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A Transcriptional Enhancer Required for the Differential Expression of the Human Estrogen Receptor in Breast Cancers

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Breast cancers lacking estrogen receptor (ER) expression have an adverse prognosis and fail to respond to endocrine therapy. We have identified a transcriptional enhancer in the human ER gene which is differentially active in ER-positive (ER^+) and ER-negative (ER^-) human breast cancer cell lines. Enhancer function was mapped to a 35-bp element located from −3778 to −3744 upstream of the major human ER mRNA start site, which we have termed ER-EH0 (for estrogen receptor enhancer). Gel retardation assays with ER^+ and ER^- cell lines identified multiple DNA-protein complexes which specifically form on this enhancer. One of these complexes could be supershifted by anti-Jun or anti-Fos antibodies, identifying it as an AP-1-containing complex. Methylation interference assays suggest binding of factors to both the AP-1 site and adjacent base pairs. Enhancer activity requires both the AP-1 site and these adjacent sequences. Mutations introduced into ER-EH0 and the recently described proximal promoter element ERF-1 in the context of the full-length promoter confirm ER-EH0 as the dominant *cis*-acting element involved in differential ER expression.

Estrogen receptor (ER) status is used clinically both as a prognostic factor and as a target in the therapy of breast cancer. Approximately half of all breast cancers express high levels of ER. The benefits of endocrine therapy are limited, for the most part, to patients with ER-positive (ER^+) tumors (10, 11, 15). We have been analyzing the ER gene to identify the regulatory elements which are involved in ER expression and to determine whether these elements are responsible for the difference in ER expression between ER^+ and ER-negative (ER^-) breast tumors.

ER expression has been shown to be regulated at the mRNA level both in human breast cancer cell lines and in tumors (2, 12, 24, 29). Southern blot analysis has revealed that the absence of ER expression in these cell lines or tumors was not due to deletions or other gross structural alterations in the ER gene (17, 20). Comparison of ER^+ and ER^- human breast cancer cells has shown evidence for regulation at the level of gene transcription in nuclear run-on assays (29). Some studies have shown that cells lacking ER mRNA have a higher capacity to methylate DNA and display extensive methylation of the CpG island in the 5′ promoter region of the ER gene (19, 23). In addition, treatment of ER^- cells with inhibitors of DNA methylation such as 5-azacytidine was shown to partially induce ER expression (5). However, it is unclear whether increased methylation of the ER gene is the consequence or the cause of decreased expression.

ER expression is regulated at two separate promoters (16). P1 is the major ER transcriptional start site (8). An upstream promoter, P0, was found on the basis of sequence homology with the major ER mRNA start site in the mouse (22). Transcription from this upstream promoter has been detected by some investigators in the human uterus (16) and normal breast tissue (7) by reverse transcription-PCR. Other investigators have found that the upstream promoter is utilized only in breast cancer cell lines that express high levels of ER and not in primary human mammary epithelial cells. In addition, the downstream promoter accounted for most of the ER transcription in ER^+ cells (28). Normal breast epithelial cells show a very low level of ER expression (28), thus raising the possibility that the very high level of ER expression in some tumors is secondary to the function of a *trans*-acting factor not active in normal breast epithelium.

Only one *trans*-acting factor, ER factor 1 (ERF-1), has been shown to play a possible role in the differential expression of ER (4). This factor binds to an element in the 5′-untranslated leader sequence of the ER gene. In contrast, our work has focused on the 4-kb region 5′ of the major ER mRNA start site. We analyzed this region to identify *cis*-acting elements important in transcriptional regulation. We found an enhancer located approximately 3,700 bp upstream of the major transcriptional start site that is critical for the high level of ER expression in ER^+ breast cancer cell lines. This enhancer, termed ER-EH0, consists of an AP-1 site, which binds c-Fos and c-Jun, and adjacent sequences which bind another, unknown factor(s). We show in the context of the full-length ER promoter that ER-EH0 is required for the differential ER expression found in ER^+ and ER^- tumor cell lines.

MATERIALS AND METHODS

Cell lines. The MCF-7, BT-20, MDA-MB-231, and HeLa cell lines were obtained from the American Type Culture Collection (Rockville, Md.). Cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (GIBCO-Bethesda Research Laboratories), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator.

Plasmid constructions. A genomic clone spanning the ER transcriptional start site to −750 upstream was kindly provided by P. Chambon. In addition, we have cloned and sequenced approximately 4 kb of the genomic sequence 5′ to the major ER start site from a human placenta genomic library obtained from Clontech. Chloramphenicol acetyltransferase (CAT) expression plasmids pBLCAT2 and pBLCAT3 (18) were used to test the activity of the upstream ER regulatory sequences. pBLCAT2 contains the minimal herpesvirus thymidine kinase (TK) promoter, which has low basal activity in the absence of additional positive regulatory elements, while pBLCAT3 contains neither a promoter nor an enhancer.

