Identification of a New Subclass of Alu DNA Repeats Which Can Function as Estrogen Receptor-dependent Transcriptional Enhancers*

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We have utilized a genetic selection system in yeast to identify novel estrogen-responsive genes within the human genome and to define the sequences in the BRCA-1 gene responsible for its estrogen responsiveness. This approach led to the identification of a new subclass within the Alu family of DNA repeats which have diverged from known Alu sequences and have acquired the ability to function as estrogen receptor-dependent enhancers. Importantly, these new elements confer receptor-dependent estrogen responsiveness to a heterologous promoter when assayed in mammalian cells. This transcriptional activity can be attenuated by the addition of either of three different classes of estrogen receptor antagonists, indicating that these elements function as classical estrogen receptor-dependent enhancers. Furthermore, this enhancer activity is restricted to a specific subset of DNA repeats because consensus Alu elements of four major subfamilies do not respond to the estrogen receptor. Previously, most Alu sequences have been considered to be functionally inert. However, this work provides strong evidence that a significant subset can confer estrogen responsiveness upon a promoter within which they are located. Clearly, Alu sequences must now be considered as important contributors to the regulation of gene transcription in estrogen receptor-containing cells.

The steroid hormone estrogen is a key intracellular modulator of the processes involved in establishment and maintenance of female reproductive function (1). In addition, its actions play an important role in maintaining female cardiovascular tone and regulating bone cell differentiation (2). In pathological states, the mitogenic activity of estrogen facilitates progression of breast cancers (3) and is implicated in the abnormalities of uterine function observed in endometriosis and possibly uterine fibroids (4). These actions of estrogen all appear to be mediated through specific high affinity receptors located within target cell nuclei (1). Molecular cloning has revealed that the estrogen receptor (ER)† is a member of a superfamily of receptors which mediate the nuclear actions of the sex steroids, retinoic acid, vitamin D₃, and thyroid hormones (5). In the absence of hormone, ER resides in a latent form in target cell nuclei associated within a large macromolecular complex comprising heat shock protein 90 (hsp90), hsp70, p59, and other proteins (6). Upon ligand binding, the receptor undergoes a dramatic conformational change (7, 8), initiating a cascade of events leading ultimately to the association of an ER dimer with specific estrogen response elements (EREs) within the regulatory regions of target genes (9). The mechanism by which the bound receptor modulates gene transcription is unknown.

Because of its diverse biological functions and the implied complexity of its targets, there has been a keen interest in defining the genes which are regulated by estrogen. To date, a relatively small number of genes have been identified in humans which are modulated directly by the estrogen receptor. These include the genes for PS-2, the progesterone receptor, oxytocin, c-fos, and α₂-globulin (10). However, it was the dissection of the Xenopus laevis vitellogenin A2 promoter which led to the definition of a consensus ERE, the palindromic sequence GGTCACgTGACC, which confers estrogen responsiveness to a heterologous promoter (11). To date, only a single estrogen-responsive gene within human genomic DNA has been shown to contain this consensus sequence (12). In fact, recent studies have indicated that sequences distinct from the vitellogenin ERE, which function only in specific cell and promoter contexts, may be more important in facilitating ER function (13, 14). Given the complexity of the biological events that occur in response to estrogen, it is likely that a large number of genes which are directly regulated by this hormone remain to be identified. As a consequence of the need to define the targets of ER action in mammalian cells, we have developed a genetic screen in Saccharomyces cerevisiae to identify novel estrogen-responsive genes within human genomic DNA.

MATERIALS AND METHODS

Biochemicals—DNA restriction and modification enzymes were obtained from Boehringer Mannheim, New England Biolabs, or Stratagene. Polymerase chain reaction reagents were obtained from Perkin-Elmer Cetus or Promega Corp. 17β-Estradiol was purchased from Sigma. The estrogen receptor antagonist ICI182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). Keoxifen was obtained from Eli Lilly Inc. Oligonucleotides were synthesized by the Duke University DNA Synthesis Core Facility. Screening for Estrogen Receptor-dependent Enhancers—Two libraries were created by inserting Sau3A-digested genomic DNA fragments, isolated from either MCF-7 cells or the P1141 clone containing approx.

