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Transcriptional Regulation of Estrogen Receptor in Breast Carcinomas

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An important transcriptional regulatory element of the estrogen receptor (ER) gene that binds a protein expressed in ER-positive breast carcinomas has been identified. Using a transient expression assay, we identified a 75-bp region of the 5′ untranslated leader of the ER gene which augments expression from the ER promoter. This region contains two binding sites for a protein, estrogen receptor factor 1 (ERF-1), which is expressed in ER-positive breast carcinomas. A concatenated ERF-1 binding site probe identified a 30,000-Da protein. Low-level ERF-1 expression was detected in normal human mammary epithelial cells. Abundant ERF-1 expression was also found in endometrial carcinoma cell lines that express the ER-positive phenotype. These results indicate that ERF-1 expression represents a common mechanism of ER regulation in hormonally responsive carcinomas.

The expression of estrogen receptor (ER) is intimately associated with the biology of breast carcinomas. Breast carcinomas occurring in postmenopausal women are often ER positive (8), and many of these tumors express significantly more receptor than normal mammary epithelium does (17). ER-negative breast carcinomas are more likely to occur in young women, and these tumors carry a worse prognosis than carcinomas which express ER (3, 12). Several studies have focused on the function of ER in an attempt to explain the association between ER expression and tumor biology. Mutations in the ER gene of some breast carcinomas that render these altered ER proteins incapable of binding estrogen response elements (19, 21) and able to inhibit wild-type ER function (5, 9) have been described. Other studies, however, have found ER mutations which result in a constitutively active receptor which has also been postulated as important to the development of hormone-independent growth (2, 14). If ER function influences the oncogenic process, it is difficult to conceptualize within a single model of oncogenesis the occurrence of mutations which inhibit ER function and mutations which result in constitutive activity. An alternative hypothesis is that mechanisms regulating transcription of the ER gene influence the phenotype of breast carcinomas; within this model, ER-negative carcinomas which do not transcribe the ER gene define a subset of tumors with a more aggressive phenotype. This theory is supported by recent studies which have identified breast carcinoma cell lines that fail to transcribe an apparently normal ER gene (24). It is therefore possible that defining molecular mechanisms controlling transcription of the ER gene may provide new insight into the biology of breast carcinomas.

Transcription of ER occurs from two separate promoters, P0 and P1 (10), although no functional mapping has been previously published. P1 is the major ER transcriptional start site (7). The P1 cap site is predominantly utilized in human mammary epithelial cells (HMEC) and is the major start site in ER-positive human breast carcinomas (23). Multiple cap sites have been identified for the P0 promoter. Studies of the murine ER gene identified 10 cap sites spanning approximately 60 bases (25), and a start site at −1994 (from the P1 cap site) which would agree closely with the major murine P0 cap site was identified in human cells (16). There is possibly another ER transcriptional start site farther upstream at −3090 (16). Transcription from the P0 promoter is characteristic of human endometrial tissue and can account for 12 to 33% of ER transcription in breast carcinoma cells (23). We have now performed a functional analysis of the ER promoter and have examined DNA-binding proteins which interact with critical transcriptional control regions.

MATERIALS AND METHODS

Cell lines. HMEC were obtained from reduction mammoplasties and maintained in DFCI-1 growth medium as described previously (1). The ECC-1 ER-positive human endometrial carcinoma cell line was obtained from P. G. Satyaswaroop, Hershey, Pa. All other cell lines were obtained from American Type Culture Collection, Rockville, Md. Cells were maintained in minimal essential medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine sera (HyClone, Logan, Utah), 25 mM 4-(2-hydroxyethyl) piperazineethanesulfonic acid (HEPES), 26 mM sodium bicarbonate, 5,000 U of penicillin G (Gibco BRL) per ml, 5,000 μg of streptomycin (Gibco BRL) per ml, and 6 ng of bovine insulin (Sigma Chemical Company, St. Louis, Mo.) per ml. Cells were incubated at 37°C in 5% CO_2.

