V for 3 h at 4 °C and dried under vacuum. Results were visualized using Bio-Image Analyzer BAS2000 (Fuji Film Co. Ltd, Tokyo).

Preparation of RNA and RT–PCR

Total RNAs were prepared from 1–5 × 10^6 cells according to the method of Chomczynski and Sacchi (24). Semi-quantitative RT–PCR was carried out using the GeneAmp RNA PCR Kit (Takara Shuzo Co. Ltd, Tokyo) as previously reported (25).

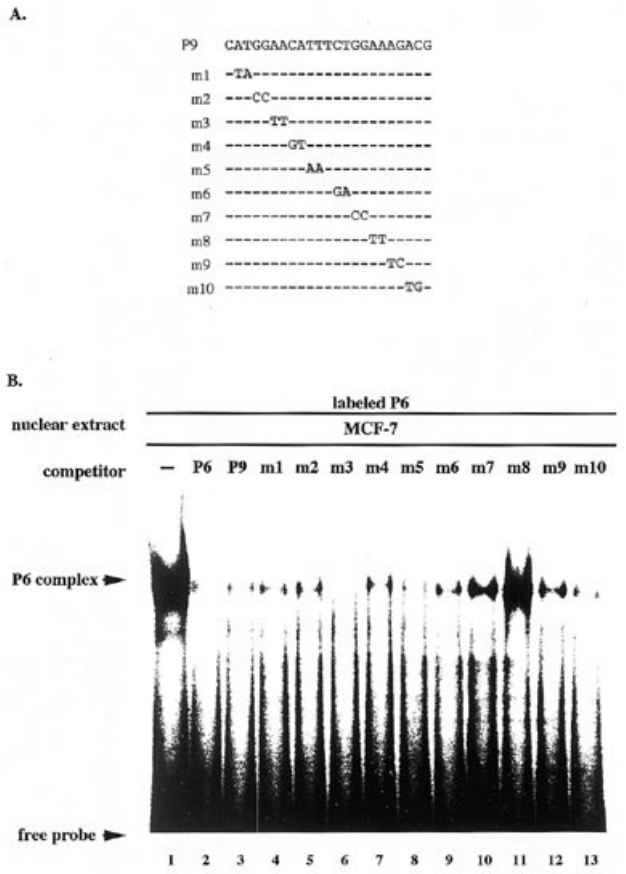

Figure 4. Mutational analysis of the ERBF-1 binding site. (**A**) Sequences of the sense strand of the oligonucleotides used in EMSA. Each mutation site is indicated by capital letters and wild-type sequences by short bars. (**B**) EMSA using nuclear extract prepared from MCF-7 cells with the radiolabeled P6 probe was performed in the presence of a series of mutant oligonucleotides as described in Materials and Methods. Lane 1, no competitor; lane 2, P6 oligonucleotides; lane 3, P9 oligonucleotides; lanes 4–13, mutant probes as shown by their names. Each lane contained 100 times molar excess of cold probe.