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† The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; bp, base pair(s); PS, primate-specific; AS, anthropoid-specific; CS, catterhine-specific; HS, human-specific.
imimately 50 kilobases of the 5’ end of the BRCA-1 locus (15), into the BamHI site of pBM2389. This low copy (ARS/CEN4) parent vector contains the TRP1 and AmpRK markers allowing selection in yeast or bacteria. In addition, it contains an enhancerless GAL1 promoter fused to the HIS3 coding sequence. Insertion of a functionally active enhancer into this vector and its subsequent transformation into yeast permits histidine prototrophy in a mutant his3 background. The libraries generated were transformed into the yeast strain YPH500 (MATa ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200 leu2Δ1) (16) containing pRS-415-hER, a low copy plasmid expressing the wild-type human estrogen receptor under control of a copper-inducible promoter (CUP1) (17). Transformants were plated on minimal media containing 100 mM 17β-
estradiol, uracil, and 1 mM aminotriazole, a competitive inhibitor of the HIS3 gene product, to select against sequences which resulted in a weak activation of the HIS3 gene fusion. Colonies which grew under these conditions were picked from these primary plates and replicated onto secondary plates under three different conditions: 1) −histidine, +5 mM aminotriazole; 2) −histidine, +100 mM 17β-estradiol, +5 mM aminotriazole; 3) −histidine, +5 mM aminotriazole. Colonies which grew under conditions 1 and 2, but not 3, were considered to contain estrogen-dependent sequences and were selected for further analysis. For comparative purposes, a strain containing a plasmid in which the X. laevis vitellogenin A2 ERE was ligated into the BamHI site of pBM2389 was also used (13).

Cell Culture and Co-transfection Assays—HepG2 cells were cultured in 24-well gelatin-coated plates overnight in modified Eagle’s medium (Life Technologies, Inc.) plus 10% fetal calf serum (Life Technologies, Inc.). Before transfection, the medium was changed to phenol red free media plus 10% charcoal/dextran-treated serum (Hyclone Inc.). DNA was introduced into the cells using LipofectAMINE reagent (Life Technologies, Inc.) as described by the manufacturer. Briefly, each transfection was performed in triplicate using 3 μg of total DNA. Each transfection contained 1000 ng of pRST7-hER (13, 18), 1500 ng of luciferase reporter, and 500 ng of pCMV-β-Gal to normalize for transfection efficiency (19). Transfection of this DNA into HepG2 cells with LipofectAMINE was allowed to proceed for 3 h, then cells were washed, fresh medium was added, and the cells were cultured for 16 h. At this time, a 24-h incubation with hormones and antimetabolites was initiated. Subsequently, the cells were lysed and assayed for luciferase and β-galactosidase. Luciferase activity was normalized for transfection efficiency by concomitant assay of β-galactosidase activity. Relative response is calculated by dividing normalized response at 100 mM 17β-
estradiol with that measured in the absence of hormone (basal activity). RESULTS AND DISCUSSION