Plasmid construction and transfection. The region of DNA in the 5′ end of the ER gene was isolated from a human genomic lambda library (Stratagene, La Jolla, Calif.). The 5′ end was sequenced, and a set of primers was constructed, all of which contained a *Bgl*II site for use as upstream primers. All 3′ oligonucleotides contained a *Hin*dIII site. These oligonucleotide primers were used in PCR with cloned DNA as the template. Primers used to generate 5′ deletion constructs were ER724 (5′-TACAGATCTG TGGTCCAACA TAAACACA), ER586 (5′-TGCAGATCTT CCTATATGTA TACCC), ER464 (5′-CATTAGA TCT GCCCTATCTC GGTTACAGTG T), ER375 (5′-GGGGAGATCT AA CAGAAAGA GAGACAA), ER289 (5′-CCCTAGATCT GTCTTTCGCG TT TAT), ER122 (5′-GGGAGATCTG CCTGGAGTGA TGTTTAAG), ER40 (5′-TATGAGATCT GGAGACCAGT ACTTAAAG), and ER0 (5′-CCCAGATC TG GCGGAGGGCG TTCG). Primers used to generate specific 3′ ends were +230 (5′-CATAAGCTTG GTCCGTGGCC GCGGGCAGGG T), +210 (5′-C GGGAAGCTT GCAGACCGTG TCCCCGCAGG), +135 (5′-GCCCAAGC TT AGAGGCGACG CAGCGCA), and +0 (5′-CGCCAAGCTT CCTGGGCT CC CGGGCCTC).

PCR products were then subcloned into the *Bgl*II-*Hin*dIII sites of the luciferase reporter plasmid pGL2-Basic (Promega, Madison, Wis.). To construct the plasmids with 3,500 bp upstream of P1, the 5′ region was first subcloned into pBluescript from the genomic lambda clone as a *Sal*I-*Not*I fragment, using the *Sal*I site in lambda DASH and the *Not*I site in the first exon of ER. The ER724-xLUC constructs were each digested with *Xho*I-*Nde*I, removing the 5′ region of the ER gene. This region was then replaced with the larger 5′ flanking region as an *Xho*I-*Nde*I fragment from the ER gene cloned in pBluescript. In this construct, the *Xho*I site 6 bp upstream of the *Sal*I site in pBluescript was used and

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was inserted into the *Xho*I site 4 bp upstream of the *Bgl*II site in pGL2-Basic. Each of these plasmids then contained 3,500 bp upstream of P1.

Plasmid DNA was prepared by alkaline lysis and purified by using polyethylene glycol as described previously (18). All cells were transfected by the calcium phosphate precipitation procedure (6). In these experiments, 30 μg of cloned plasmid DNA was used in transfections of 100-mm-diameter plates with cells at 50 to 60% confluence. In each transfection, 2 μg of a Rous sarcoma virus-driven chloramphenicol acetyltransferase (CAT) expression vector was cotransfected, and cells were assayed for luciferase and CAT expression 48 h after transfection. Values presented are luciferase units as measured on a luminometer corrected for transfection efficiency, as determined by CAT assay. Similar results were obtained when 15 μg of plasmid DNA was used in transfections.

Gel retardation assay. Cells were collected by dislodging with a policeman or trypsinization. Cell pellets were washed with 1× phosphate-buffered saline and then resuspended at 10^8 cells per ml in microextraction buffer (450 mM NaCl, 20 mM HEPES [pH 7.7], 25% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, proteinase inhibitors). Cells were sonicated, and cellular debris was pelleted at 14,000 rpm in a microcentrifuge. Protein concentration of the supernatant was determined by using a Bio-Rad (Hercules, Calif.) protein assay and ran in the range of 5 to 15 mg/ml. Extracts were stored at −80°C until use.

Ten micrograms of whole-cell extract was incubated in 1× binding buffer (40 mM KCl, 20 mM HEPES [pH 7.7], 1 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM dithiothreitol)–4% Ficoll–40 μg of poly(dI-dC) per ml–0.1 ng of radiolabelled probe in a volume of 25 μl at room temperature for 45 min. Reaction mixtures were then loaded on a 4% acrylamide gel in 0.25× Tris-borate-EDTA and electrophoresed at 270 V at 4°C. Gels were dried and exposed to X-ray film.