(i) Constructs in the enhancer region. In preliminary experiments, the genomic *Bam*HI fragment extending from −4100 to −2765 was found to possess enhancer activity when inserted into pBLCAT2. This fragment was selected for further analysis. Oligonucleotide primers were designed which would generate a series of overlapping deletions when the PCR was carried out with the cloned

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DNA as the template. The upstream primers were ER-4099 (5′-GGAAGCTT GGATCCTGAAGCAGTA), ER-3911 (5′-GGAAGCTTGCCTCTTGATTAG GTG), ER-3804 (5′-GGAAGCTTGAAGTTGAGAGGAACACCATG), and ER-2985 (5′-GGAAGCTTCAGTAACTGCATAGTGTCC). The downstream primers were ER-2765 (5′-GGCTCGAGGGATCCACCTCTTGA), ER-2983 (5′-GGCTCGAGCTGAGAGATTTATGAGTG), ER-3327 (5′-GGCTCGAGA TCTGCAATTGTTCACTA), ER-3705 (5′-GGCTCGAGGAATGTGTCTCCA GCC), ER-3784 (5′-GGCTCGAGCATGGTGTTCCTCTCAACTTCC), and ER-3899 (5′-GGCTCGAGCTAATCAAGAGGCAATTCC).

The upstream primers all contained a *Hin*dIII site. Downstream primers ER-2765, ER-2983, ER-3705, and ER-3899 contained an *Xho*I site. ER-3784 contained a *Bam*HI site. The PCR products were subcloned, as appropriate, into the *Hin*dIII-(*Xho*I-compatible) *Sal*I or *Hin*dIII-*Bam*HI sites of the pBLCAT2 vector.

(ii) Constructs containing the ER promoter. The upstream primers were ER-3911 (described above) and ER-335 (5′-GGCACGGGGCACATAAGGCA GCACATTAGAGAAAGCCGG). The downstream primers were ER-2765 (described above) and ER+212 (5′-GGCTCGAGGCAGACCGTGTCCCCGCAG G), which included an *Xho*I site. ER+212 corresponds to primer +210 used by deConinck et al. (4).

The construct designated ERFL was constructed as follows. First, the fragment containing the promoter was amplified by PCR with ER-335 and ER+212 as the primers. The PCR product was digested with *Bam*HI and *Xho*I and subcloned into the appropriate sites of the pBLCAT3 vector to yield the construct ERP. Second, the most upstream fragment was amplified with ER-3911 and ER-2765 as the primers, and the product was digested with *Hin*dIII and *Bam*HI and inserted into the appropriate sites of ERP to yield the plasmid pTZQ10ERP. Finally, the middle fragment, −2969 to −290, was excised from the original genomic subclone by using *Bam*HI and inserted into pTZQ10ERP to yield ERFL.

Mutations in both the AP-1 and ERF-1 sites were made by using the Promega Altered Sites II in vitro mutagenesis system. Mutations in the ERF-1 site were based on construct ER3500-230p1d1, described by deConinck et al. (4). The mutation primers were ERF-1 distal site, 5′-GCAGACCGTGTCCCCGaAttcC AGAAGGCTCAGAAACC, and ERF-1 proximal site, 5′-AGAGCACAGCCC GAttTTAaAGGCGACGCAGCGC, where the small letters refer to the nucleotides which were changed. One additional nucleotide in the proximal site and two additional nucleotides in the distal site were mutated in our constructs to generate the restriction sites used to analyze the constructs. Gel shifts assays confirmed that these mutant primers could not compete with the wild-type primers for DNA binding (data not shown) and were therefore equivalent to the previous mutations (4). Primer EHmAP (see Fig. 2) was used to mutate the AP-1 site.

Transfection. Plasmid DNAs were prepared by alkaline lysis and doubly purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (25). All transfections were done by the calcium phosphate precipitation method (1). A 10-μg sample of cloned plasmid DNA was used in each transfection. A 2-μg sample of a cytomegalovirus-driven β-galactosidase (β-gal) expression vector was cotransfected for use as an internal control. The cells were grown in 100-mm-diameter dishes and were 30% confluent for transfection. Transfected cells were collected with a cell scraper and assayed for CAT and β-gal expression 72 h after transfection. Procedures for the CAT and β-gal assays have been previously described (1). CAT activity was quantified by PhosphorImager (Molecular Dynamics) analysis and corrected for transfection efficiency as determined by the β-gal assay.

Gel retardation assay. To prepare nuclear extracts, cultured MCF-7 and MDA-MB-231 cells were collected with a scraper, washed with phosphate-buffered saline, and swelled at 4° in hypotonic buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.9], 15 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol [DTT]). The swollen cells were homogenized, and the nuclei were pelleted and resuspended in low-salt buffer (20 mM HEPES [pH 7.9], 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) at 4°C. Nuclear proteins were extracted by dropwise addition of high-salt buffer (same as low-salt buffer, except that the KCl concentration was 1.2 M). The nuclei were then removed by centrifugation. The nuclear extract was dialyzed against 20 mM HEPES (pH 7.9)–20% glycerol–100 mM KCl–0.2 mM EDTA–0.2 mM PMSF–0.5 mM DTT at 4°C, and the precipitated protein was removed by centrifugation. Lysates of Sf9 insect cells infected with wild-type or recombinant baculovirus expressing c-*fos* or c-*jun* were generated as previously described (13).