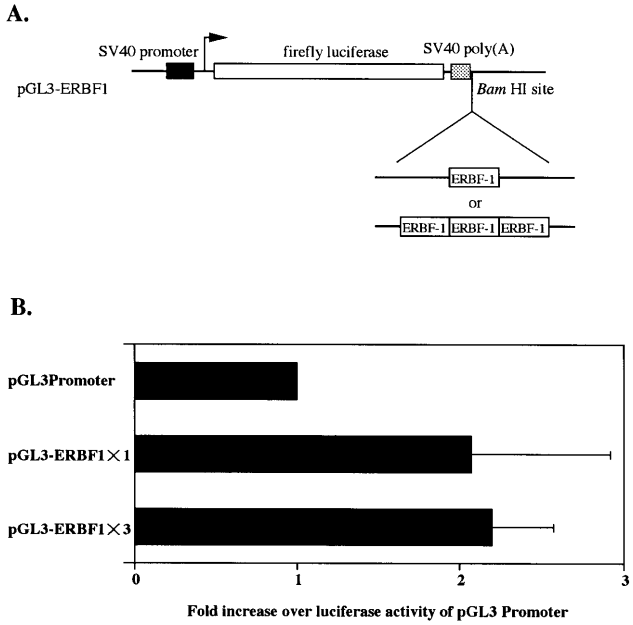

Figure 5. Functional assay of ERBF-1. (**A**) Schematic diagrams of the constructs used for the functional analysis of the ERBF-1 element. Either single or three copies in tandem of the double-stranded oligonucleotides of ERBF-1 fragments were ligated into the *Bam*HI site of a reporter plasmid pGL3-Promoter. (**B**) Effects of the ERBF-1 element on a heterogeneous SV40 promoter activity. All the constructs were transiently transfected into MCF-7 cells as described in Materials and Methods. Luciferase activities were measured as described in the legend to Figure 2. All the measured luciferase activities were normalized against protein concentration. Data are presented as fold increase in induction over that obtained with the parental pGL3-Promoter vector. The results shown are averages of three independent experiments for each plasmid. The error bars indicate standard deviations.

Oligonucleotides used in the amplification were as follows: hER1, 5′-CTC GCA CAT GCG AGC ACA TT-3′, and hER8, 5′-GCT CGT TCC CTT GGA TCT GA-3′, for type B ER mRNA; hER9, 5′-GGT ACT GGC CAA TCT TTC TC-3′, and hER13, 5′-TAA CCT CGG GCT GTG CTC TT-3′, for type A ER mRNA; hER10, 5′-AAC GCG CAG GTC TAC GGT CA-3′, and hER9 for total ER mRNA; GAP1, 5′-ACA TCG CTC AGA CAC CAT GG-3′, and GAP2, 5′-GTA GTT GAG GTC AAT GAA GGG-3′, for glyceraldehyde phosphate dehydrogenase (GAPDH) (26). These primers were designed to sandwich one intron for specific detection of mRNA. Prepared total RNA (2 μg) was reverse transcribed to synthesize cDNA using AMV reverse transcriptase (Takara) with random hexamers at 42°C for 30 min and then subjected to PCR amplification with specific primers (0.4 μg) and 3 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) in 50 μl mixtures consisting of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.2 mM dNTPs (dATP, dTTP, dGTP and dCTP). PCR comprised 25 cycles for GAPDH using primers GAP1 and GAP2 and 30 cycles for each ER mRNA using the primers shown in Figure 1, with denaturing at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min in each cycle using a GeneAmp™ PCR System 9600 (Perkin Elmer-Cetus). The PCR products were then subjected to 5% polyacrylamide gel electrophoresis and the radioactivity was quantified by autoradiography with a Bio-Image Analyzer BAS2000.

Functional analysis of ERBF-1 element

Either single or three copies of ERBF-1 binding elements in tandem were subcloned into the *Bam*HI site of a reporter plasmid pGL3-Promoter (Promega), to give plasmids pGL3-Pro-ERBF1-1 and pGL3-Pro-ERBF1-3, respectively (Fig. 5A). The sequence of the oligonucleotide encoding ERBF-1 element was as follows: 5′-GAT CTC ATG GAA CAT TTC TGG AAA GAC GTT CTT GAT G-3′. All constructs were confirmed by sequencing as described above.

RESULTS

Identification of a transcriptional enhancer element in the ERα promoter B

Since the distal promoter B activity is essential for the over-expression of ERα protein in human mammary tumor, we concentrated on studying the function of promoter B in order to elucidate the mechanisms of the tumor-specific regulation of ERα gene expression. To analyze this, we cloned the 5′ flanking

region of the human ERα gene spanning from –3284 to –1864 bp relative to the proximal transcription start site which contains 5′ flanks as well as the upstream exon 1a (Fig. 1). The amplified fragment was cloned into a promoter-less reporter plasmid pGL2 Basic and the resultant plasmid was transfected into ER-positive breast cancer cell line MCF-7, after which luciferase activity was measured. As shown in Figure 2, this construct revealed a weak but apparent promoter activity compared with the parental vector pGL2 Basic, indicating that this region contains elements important for the basal level expression of B type ER mRNA. Computer analysis of this region showed the existence of numerous putative binding sites for known transcription factors, as shown in Figure 1. Thus, various deletions from the 5′-ends of the amplified fragment were prepared to divide these putative elements, as described in Materials and Methods; they were also analyzed by transient transfection. Deletion from the 5′-ends gradually decreased reporter activities in MCF-7 cells, indicating that multiple elements in this region are involved in promoter B activity (Fig. 2A). Next we explored the 3′-downstream region: two deletion constructs from the 3′-ends were prepared and analyzed (Fig. 2B). Notably, the 39 bp deletion spanning from –1905 to –1864 bp was the only deletion that almost completely abolished promoter B activity in MCF-7 cells, indicating that an important *cis*-acting element is present in this region (Fig. 2B). Moreover, and interestingly enough, further deletion up to –1965 bp partially recovered promoter activity, suggesting the existence of a negative element in this region.