Previously our laboratory has shown that ER can function as a hormone-dependent transcription factor in yeast and used this observation to develop a functional screen based on nutritional prototrophy to identify novel estrogen response elements from a library of random oligonucleotides (13). The fact that these elements identified in yeast function analogously in mammalian cells indicated that this approach could be used to identify fragments from human genomic DNA which permitted ER action. To accomplish this, we constructed a library of genomic fragments from Sau3A-digested MCF-7 cell genomic DNA. Size-fractionated DNA was cloned into the promoter of an enhancerless GAL1-HIS3 fusion contained within the pBM2389 vector (Fig. 1) (13, 20). A library of over 150,000 recombinants with an average insert length of 300 bp was obtained. Following amplification in Escherichia coli, the plasmid library was transformed into a yeast strain, YPH500, containing the plasmid pRS415-hER expressing the wild-type human ER under control of the copper-inducible CUP1 promoter. Transformed yeast were then plated on minimal media plates containing 17β-estradiol and uracil, but lacking histidine. In addition, we included aminotriazole, a competitive inhibitor of the HIS3 gene product, to enhance the stringency of the screen and eliminate background growth due to leakiness of the GAL1 promoter. Using this approach, we identified greater than 500 colonies which grew in the absence of added histidine and contained putative enhancers within the cloned DNA. Because it was likely that this primary screen would identify ER-independent enhancers, those which operate in response to endogenous yeast transcription factors, we performed a secondary screen to determine estrogen dependence of the selected clones. For this purpose, the colonies obtained from the primary screen were assayed for their ability to grow on minimal media plates containing either no histidine, histidine alone, or histidine + 17β-estradiol. A vector containing the consensus vitellogenin A2 ERE within pBM2389 was used as a positive control for this assay (indicated by squares in Fig. 1). The results of one of these secondary screens are shown in Fig. 1. Those clones which were determined to be estrogen-responsive are indicated (circles in Fig. 1). We identified 16 clones which contained sequences from MCF-7 cell genomic DNA which permitted activation of transcription by 17β-estradiol-activated ER.

Before proceeding with a detailed analysis of these estrogen-dependent clones, we wanted to determine whether or not they would function as estrogen-responsive enhancers in mammalian cells. To this end, plasmids were isolated from yeast, and their inserts were cloned directly into the BamHI site of pBL-TK-Luc which contains the ERE-negative thymidine kinase promoter fused to the firefly luciferase gene (18). The ER-dependent enhancer activity within each plasmid was then assayed in HepG2 cells following co-transfection of an ER expression vector (pRST7ER) and subsequent analysis of luciferase activity following administration of 17β-estradiol. As a positive control, we assayed the ER-dependent enhancer activity of a single copy of the consensus vitellogenin ERE inserted into the identical site within pBL-TK-Luc (TK-ERE-Luc). The results of this analysis, shown in Fig. 2A, indicate that TK-ERE-Luc exhibited a significant basal transcriptional activity which was induced 3-fold in the presence of added 17β-estradiol. Although greater inductions are obtained using multimerized copies of this ERE (data not shown and Ref. 13), we felt that only a comparison of the activity of a single-copy ERE to novel enhancers was relevant in this instance. This analysis revealed
that most of the sequences identified in yeast permitted significant ER-dependent enhancer activity when compared to TK-ERE-Luc. Notably, estrogen-responsive fragment-2 (ERF-2), ERF-3, ERF-9, ERF-10, ERF-15, ERF-16, and ERF-17 all had induction values greater than the TK-ERE-Luc control (Fig. 2A). Interestingly, several clones which function in yeast do not appear to function in HepG2 cells. It is possible that these may represent estrogen-responsive sequences which operate only in cooperation with additional yeast factors or that their activity is not manifest in the cell and promoter background used for these initial studies. For the purposes of this study, however, we limited our analyses to those clones which functioned as ER-dependent enhancers in HepG2 cells.

In order to demonstrate that the estrogen-induced enhancer activity of the isolated genomic clones represented a classical ER-mediated signal transduction pathway. More importantly, however, these results validate the general approach used to detect novel estrogen-responsive enhancers.