The [^{32}P]dCTP (3,000 Ci/mmol)-labeled probes (100,000 cpm; 10^8 cpm/μg) were incubated with 10 μg of nuclear extract (or insect cell lysate) and 0.5 μg of poly(dI-dC) in 1× binding buffer (10 mM Tris · HCl [pH 7.5], 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl) in a final volume of 10 μl. The reaction mixture was incubated for 20 min at room temperature, and the DNA-protein complex was separated from the unbound probe on a 4% polyacrylamide gel in 1× TBE (45 mM Tris-borate [pH 8.2], 1 mM EDTA). For supershift experiments, polyclonal antibodies against c-Fos and c-Jun (Upstate Biotechnology Incorporated, Lake Placid, N.Y.) were added at a 1:2 dilution.

Methylation interference. The double-stranded DNA probe was labeled at one end with [α-^{32}P]dCTP and [α-^{32}P]dGTP. The labeled probe (1×10^6 to 2×10^6 cpm) was methylated with dimethyl sulfate. Gel shift assays were then carried out as described above, with MCF-7 nuclear extracts incubated with the methylated probe. The DNA-protein complexes were separated on a 4% gel and purified with a Mermaid Kit (Bio 101). The DNA was cleaved with piperidine at 95°C for 30 min. These fragments were then electrophoresed on a 6% polyacrylamide sequencing gel and autoradiographed (1).

RESULTS

Identification of a transcriptional enhancer element in the ER gene. MCF-7, an ER$^+$ breast cancer cell line expressing high levels of ER mRNA, and MDA-MB-231, an ER$^-$ breast cancer cell line which does not express detectable ER mRNA as determined by Northern blot analysis, were chosen initially for these experiments. To study the factors which regulate ER expression, we cloned and sequenced approximately 4 kb of the human genomic sequence upstream of the major ER transcriptional start site, defined as the P1 cap site by deConinck et al. (Fig. 1A) (4). Fragments from −4100 to −2765, −2765 to −291, and −291 to +120 were cloned in front of a minimal TK promoter in the pBLCAT2 vector (18) and transfected into MCF-7 and MDA-MB-231 cells. Strong CAT activity was detected only in construct E0, containing the sequence from −4100 to −2765, when transfected into MCF-7 cells (Fig. 1B). To identify the *cis*-acting elements responsible for this activity, synthetic oligonucleotides were used to PCR amplify portions of this region. These were inserted into pBLCAT2 to generate a series of overlapping deletion constructs (Fig. 1A). The plasmids were then transfected into both MCF-7 and MDA-MB-231 cells, and CAT activity was determined as shown in Fig. 1B. Fragment E3, spanning −4100 to −3705, produced a 37-fold increase in CAT activity in MCF-7 cells compared to the control pBLCAT2 vector alone. When E3 was divided approximately in half, downstream fragment E5, spanning −3911 to −3705, was active. Activation also occurred when this fragment was inserted in the opposite orientation relative to the CAT gene (E5R), suggesting that this region contained an enhancer element. When E5 was further divided, downstream 100-bp fragment E7, spanning −3804 to −3705, yielded full activity while upstream 100-bp fragment E6 possessed minimal activity. Significantly, all of the fragments containing enhancer activity were markedly more active in ER$^+$ MCF-7 cells than in ER$^-$ MDA-MB-231 cells. Interestingly, longer fragments E1 and E2, extending from −4100 to −2983 and −3327, respectively, had significantly lower activity than either E3 or the entire piece extending from −4100 to −2765. This suggests that the region between −3705 and −3327 contains a negative regulatory element. The reduction in activity seen when E1 was compared to E0 suggested that there might be an additional positive element located between −2985 and −2765. However, no independent enhancer activity was detected when this fragment was inserted into pBLCAT2 to yield E8 (Fig. 1B). In addition, another fragment extending from −2765 further upstream to −3327 was also negative (data not shown).

Gel retardation analysis of the enhancer fragment. Once we had identified 100-bp fragment E7 as a strong-enhancer-containing element involved in the differential regulation of ER expression, DNA binding assays were used to define the *trans*-acting factors which bind to this element and to identify bases critical for binding in this region. The nucleotide sequence of E7 is shown in Fig. 2A. Inspection of the sequence revealed the presence of a potential AP-1 site between −3773 and −3766. Accordingly, double-stranded oligonucleotides corresponding to smaller parts of this region were synthesized and cloned into pBLCAT2 (Fig. 2A). A 35-bp fragment, EH0, which contained the putative AP-1 site was found to display 80% of the activity of E7 in MCF-7 cells, as described in detail below. EH0 was labeled with ^{32}P and used as a probe in gel shift assays with nuclear extracts from MCF-7 and MDA-MB-231 cells. Three