Double-stranded DNA oligonucleotides were used for gel shift competition and were prepared by separately synthesizing each strand of the oligonucleotide, using a Gene Assembler Special (Pharmacia LKB, Alameda, Calif.). Oligonucleotides were mixed in equal molar ratio in 0.3 M sodium acetate, boiled for 10 min, and cooled slowly to room temperature. Double-stranded oligonucleotide DNA was then ethanol precipitated and resuspended in water at a concentration of 0.2 mg/ml. These double-stranded oligonucleotides were then used in gel shift competitions at 500 M excess.

Southwestern (DNA-protein) blotting. Approximately 70 μg of total cell protein was electrophoresed in a sodium dodecyl sulfate–12% polyacrylamide gel, transferred to nitrocellulose, and renatured by using guanidine hydrochloride as described previously (18). Probes were labelled with [α-^{32}P]dCTP, using a random primer kit (Boehringer Mannheim, Indianapolis, Ind.).

RESULTS

Functional analysis of the ER promoter. To elucidate the mechanisms which regulate transcription of the ER gene, we have performed a functional analysis of the ER promoter. A human genomic lambda library was screened with a probe from the 5′ flanking region of the ER gene. A genomic clone which contained 3,500 bp upstream of the P1 cap site for ER, the entire first coding exon, and approximately 10 kbp of the first intron was obtained. This 5′ flanking region encompasses 500 bp upstream of the farthest ER cap site identified. Regions of this clone were then subcloned into the luciferase reporter vector pGL2-Basic. Figure 1 shows a diagram of the promoter constructs. The major ER mRNA beginning at P1 contains a 230-base untranslated 5′ leader sequence (7). In the first set of constructs, all inserts contained 210 bp of the untranslated leader, and a nested set of 5′ deletions was generated from 3,500 bp down to the P1 cap site at +1 (Fig. 1A). In a second set of promoter constructs, the 5′ end remained at 3,500 bp, and 3′ deletions were constructed, beginning at +230 and progressively deleting portions of the leader by sequentially bringing the luciferase gene closer to the P1 cap site (Fig. 1B).