In some experiments, transfection was also performed with an internal control plasmid, pRL-TK, as described in Materials and Methods and the pattern of relative expression compared with the internal control was essentially identical to that shown in Figure 2A and B (data not shown).

Electrophoresis mobility shift assay (EMSA) of the enhancer elements

Since the 3′-downstream region spanning from –1905 to –1864 bp in the non-coding exon 1a was indispensable for promoter B activity in transient expression analysis, we next sought to determine whether any nuclear protein bound to this element by EMSA. The double-stranded oligonucleotide probe P6 spanning from –1901 to –1863 (Fig. 3A) was incubated with nuclear extracts prepared from MCF-7 cells and was resolved in a non-denaturing polyacrylamide gel. A strong band, designated P6 complex, was formed (Fig. 3B): P6 complex was specific for this element, because excess amounts of cold oligonucleotides encoding binding sites for AP-1 or Sp-1 could not compete it off (data not shown). To identify which part of the element is important for the formation of the specific complex, we synthesized three overlapping oligonucleotides, P9–P11 (Fig. 3A), which were used in competition experiments. EMSA was performed as described above in the presence of 50, 100 and 200 times molar excesses for each of the cold oligonucleotides. The P6 complex may contain two closely migrating bands. The major one, migrating more slowly, was selectively competed out by P6 oligonucleotide, while the minor one was severely affected by oligonucleotide P11. Thereafter, we referred to the major band which was bound tightly to the 5′-portion of the P6 oligonucleotide as P6 complex.

Moreover, mutational analysis was also performed using a series of mutant oligonucleotides, containing a 2 bp substitution mutation in each oligonucleotide, as indicated in Figure 4A. EMSA was performed using labeled P6 oligonucleotides with nuclear extracts from MCF-7 cells in the presence of a 200 times molar excess of each oligonucleotide. As shown in Figure 4B, substitution of nucleotides AA by TT (m8) abolished interference activity, indicating that these nucleotides are most important for the specific interaction. In addition, two other mutant oligonucleotides, m7 and m9, with mutation sites adjacent to those of m8, showed reduced competition activity, suggesting that these nucleotides may also participate in the specific interaction. Taken together, the results showed that the sequence GGAAAG was an important binding sequence for the formation of P6 complex and we named this element ERBF-1, for estrogen receptor promoter B associated factor-1.

Functional assay of ERBF-1

To test whether the ERBF-1 element can functionally transactivate a heterogeneous promoter, single or three copies in tandem of double-stranded oligonucleotides containing ERBF-1 sequence were subcloned into a reporter plasmid pGL3-Promoter driven by the SV40 promoter, as shown in Figure 5A. These constructs were transiently transfected into MCF-7 cells and the luciferase activities were measured. As shown in Figure 5, pGL3-Pro-ERBF1-1 and pGL3-Pro-ERBF1-3 showed 2.1 and 2.2 times higher reporter activity, respectively, compared with the parental pGL3-Promoter. The same transfection experiment using MDA-MB-231 cells (ER-negative) did not show any enhancement of the activity (data not shown). These results indicate that this element enhances the transcription even with a heterogeneous promoter, although the enhancing activity is low.