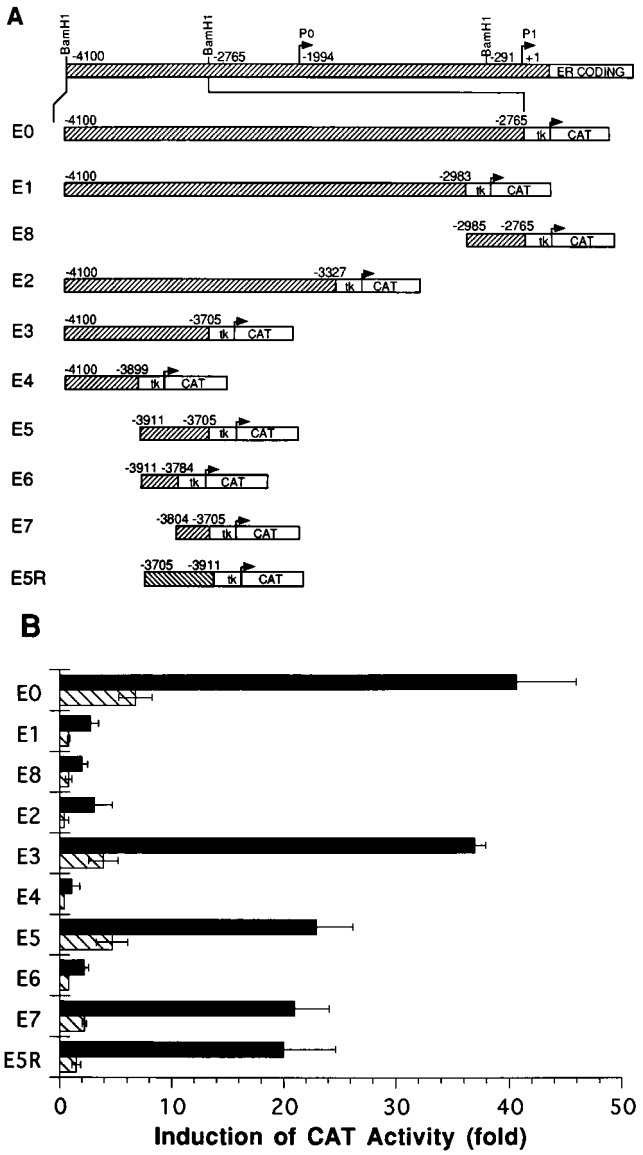

FIG. 1. Identification of *cis*-acting elements in the ER gene. (A) Diagrams of ER promoter constructs cloned in CAT expression vector pBLCAT2. All of the plasmids contained a CAT reporter gene driven by a TK promoter. Hatched bars represent the DNA sequence derived from the ER gene. All of the numbers above the bars correspond to distances from the major transcription start site, +1. (B) CAT activities from putative ER enhancer constructs. Plasmids containing various fragments of the ER gene were transfected into MCF-7 (ER^+, solid bars) or MDA-MB-231 (ER^-, hatched bars) breast cancer cells. CAT activity was quantitated by PhosphorImager analysis. The data are fold inductions over that obtained with the pBLCAT2 vector alone, which was normalized to 1. All data were corrected for transfection efficiency. The results shown are averages of 4 to 10 transfection experiments for each plasmid. The error bars show standard deviations.

specific bands, designated B1, B2, and B3, were repeatedly observed (Fig. 2B). Competition assays were then carried out to identify possible transcription factor binding sites within this region. The formation of all three complexes was specifically competed with a 50-fold molar excess of unlabeled EH0 (Fig. 2B). EH1, containing the AP-1 site and the immediate flanking region from −3784 to −3760, competed away the B3 complex efficiently, suggesting that the B3 complex might be composed of AP-1 binding factors. EH1 only partially competed B1 and B2. EH2 (−3804 to −3785) showed no competition. Meanwhile, EH3 (−3759 to −3730) slightly competed away B1, and EH4 (−3764 to −3736) specifically competed away the B2 band. Together, the data indicate that the region downstream of the putative AP-1 sequence is important for B1 and B2 complex formation. While all three complexes were detected with both nuclear extracts, there was a very clear difference between the two cells lines in the relative intensity of the complexes, as shown in Fig. 2B.

c-Fos and c-Jun are involved in the AP-1 binding complex. Many transcription factors are known to bind to an AP-1 site, including c-Fos and c-Jun (3). When a gel shift experiment with EH0 and purified, baculovirus-expressed Fos and Jun proteins was performed, a single specific band was present which had a mobility similar to that of B3 (Fig. 2C, third lane from the left). In a supershift experiment, polyclonal antiserum to c-Jun completely shifted this band, while polyclonal antiserum to c-Fos partially shifted the band (data not shown). This was reasonable, since the protein complexes at an AP-1 site are known to involve homodimers of Jun or heterodimers of Jun and Fos but not homodimers of Fos. These experiments showed that ER-EH0 was capable of binding Fos and Jun. To determine whether the complexes formed with the nuclear extracts in Fig. 2B were due to binding of these proteins, a similar supershift experiment was performed. Nuclear extract from MCF-7 cells was incubated with antiserum to either c-Fos or c-Jun, and labeled EH0 was added. As shown in Fig. 3, both antisera produced shifted bands, indicating the presence of Fos- and Jun-containing complexes (SF and SJ). Other AP-1 binding factors may also bind EH0, since only a portion of the B3 complex was supershifted by anti-Fos or anti-Jun antibodies.

Mutations alter transcription factor binding. To further characterize the sequences required for complex formation, oligonucleotides EHmF and EHmAP (Fig. 2A) were used to introduce mutations into the enhancer region. In EHmF, the AP-1 site was intact but the entire flanking region was replaced with an unrelated sequence. When EHmF was used as the probe in gel shift assays (Fig. 2C), B3 was the predominant complex seen. On the other hand, when EHmAP, containing a double point mutation in the AP-1 site, was used as the probe (Fig. 2C), no B3 complex was formed while B2 was unchanged. When baculovirus-produced c-Fos and c-Jun were tested in place of the nuclear extracts, the mAP mutation, but not the mF mutation, eliminated the formation of the B3 complex. These findings confirm that the B3 complex results from AP-1 binding and that the B2 complex is formed by factors binding to the 3′-flanking region of the AP-1 site.