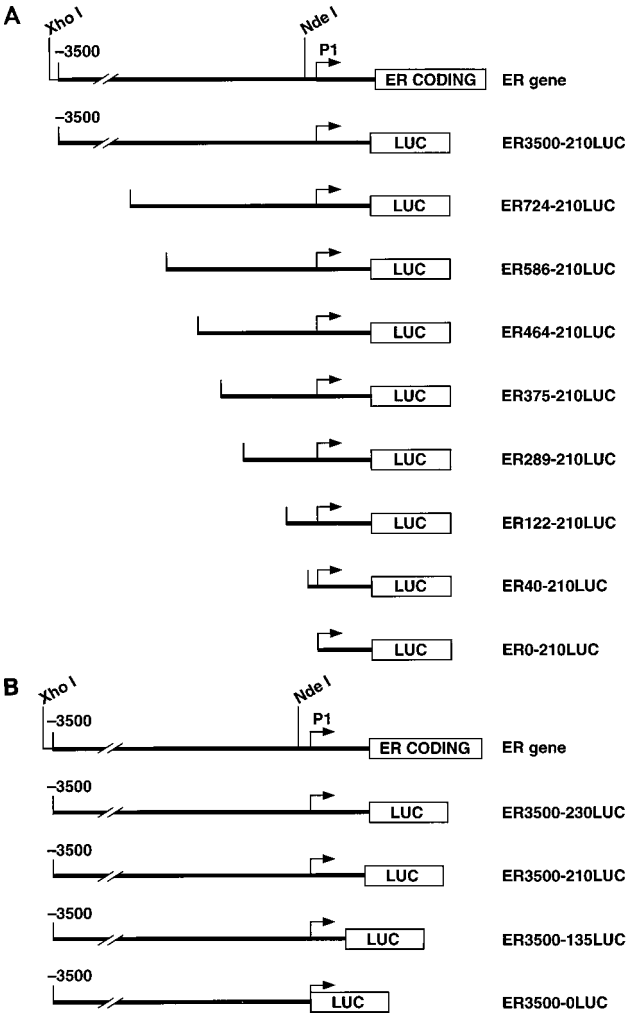

FIG. 1. Diagrams of ER promoter constructs cloned in luciferase expression vector pGL2-Basic. Dark line, DNA sequence derived from the ER gene; arrow, location of the P1 transcriptional start site. All numbers correspond to distance from the P1 cap site. Locations of the *Nde*I site at −45 and *Xho*I site (in the vector) are shown. The ER coding region is indicated in a box. The luciferase-coding region is designated by a box labelled LUC. In plasmid names, the first number corresponds to DNA length upstream of P1 and the second number indicates DNA length downstream of P1. (A) 5′ deletion constructs. All constructs contain 210 bp of the 230-bp 5′ untranslated leader. (B) 3′ deletion constructs. All constructs contain 3,500 bp upstream of P1 and variable portions of the untranslated leader.

These constructs were tested for luciferase expression upon transfection into two breast carcinoma cell lines (Fig. 2). T47D is an ER-positive breast carcinoma line in which approximately 90% of the ER mRNA begins at the P1 cap site (23). The ER-negative carcinoma line MDA-MB-231 was also used since we have previously shown this cell line to lack transcription of the ER gene (24). Luciferase activities in these two cell lines were strikingly different (Fig. 2). In ER-positive T47D cells, the full-length construct gave excellent expression. Progressive deletion of the 5′ end of the gene failed to significantly alter expression, although there was a reproducible decline of expression upon deletion of the last 40 bp of the 5′ flanking region, which contains a TATA element at +25 (Fig. 2A). The pattern of expression in ER-negative MDA-MB-231 cells was qualitatively and quantitatively different. The full-length construct expressed poorly and gave values an order of magnitude less than in T47D cells. This level of expression was only three times the expression from the negative control vector pGL2-Basic, which does not contain a promoter. Progressive deletion of the upstream sequences improved expression, and no significant decline occurred when the TATA element upstream of the P1 cap site was deleted. Results from the 3′ deletion constructs are shown in Fig. 2B. Deletions from the 3′ end of the leader identified a sequence between +210 and +135 which augmented expression of the ER promoter in ER-positive T47D cells. Analysis of these constructs in ER-negative MDA-MB-231 cells was also performed, and the effect of this region appeared to be cell line specific. We therefore searched