Given that ERF-3 was potentially derived from a novel estrogen-responsive gene, it was sequenced to determine if it contained any similarities to known EREs (Fig. 2D). We were surprised to find that most of the sequence (178 bp out of 253 bp total) corresponded to the Alu family of repetitive DNA (represented in bold letters). The remaining 75 bp do not appear to be related to any known Alu sequence. We are unsure whether or not these sequences are contiguous within the intact genome; however, it is possible that they are and represent a genetically altered Alu.
 genomic DNA in the construction of our library, it is suspect that the divergence from Alu consensus occurs close to a Sau3A restriction site. Nevertheless, within the sequence which was homologous to Alu DNA, we identified an imperfect ERE (5'-GGTCAanTGGTC-3') which diverged from the vitellogenin A2 ERE by 2 base pairs in the right arm of the palindrome (underlined in Fig. 2D) and a half-site (5'-TGACC-3') located 9 bp downstream of the imperfect ERE. Additionally, two half-sites separated by 22 bp were found in the non-Alu sequence (underlined in Fig. 2D). Because both the Alu-related and non-related sequences within ERF-3 contained potential EREs, we cloned them separately into pB-L-TK-Luc. In this way we were able to determine that each in fact functioned independently as ER-dependent enhancers (Fig. 2D). These data suggest that the imperfect ERE within the Alu sequence (ERF-3a) functions as a genuine estrogen-inducible enhancer. The activity of the composite clone (ERF-3) seems to represent synergism between the two independent estrogen-responsive sequences and may be functioning in a manner similar to the vitellogenin B2 ERE where two imperfect palindromes cooperate to yield a functionally important element (21). Unlike our case, however, the individual B2-derived palindromes do not demonstrate independent activity. The surprising finding that an independent functional ERE was located wholly within a highly repetitive Alu DNA sequence defines a role for this repetitive DNA here-tofore unrecognized.

It is unlikely that all Alu repeats function as ER-dependent enhancers due to their abundance. We were interested therefore in determining what specifically within the ERF-3-derived Alu was responsible for ER responsiveness. Fortuitously, we got the answer when two independent projects converged. We have determined that the newly identified BRCA-1 (breast cancer susceptibility gene) gene is positively regulated by 17β-estradiol in a series of cultured breast cancer cells.2 Because of the potential link between estrogens and breast cancer, we wished to determine the molecular basis of the observed 17β-estradiol-induced up-regulation. Specifically, we were interested in mapping the sequences within the BRCA-1 locus responsible for this activity. To this end we constructed a library in the pBM2389 vector of DNA fragments derived from a P1 clone (p1141) which contained a large portion of the 5' end of the BRCA-1 locus (15). This library was transformed into YPH500, and estrogen-dependent colonies were isolated as described previously. In this manner, we identified one clone (BCER-1) which functioned as an ER-dependent enhancer in transfected mammalian cells. Sequencing of this clone revealed that it was in fact an Alu sequence which displayed 100% sequence identity in the region corresponding to the ERE-related imperfect palindrome within ERF-3. This Alu repeat was subcloned into pB-L-TK-Luc and tested in HepG2 cells. The results shown in Fig. 3A indicate that this Alu element functioned as an ER-dependent enhancer manifesting 75% of the activity of the TK-ERE-Luc control when assayed in the presence of 17β-estradiol. As observed previously, with ERF-3, all three distinct classes of ER antagonists can effectively inhibit 17β-estradiol-induced activation of the BCER-1-derived element when presented in a 10-fold molar excess (Fig. 3B). Thus, we have independently identified another Alu sequence from a different source of genomic DNA which can function as an independent enhancer in the presence of estradiol-activated ER. The Alu family of repeat sequences can no longer be considered to be functionally inert, but in fact can function as efficient ER-dependent enhancers, the efficacy of which is influenced by promoter context.