Methylation interference defines critical contacts in both the AP-1 and flanking sequences. A methylation interference assay was performed on the gel shift bands from Fig. 2 to further define the specific residues within the enhancer which are necessary for formation of the DNA-protein complexes. The positions of eight critical G residues are indicated by the arrows in Fig. 4. The B3 complex involved five G residues on the sense strand, three of which were in the AP-1 site (Fig. 4A), and two G residues on the antisense strand, both within the AP-1 site (Fig. 4B), whose methylation interfered with binding. On the other hand, the B1 and B2 bands involved two important G residues on the sense strand (Fig. 4A) and the B2 band involved one important G residue on the antisense strand (Fig. 4B) whose methylation interfered with binding. These three residues were all located outside of the AP-1 site. There were no G residues within the AP-1 site whose methylation interfered with binding in the B1 and B2 complexes. These data support a model in which B3 is composed of factors which bind

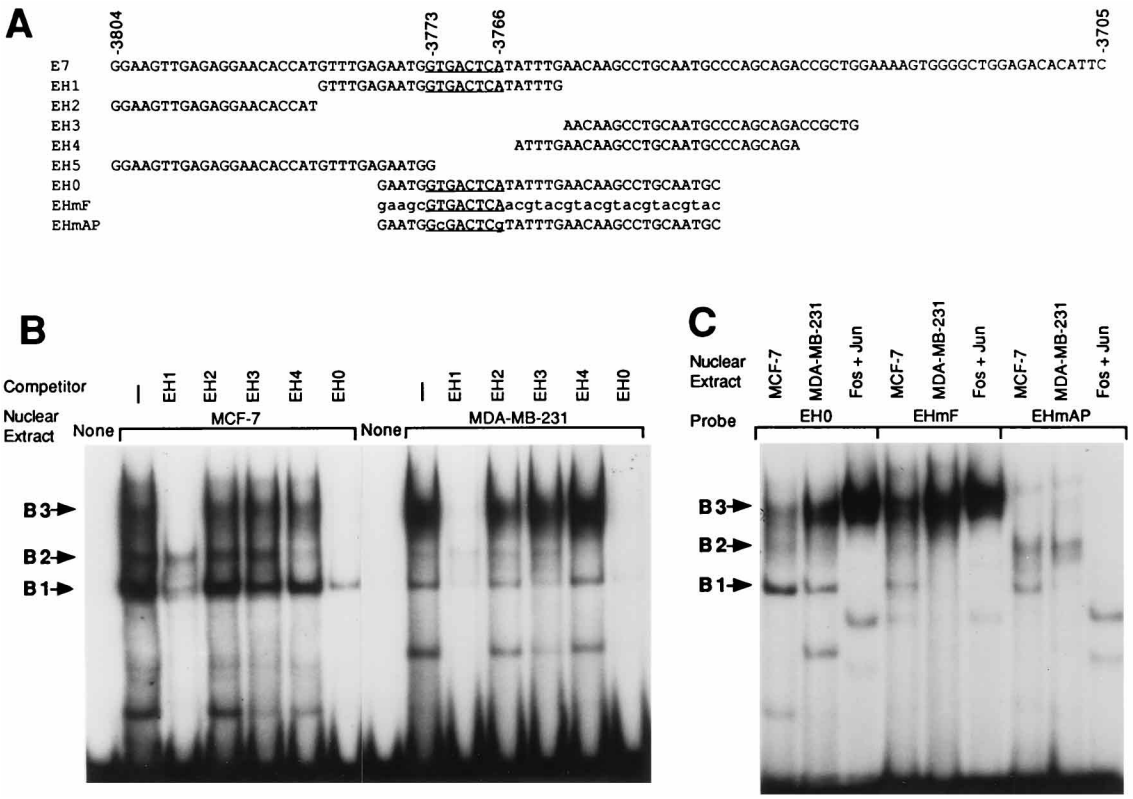

FIG. 2. Multiple protein-DNA complexes form on ER-EH0. (A) Sequences of oligonucleotides from −3804 to −3705 used in gel shift assays. The AP-1 site is underlined, and the mutations are in lowercase letters. (B) Gel shift assay with radiolabeled EH0 as the probe. The probe was incubated with MCF-7 and MBA-MB-231 nuclear extracts as indicated. Three specific complexes were formed, B1, B2, and B3. Fiftyfold molar excesses of unlabeled oligonucleotides from sequences of the ER gene, as shown in panel A, were used as competitors. (C) Gel shift assays with labeled wild-type EH0 or mutant EHmF or EHmAP as the probe. MCF-7 and MDA-MB-231 nuclear extracts and purified, baculovirus-expressed c-Fos and c-Jun were tested.

primarily to the AP-1 portion of the enhancer and, in addition, to the 3′-flanking region. B1 and B2 are composed of factors which bind primarily to the flanking sequence and do not appear to contact the AP-1 site.