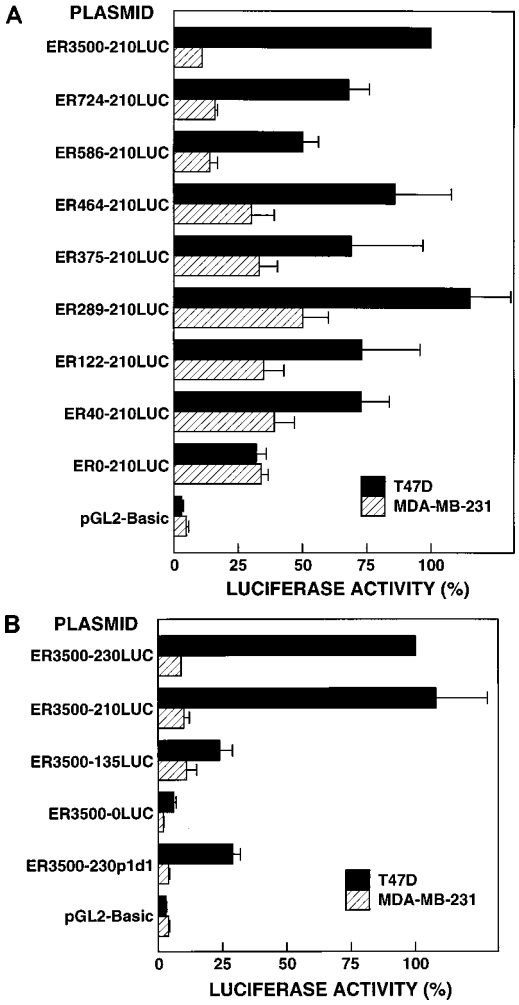

FIG. 2. Luciferase activity from ER promoter constructs. Plasmid DNA was transfected into T47D (ER-positive) or MDA-MB-231 (ER-negative) breast carcinoma cells and assayed for luciferase activity. Data presented are corrected for transfection efficiency. Data were normalized to the activity obtained from the largest plasmid in T47D cells, which was considered 100%. Standard errors are shown by error bars. (A) Representative results of expression from 5′ deletion constructs. Values shown are averages of four separate transfection experiments. (B) Representative results of expression from 3′ deletion constructs. Values shown are averages of eight separate transfections. Data for ER3500-230p1d1 are averages of two separate transfections performed in triplicate and are representative of other transfections performed.

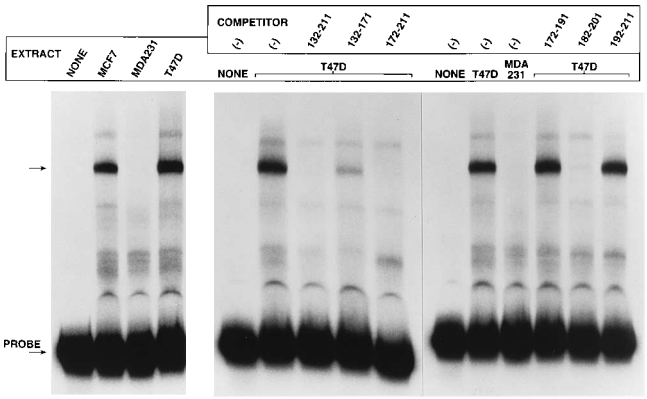

FIG. 3. Gel shift assay using the 80-bp probe. The gel shift assay was performed with whole-cell extracts from cells as shown. The probe is a radiolabelled 80-bp DNA fragment from ER promoter sequences from +132 to +211. Gel shift competition involved addition of a 500-fold molar excess of unlabelled competitor from sequences of the ER gene as shown. In (−) lanes, no competitor was added.

for binding proteins that interact with this region of the ER gene.

Identification of ERF-1 by gel shift. An 80-bp probe was prepared from sequences from +132 to +211 of the ER promoter. This probe was used in gel shift assays with extracts prepared from the ER-positive breast carcinoma cell lines MCF7 and T47D and the ER-negative cell line MDA-MB-231. A prominent shift band was found only in extracts from the ER-positive cell lines (Fig. 3). This complex is referred to as estrogen receptor factor 1 (ERF-1). Gel shift competitions were used to identify two ERF-1 binding sites within this 80-bp region. An unlabelled competitor prepared from the entire 80-bp probe efficiently competes for binding (Fig. 3). The region from +132 to +171 partially competes, and the region from +172 to +211 competes efficiently. Within the region from +172 to +211, the sequences from +182 to +201 compete efficiently for binding (Fig. 3). Sequences from neither +172 to +191 nor +192 to +211 demonstrate any competition. These results indicate that a DNA-binding protein, ERF-1, is expressed in ER-positive breast carcinoma cells and interacts with a region of the leader with transcriptional activity. ERF-1 binds to two sites in this region, a distal (high-affinity) site and a proximal (low-affinity) site.