Expression of ERBF-1 binding protein correlated well with endogenous promoter B activity of ER gene in cancer cell lines

Next, to determine whether the ERBF-1 binding protein expression is restricted to certain types of cells, we performed EMSA using P6 oligonucleotide probes with various nuclear extracts prepared from mammalian cell lines, as indicated in Figure 6. Specific strong bands showing the same migration as that of MCF-7 cells were observed in another ER-positive breast cancer cell line, T-47-D, and lung adenocarcinoma cell line, PC-9. On the other hand, no specific complex was formed in ER-positive breast cancer cell line ZR-75-1, ER-negative breast cancer cells BT-20 and MDA-MB-231, cervical cancer cell line HeLa, hepatoma cell line HepG2, renal carcinoma cell line Cos-7 and myeloma cell line U937 (Fig. 6).

To study the relationship between the cell type-specific formation of ERBF-1 complex and promoter B activity, we next analyzed the expression of total, A type and B type ERα mRNA in various cells. Total RNAs from cultured MCF-7, T-47-D, ZR-75-1, MDA-MB-231 and PC-9 cells were prepared and RT–PCR was carried out to detect each type as well as total ERα mRNA expression (Fig. 7). As shown, total ERα mRNA was expressed in MCF-7, T-47-D, ZR-75-1 and PC-9 cells, but not in MDA-MB-231 cells. As reported previously, MCF-7 and T-47-D cells showed expression of both type A and type B ER mRNA. Most notably, PC-9 cells expressed only B type mRNA, while ZR-75-1 cells showed only type A mRNA, indicating a strong correlation between ERBF-1 complex formation and promoter B activity.

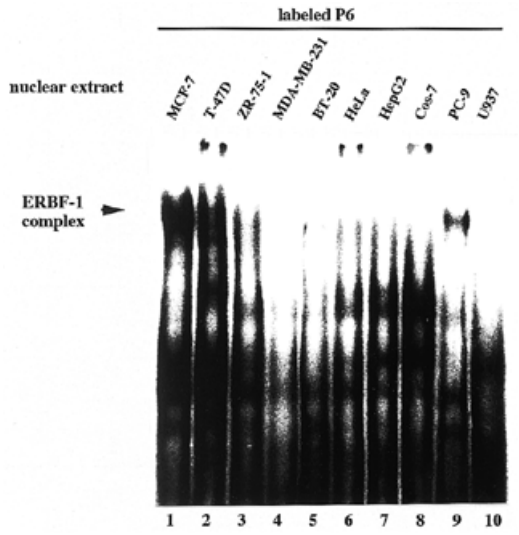

Figure 6. Cell type-specific expression of ERBF-1 binding factor. Annealed P6 oligonucleotides were radiolabeled by fill-in reaction using [α-^{32}P]dCTP. The probe was incubated with 7 µg of cell extract on ice for 30 min and electrophoresed in a 5% polyacrylamide gel. Lane 1, nuclear extracts of MCF-7 cell; lane 2, T-47-D; lane 3, ZR-75-1; lane 4, MDA-MB-231; lane 5, BT-20; lane 6, HeLa; lane 7, HepG2; lane 8, Cos-7; lane 9, PC-9; lane 10, U937.

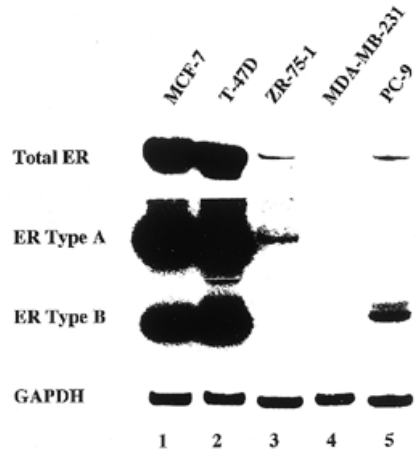

Figure 7. ERα mRNA expression in various cell lines detected by RT–PCR. Total RNAs were prepared from cultured cells: MCF-7, T-47D, ZR-75-1, MDA-MB-231 and PC-9. Semi-quantitative RT–PCRs were carried out to detect total, A type, and B type mRNAs of the ERα gene as described in Materials and Methods.

DISCUSSION

ERα is present in a variety of normal as well as cancer tissues at different levels of expression. Among these, ER-positive breast cancer is a representative of a high expression tissue of ERα, while the expression level in normal mammary gland is relatively low, when examined by immunohistochemical staining. Enhanced expression of ERα protein is one of the most important factors for the development, as well as the prognosis, of breast cancer. Previously, we have shown that this overexpression of ERα is mainly due to the enhanced expression of type B ER mRNA transcribed from the distal promoter B, while the type A promoter is dominant in normal mammary gland. Thus, unwrapping the mechanisms of preferential usage of promoter B of the ERα gene in breast cancer is a vital step in understanding breast carcinogenesis.