Functional assays confirm that ER-EH0 is active. The oligonucleotides used in the gel shift assays were cloned into pBLCAT2, and the recombinant plasmids were tested for CAT activity in both MCF-7 and MDA-MB-231 cells. As shown in Fig. 5, 35-bp fragment EH0, which contains the AP-1 site, displayed 80% of the activity of E7. We therefore defined this fragment as the core enhancer sequence, which we termed ER-EH0 (for estrogen receptor enhancer). The CAT assays confirmed that both the AP-1 site and the flanking regions are essential for full activity of the enhancer in MCF-7 cells. Removal of the AP-1 site (EH4 and EH5) or mutation of the AP-1 sequence (EHmAP) significantly reduced but did not completely eliminate enhancer activity. Likewise, mutation of the flanking sequence (EHmF) reduced the activity of this enhancer.

ER-EH0 is critical for differential ER expression. ER-EH0 was identified by using constructs containing the TK minimal promoter. We next wanted to examine the function of the enhancer in the context of the full-length ER promoter. The construct ERFL contains the entire ER promoter sequence (−3911 to +212), including ER-EH0, inserted into CAT expression vector pBLCAT3 (Fig. 6A, ERFL). This construct was transfected into MCF-7 cells, into MDA-MB-231 cells, and into two additional cell lines. BT-20 is a breast cancer cell line which expresses ER mRNA, but to a lesser extent than

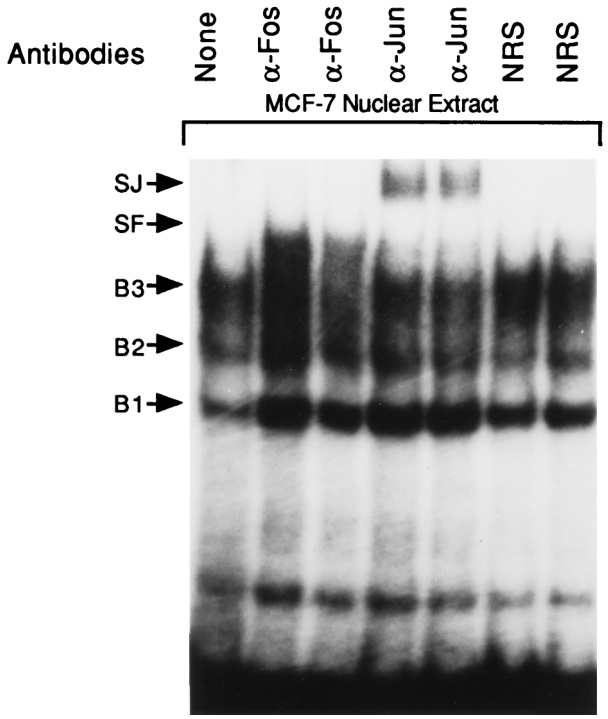

FIG. 3. Fos and Jun bind the ER enhancer. Radiolabeled EH0 was incubated with MCF-7 nuclear extract in the absence or presence of polyclonal anti-c-Fos or anti-c-Jun antibodies. Normal rabbit serum (NRS) was used as a control.

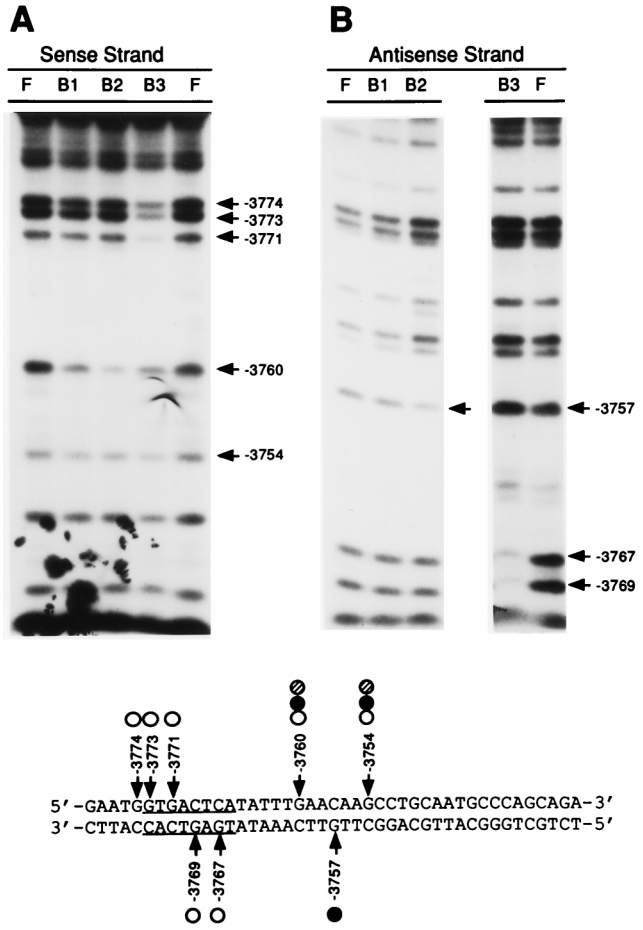

FIG. 4. Identification of critical residues for ER enhancer complex formation. For methylation interference, each strand of the 43-bp probe (−3778 to −3736) was labeled at one end. (A) Sense strand. (B) Antisense strand. The probes were then methylated with dimethyl sulfate and incubated with MCF-7 nuclear extracts. The bound (B) probe and free (F) probe were separated on a 4% nondenaturing polyacrylamide gel. DNA was then cleaved with piperidine, and fragments were analyzed on an 8% sequencing gel. The positions of the G residues at which methylation interfered with binding are indicated by arrows. Hatched circles represent Gs involved in B1 complexes. Solid circles represent Gs involved in B2 complexes, and open circles represent Gs involved in B3 complexes.