ERF-1 binding sites. The distal binding site was mapped precisely, using gel shift competition with oligonucleotides containing mutations within the sequences from +182 to +201. These results are shown in Fig. 4A. This region contains the sequence CCCTGCGGGG, which is an imperfect palindrome. The wild-type sequence of this distal site competes efficiently. Mutations d1 and d3 disrupt the imperfect palindrome and destroy the ability of the oligonucleotide to compete. Mutations d2 and d4 do not alter the 10-bp imperfect palindrome and retain the ability to compete for binding, although d4 is slightly less efficient than the wild-type distal site. Mutation d5 changes the T at +192 to a C and converts the sequence to a perfect palindrome but partially weakens the ability to efficiently compete. The sequence between +132 and +171, which demonstrated weak competition (Fig. 3), was found to contain a second ERF-1 site located at +130 to +149 (Fig. 4B). Within this region, a related sequence can be found, and homologous mutations as used for the distal site have identical consequences for binding. Interestingly, insertion of a G between +140 and +141 creates a site identical to the distal site. This mutation improves the ability of the weak proximal site to compete.

Mutation of ERF-1 sites affects expression. Because the mutation p1 destroys binding to the proximal site and d1 destroys binding to the distal site, these two mutations were built into the expression vector ER3500-230LUC, and the resulting construct was called ER3500-230p1d1. This new vector is identical to ER3500-230LUC except for the two mutations within the proximal and distal ERF-1 binding sites. Expression from this construct is shown in Fig. 2B. Mutation of these ERF-1 sites has an effect on expression similar to that of deletion of the region from +135 to +210. These results strongly suggest that ERF-1 is a transcription factor which is expressed in ER-positive breast carcinomas and which functions by binding to two sites in the untranslated leader of the ER gene.

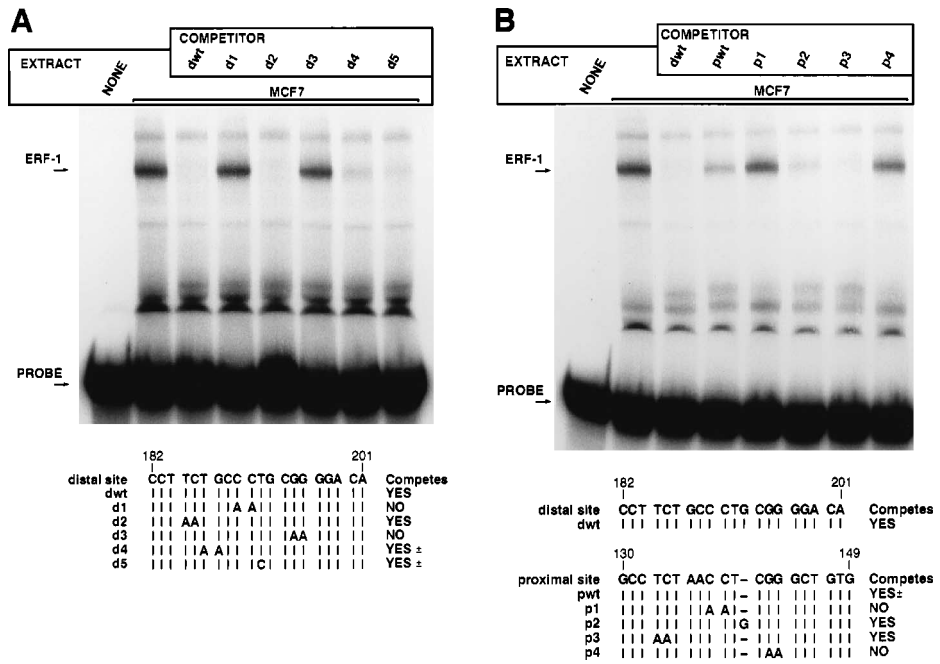

FIG. 4. Mapping of ERF-1 binding sites. Gel shift competitions were performed with whole-cell extracts from cells as shown. The probe used in both panels is a 72-bp DNA fragment from ER promoter sequences from +130 to +201. (A) Competitions using mutant distal binding sites. The sequence of the wild-type distal site (dwt) and position of the mutated sequence are shown for each mutant sequence d1 to d5. (B) Proximal binding site defined by using proximal mutants p1 to p4. Below the gel are shown sequences of wild-type distal site (dwt) and proximal site (pwt) sequences aligned to highlight homology. Sequences of the wild-type proximal site sequence and the four proximal site mutants p1 to p4 are shown.