In this study we characterized the function of promoter B of the human ERα gene by transient transfection experiments in breast cancer cell line MCF-7 and identified a novel *cis*-element, ERBF-1, in the non-coding exon 1a of this gene. Deletion of this element resulted in almost complete loss of promoter activity (Fig. 2), while multiple or single copies transactivated a heterogeneous SV40 promoter (Fig. 5), indicating that this element can function as an enhancer in MCF-7 cells. EMSA showed a specific DNA–protein complex formed with the ERBF-1 element with nuclear extracts prepared from MCF-7, T-47-D and PC-9 cells, where endogenous expression of type B mRNA was observed, while another ER-positive breast cancer cell, ZR-75-1, where the type A promoter was selectively utilized, did not contain nuclear protein bound to this element. Considering these results together, ERBF-1 binding protein could be the determining factor for the expression of type B mRNA.

Mutational analysis of the ERBF-1 element revealed that the GGAAAG sequence is important for the binding of a specific nuclear protein. In this sequence, the underlined nucleotides AA were found to be most crucial for the binding of ERBF-1. It has been shown that oncogenic transcription factor Ets family proteins bind as a monomer to DNA sequences containing a purine-rich core GGAA/T (27,28). Recently, two members of this family, PEA3/E1AF and ESX, have been shown to be expressed in breast cancer cells (29–31). The former factor is expressed in various breast cancer cell lines, including MDA-MB-231 cells but not MCF-7 cells, while expression of the latter has been observed in ZR-75-1 cells. Since this cell type-specific expression pattern was different from that of ERBF-1 binding protein determined by EMSA in this study, neither of these factors can be a candidate for the ERBF-1 binding protein; the identification of the ERBF-1 binding protein will be required for understanding the switching mechanisms of alternative usage of ERα gene promoters in breast cancer.

In addition to the enhancer element ERBF-1, we found in exon 1a spanning from –1964 to –1904 located upstream of ERBF-1, another functionally important region which suppresses promoter activity in transient transfection analysis. Interestingly, this region contained a pyrimidine-rich sequence (–1946 to –1922) which is also well conserved among human, mouse and rat ERα gene promoters. Many *cis*-acting silencer elements containing this pyrimidine-rich sequence, represented by those of the c-*myc* gene immediately downstream of the transcription start sites on the non-coding strand, have been reported (32) and these sites are known to function as RNA polymerase II pausing regions (33). Since the type B promoter of the human ERα gene is silent, or weak at most, in the normal mammary gland, silencer elements may participate in regulation of the promoter. A further investigation will be needed to identify the silencer elements.

Moreover, several consensus binding sequences of known transcription factors, such as Sp-1, AP-1, NF-κB and the E-box, were found in the 5′ flanking region: some of them were examined by EMSA and their binding to the sequences was confirmed (data not shown). Further analysis of the mutual interaction of these factors with ERBF-1, as well as with a silencer element in exon 1a, will be required.

Interestingly, EMSA also revealed the presence of ERBF-1 binding protein in the PC-9 lung adenocarcinoma cell line, where type B mRNA expression was observed by RT–PCR analysis. Since the possible involvement of the estrogen receptor in the development of lung carcinoma has been postulated (34,35), it would be interesting to investigate the expression of ERα in not only adenocarcinoma cell lines but also lung cancer tissues.

In conclusion, we identified a novel *cis*-acting element, which interacts with a specific nuclear protein (named ERBF-1) found only in type B ER-positive cell lines. Functional analysis showed that the element was essential for basal level expression of type B mRNA and was capable of enhancing a heterogeneous SV40 promoter. These results indicate that ERBF-1 may be the determining factor for the tumor-specific induction of the B type promoter of the human ERα gene. Identification of ERBF-1 protein may yield a clue that would aid in the development of a new tool for diagnosis of early stage breast cancer along with chemopreventive agents to selectively suppress the B type promoter of the ERα gene.

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