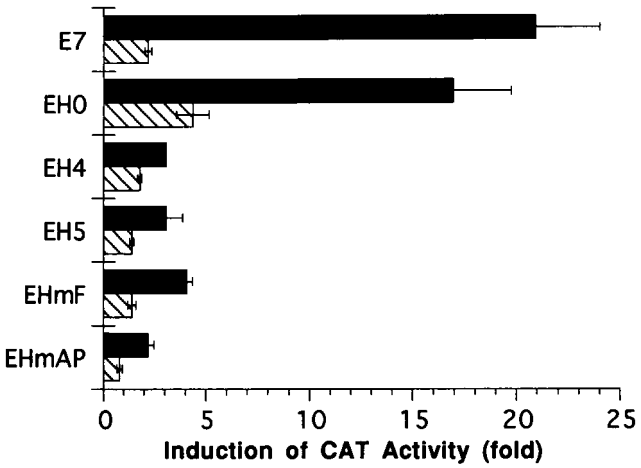

FIG. 5. Definition of a minimal ER enhancer. Double-stranded oligonucleotides spanning different parts of the region between −3804 and −3705 were cloned into the pBLCAT2 vector (Fig. 2A). Plasmids were transfected into MCF-7 (ER^+) or MDA-MB-231 (ER^-) breast cancer cells. CAT activity was quantitated by PhosphorImager analysis. The data presented are fold inductions over that obtained with the pBLCAT2 vector alone, which was normalized to 1. All data were corrected for transfection efficiency. The results shown are averages of 4 to 10 transfection experiments for each plasmid.

does MCF-7 (28). HeLa is an ER^- cell line derived from a cervical carcinoma. ERFL was active in both MCF-7 and BT-20 cells. In contrast, the full-length ER promoter was not active in MDA-MB-231 or HeLa cells (Fig. 6B, ERFL). There was a 15-fold difference in activity between MCF-7 and MDA-MB-231 cells (Fig. 6B, ERFL).

When the ERFL construct contained the mutant AP-1 site (EH-mAP) described in Fig. 2A, there was a dramatic reduction in CAT activity of approximately 60% in both ER^+ cell lines MCF-7 and BT-20 (Fig. 6B, ERFLmAP). However, there was essentially no change in activity in ER^- cell lines MDA-MB-231 and HeLa (Fig. 6B, ERFLmAP). Thus, the difference in fold induction between MCF-7 and MDA-MB-231 cells was decreased from 15-fold to 5.8-fold by introduction of the AP-1 site mutation.

It has recently been proposed that the differential expression of ER in ER^+ versus ER^- cells is due to a *trans*-acting factor termed ERF-1, which binds to an element in the 5′-untranslated region of the ER gene (4). To determine the relative contributions of the ER-EH0 enhancer element and the ERF-1 binding element to the differential expression of ER, additional mutant constructs were tested. ERFLmERF contained a mutant, nonfunctional ERF-1 binding element. ERFLmAPmERF contained both the mutant ERF-1 and mutant AP-1 sites. The activity of these constructs was compared to that of the ERFL and ERFLmAP constructs described above. As shown in Fig. 6B, there was little difference in activity between the mutant ERFLmERF and wild-type ERFL constructs in any of the cell lines. Furthermore, when the activity was reduced in the ER^+ cells by mutating the AP-1 site (ERFLmAP), minimal additional reduction was caused by addition of the ERF-1 mutation (ERFLmAPmERF). Therefore, in the context of the full-length ER promoter, it appears that the upstream ER-EH0 enhancer is the primary *cis*-acting element involved in differential expression of ER.

DISCUSSION

ER expression status in breast cancer strongly correlates with disease prognosis and with the response to endocrine therapy. This differential expression of ER is regulated at the transcriptional level (2, 12, 24, 29). Our analysis of the ER gene reveals a transcriptional enhancer element between −3778 and −3744 upstream of the major transcription start site. We have named this enhancer ER-EH0. This element appears to play a major role in the induction of expression from the ER promoter. It significantly enhances the activity of either the heterologeous herpesvirus TK promoter or, more importantly, the natural, full-length ER promoter only in ER^+ breast cancer cells. ER-EH0 consists of a core AP-1 site and a 3′-flanking region. Mutations in either of these sites severely reduce the activity of the enhancer.