Identification of ERF-1 by Southwestern blotting. A complex formed in a gel shift assay often contains a number of proteins. To define the specific ERF-1-binding protein, protein blots of crude cell extracts were prepared from MCF7 and MDA-MB-231 cells. A blot was reacted with a radiolabelled DNA probe from sequences from +100 to +230 of the ER leader. This probe identified a 35-kDa protein present in both MCF7 and MDA-MB-231 cells and a second protein of approximately 30 kDa only found in MCF7 cells (Fig. 5). An identical blot was reacted with a probe prepared from a concatenated high-affinity distal ERF-1 binding site. This probe identified only the p30 protein found in MCF7 cells. These results indicate that the specific ERF-1-binding protein is a 30-kDa protein expressed in MCF7 but not MDA-MB-231 cells. Alternatively, the protein may be expressed in MDA-MB-231 cells but is modified in such a way as to prevent DNA binding.

ERF-1 expression in breast and endometrial carcinomas. To determine which cell lines express ERF-1, a panel of human cell lines was analyzed for ERF-1 expression in the gel shift assay. These results are shown in Fig. 6. Abundant ERF-1 expression was found in all ER-positive breast carcinoma cell lines tested, MCF7, T47D, and BT20. Low levels of ERF-1 were detected in normal HMEC. The ERF-1 complex from HMEC demonstrated a binding sequence specificity identical to that of the complex in MCF7 and T47D cells (data not shown). HBL-100 is an ER-negative breast carcinoma line which appears to express low amounts of ERF-1 comparable to those expressed by HMEC. The lack of expression of ER in HBL-100 cells could be due to any one of a number of reasons, e.g., methylation of DNA, deletion of the ER gene, and rapid degradation of mRNA.

Expression of ERF-1 was also examined in other cell lines, including a panel of human endometrial carcinoma lines. The RL95-2 cell line is an endometrial adenocarcinoma line which is reportedly ER positive (22). This cell line makes abundant ERF-1 protein, as seen in Fig. 6B. Examination of ER expression in this stock of RL95-2 cells has failed to demonstrate ER expression (data not shown); however, a late passage of this cell line has been reported to have lost ER expression (20). Therefore, RL95-2 was derived from an ER-positive carcinoma, and loss of ER expression as the cells are maintained in culture is likely the result of DNA methylation. ECC-1 is another endometrial cell line which is ER positive, and we have confirmed expression of ER mRNA (data not shown). The ECC-1 cell line also expresses abundant ERF-1 (Fig. 6B). ERF-1 was not readily detected in HEC 1B or HEC 1A, which are both ER-negative human endometrial carcinoma cell lines (11, 13). These results suggest that abundant expression of ERF-1 represents a common mechanism for ER regulation in hormonally responsive carcinomas.

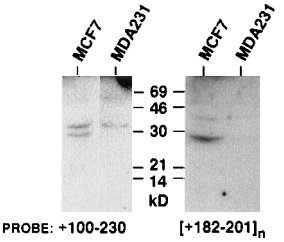

FIG. 5. Southwestern blot of protein extracts. Cell extracts from MCF7 or MDA-MB-231 cells were used on polyacrylamide gels as shown. The protein blot on the left was probed with a DNA fragment from the +100 to +230 region of the ER promoter. An identical protein blot (right) was probed with a concatenated distal ERF-1 binding site from sequences from +182 to +201 of the ER promoter.

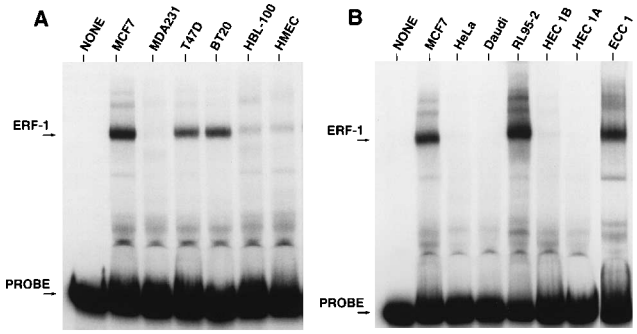

FIG. 6. ERF-1 expression determined by a gel shift assay using whole-cell extracts prepared from cell lines shown and reaction with a 72-bp probe encompassing proximal and distal ERF-1 sites. (A) Human breast carcinoma lines (ER positive: MCF7, T47D, and BT20; ER negative: MDA-MB-231, and HBL-100). (B) Other human cell lines (HeLa, cervical carcinoma cells; Daudi, lymphocytes; RL95-2, HEC 1B, HEC 1A, and ECC-1, human endometrial carcinoma cell lines) (see text for details).

DISCUSSION

Although several mechanisms are involved in controlling expression of ER in breast carcinomas, transcriptional regulation is clearly responsible for the ER-negative phenotype in some cell lines (15, 24). These data demonstrate that an important transcriptional regulatory element of the ER gene is found within the 5′ untranslated leader. This region contains two binding sites for a DNA-binding protein, ERF-1, which is abundantly expressed in ER-positive breast and endometrial carcinomas. These data indicate a role for ERF-1 in the transcriptional regulation of ER. There are likely other *cis*-acting control elements involved in the regulation of ER transcription. For example, the transcriptional mapping data also implicate positive and negative regulatory elements upstream of the P1 transcriptional start site, and there may be other regions that have not been examined in this study. However, the identification of ERF-1 offers a molecular mechanism that accounts for differences in ER expression found in breast carcinomas.

The complexity of ER transcriptional regulation is suggested by the biology of ER expression. ER is overexpressed in many ER-positive breast carcinomas that often make over 100 fmol/mg of cytosol protein, compared with 4 fmol/mg of cytosol protein in normal mammary cells (17). These differences in ER expression are reflective of mRNA levels, and there are striking differences in the levels of ER mRNA detected in different breast carcinoma lines (23). It is certainly possible that ERF-1 is responsible for the ER overexpression identified in many ER-positive carcinomas. However, more detailed experiments need to be performed to address this possibility directly.

Comparing the sequence of the ERF-1 binding site with those of known transcription factor sites fails to identify a previously identified factor (4). There can be a high degree of degeneracy in binding site sequences, but none of the known factors with GC-rich sites has a molecular mass of 30 kDa. Since ERF-1 expression is limited to ER-positive cell lines, one might wonder whether ER is part of the ERF-1 complex. Several facts make this unlikely. First, the ERF-1 site bears no resemblance to the estrogen response element. Second, BT20 cells express a truncated ER with a molecular mass of 43 kDa (2) but generate an ERF-1 complex which comigrates with complexes from other ER-positive cell lines. Third, the Southwestern blot in Fig. 4 identifies a protein of 30 kDa which is clearly distinct from the 65-kDa ER protein. Finally, attempts to supershift the ERF-1 complex with an antibody against ER have not been successful (data not shown).

The identification of ERF-1 offers new insight into our understanding of the relationship between ER expression and the biology of breast and endometrial carcinomas. ER expression defines a subset of breast cancer patients who, in general, have a better prognosis than do patients with ER-negative tumors. Because ER is a transcription factor, it has been suggested that the phenotype displayed by ER-positive breast carcinomas is due to the repertoire of genes whose expression is regulated through estrogen response elements. Alternatively, ER expression might be a marker for the degree of differentiation of a tumor, and ERF-1 might be involved in the regulation of a number of cellular genes, including the ER gene, which are critical to the differentiated phenotype. The identification of ERF-1 has immediate clinical relevance. For example, tumors that lack ERF-1 expression might define a subset of cancer patients with a prognosis different from that of patients with ER-negative tumors in which loss of expression is due to mutations within the ER gene. If ERF-1 expression is a more reliable marker of a clinically relevant phenotype, this would indicate that some ERF-1-responsive gene, other than the ER gene, is critical to the phenotype of ER-positive carcinomas. Understanding the control of ERF-1 may also provide new therapeutic approaches to the treatment of aggressive ER-negative tumors.

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