The enhancer element also appears to be directly involved in the differential expression between ER^+ and ER^- cells. For example, when 100-bp fragment E7 was placed upstream of the TK promoter, it yielded a 21-fold increase in activity relative to the TK promoter alone in MCF-7 cells but only a 3-fold increase in MDA-MB-231 cells (Fig. 5). More importantly, in the

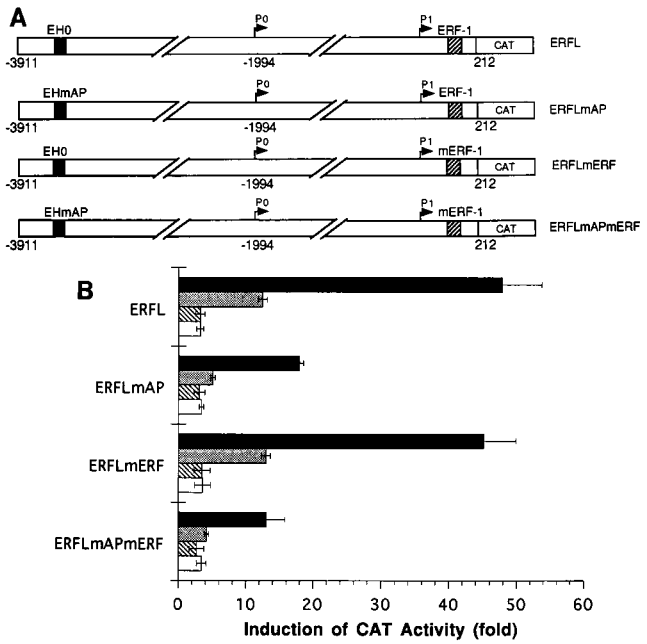

FIG. 6. ER-EH0 is functional in the context of the full-length ER promoter. (A) ER promoter constructs cloned in CAT expression vector pBLCAT3. Open boxes represent the DNA sequence derived from the ER gene. Solid boxes indicate the enhancer EH0 or the mutant EHmAP. Hatched boxes indicate ERF-1 or mutant mERF-1. All numbers below the boxes are distances from the major transcription start site, +1 (P1). The upstream promoter, P0, is also indicated. (B) CAT activities from ER promoter full-length constructs. Plasmids were transfected into MCF-7 (solid), BT-20 (shaded), MDA-MB-231 (hatched), or HeLa (open) cancer cells. CAT activity was quantitated by PhosphorImager analysis. The data presented are fold inductions over that obtained with the pBLCAT3 vector alone, which was normalized to 1. All data were corrected for transfection efficiency. The results shown are averages of 4 to 10 transfection experiments for each plasmid. The error bars show standard deviations.

context of the normal, full-length ER promoter, there was a 15-fold difference in the level of induction in MCF-7 cells compared with MDA-MB-231 cells (Fig. 6B). Mutation of the AP-1 site caused significant impairment of induction in ER^+ cells but only a minimal change in ER^- cells (Fig. 6B).

With regard to differential expression, this work also compared the relative contributions of ER-EH0 and the previously described downstream ERF-1 binding element. The results obtained with the mutant constructs in Fig. 6 suggest that the ER-EH0 element has a dominant effect on expression relative to the ERF-1 binding element in the context of the full-length ER promoter. Mutation of ER-EH0 reduced activity 60% in ER^+ cells but had little effect in ER^- cells, while mutation of the ERF-1 binding element had only a minimal effect in any of the cell lines. It is important to note that although the ERF-1 binding element was shown to be involved in differential ER expression (4), the promoter sequences used in those experiments did not include the upstream element ER-EH0.

ER-EH0 apparently consists of at least two sites. The core of ER-EH0 is a consensus AP-1 site which is essential, since mutations within this sequence significantly reduce enhancer activity (Fig. 5 and 6B). The AP-1 site bound the transcription factors c-Fos and c-Jun in gel shift experiments with either purified protein or nuclear extract (Fig. 2C), and antibodies against these proteins produced supershifted complexes (Fig. 3). The binding of these factors to the AP-1 site appears to be responsible for the formation of the complex designated B3. The sequence immediately adjacent to the AP-1 site is also necessary for full activity of the enhancer. Mutating the flanking region while leaving the AP-1 site intact results in significant impairment of enhancer activity (Fig. 5). Gel shift analysis indicates that at least one other factor is involved in binding at the 3′-flanking sequence. The B1 and B2 complexes did not form in the presence of baculovirus-produced c-Fos and c-Jun (Fig. 2C and 3). Furthermore, mutating the flanking region reduced B1 and eliminated B2 but had no effect on B3; conversely, mutating the AP-1 sequence had no effect on B2 and reduced, but did not eliminate, B1 (Fig. 2C). Finally, the methylation interference assays (Fig. 4) indicate that three Gs in the 3′-flanking region, but none of the Gs within the AP-1 sequence, are involved in the B1 and B2 complexes.

Net transcriptional activity at the ER locus is likely to result from the influence of both positive and negative factors. While this study focused primarily on the positive enhancer element, the data highlight one region of the promoter, at −3705 to −3327 (Fig. 1), which may have negative regulatory properties. This element reduces the effect of the enhancer to fourfold in MCF-7 cells and eliminates any enhancement in MDA-MB-231 cells (compare E1 and E2 to E3 in Fig. 1B). However, this negative effect may normally be suppressed since the presence of an additional sequence downstream revives the enhancement (compare E0 to E1).

Recently, an alternative pathway of ER action has been reported in which the receptor is able to stimulate transcription from a transfected promoter that contains an AP-1 site, rather than from an estrogen response element (6, 21, 26). This may occur through a protein-protein interaction with Jun or Fos which is independent of the ER DNA-binding domain (27) or through an interaction with a common coactivator (9, 14). In this context, our discovery of an active AP-1 complex in the ER enhancer suggests that the ER protein plays a role in regulating its own transcription by interacting with AP-1 pathways.

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