The Alu family of DNA repeats represents approximately 5% of the total mass of the human genome (500,000 copies) (22); as such, it is unlikely that all Alu sequences function as ER-dependent enhancers. However, the two estrogen-responsive enhancers which we have identified within ERF-3 and BCER-1 contain specific base changes which distinguish them from Alu sequences which are more abundantly distributed throughout the genome. The sequence comparisons between the Alu elements identified in our screens and the consensus sequences of major subclasses of Alu repeats are shown in Fig. 4A. These consensus Alu elements are approximately 282 bp in length, of which 49 bp are shown (22). The PS (primate-specific) consensus sequence, which is likely to represent several of the oldest Alu subfamilies, describes the majority of Alu members (22, 23). The A5 (anthropoid-specific) subfamily, which evolved next, can be identified by a diagnostic mutation indicated by the dashes representing a deletion of an A and G residue. The latest classes to evolve were the CS (catterhine-specific) and HS (human-specific), which are identified by specific mutations represented by nucleotide substitutions. The older PS subclass is distinguished from the later CS and HS subclasses by an additional mutation in the first half-site of the ERE palindrome which converts the sequence GGCCA (PS and AS) to AGCG (CS and HS). Thus, by this nomenclature, ERF-3 belongs to the AS subclass whereas BCER-1 is a member of the PS subfamily of Alu repeats. Potential estrogen-responsive sequences are underlined from which a consensus Alu ERE was derived (5'-GGTCAanTGGTC(n)TGACC-3'). More importantly, however, ERF-3 and BCER-1 differ from the consensus sequences of the 4 major classes of Alu repeats by a T residue which exists in the left arm of the imperfect palindrome within ERF-3 and

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2 P. A. Marks, A. Futreal, R. W. Wiseman, D. Iglehart, J. Norris, and D. P. McDonnell, unpublished data.
Estrogen-responsive Enhancers within Human Repetitive DNA

Fig. 4. Estrogen receptor-dependent enhancer activity is not an inherent property of all Alu sequences. A, partial sequence (in the antisense orientation) from four main subfamilies of Alu are shown along with the corresponding sequence of ERF-3a and BCER-1. Dots represent homologous sequence, slashes correspond to deletions. Underlined sequences correspond to putative ERERes from which a consensus AluERE was derived. B, the transcriptional activity of the four Alu subfamily consensus sequences were assessed in HepG2 cells in the presence of co-transfected ER. Each subfamily consensus was subcloned into pB-LTK-Luc and tested for ER-dependent enhancer activity in the presence or absence of 17β-estradiol as indicated. The methodology and data presentation were performed as described in Fig. 2.

BCER-1 instead of a C residue which is found in all the major classes of Alu repeats. Additionally, the same base change appears again in ERF-3; however, because of an additional deletion (AG, Fig. 4A), it is deemed to be a member of the AS Alu family. It is not clear whether this activating base change was present before amplification of several independently evolved subfamilies or whether these represent independent mutations occurring in subfamily members after integration. However, this change is quite common in several of the older subfamilies (23, 24), and it seems likely that some form of selection for this base change must have occurred otherwise parallel subgroups of several major subfamilies would be needed to explain its abundance.

Having determined that the Alu sequences contained within ERF-3 and BCER-1 differed from the consensus Alu sequence by either one (PS and AS), two (CS), or three bases (HS) within the 5′ half-site of the putative ERE sequence, it was important to show that the consensus Alu sequences would not function as ER-dependent enhancers. Consequently, we cloned the individual Alu consensus sequences into TK-Luc and examined their ability to confer estrogen responsiveness upon a heterologous promoter in HepG2 cells in the presence of expressed ER (Fig. 4B). The results shown indicate that neither consensus permits ER-dependent enhancer activity. We conclude, therefore, that the Alu sequences within ERF-3 and BCER-1, by acquiring specific point mutations, have evolved from the AS and PS family of repeats enabling them to function as ER-dependent transcriptional enhancers. In addition, the more recently made CS and HS Alu sequences are less likely to give rise to ER-dependent enhancers due to the acquisition of additional mutations within the 5′ half-site of the putative ERE sequence.

Although no specific function for Alu DNA repeats have been determined, their physical insertion into the coding regions of NF-1 (25) and factor IX (26) can disrupt gene function and lead to neoplasomatisms and hemophilia, respectively. In addition, Alu repeats have also been implicated as possible sources of protein variability (27). These events, however, are rare and do not reflect an intrinsic function of Alu repeats. Our evidence, which indicates that a subclass of Alu sequences possesses ER-dependent enhancer activity suggests that the presence of an Alu element in a gene can, in addition to altering the architecture of the gene, confer upon it estrogen responsiveness. Although our screens have looked specifically for estrogen-induced enhancers, we believe due to the sequence similarity of most hormone response elements that there may exist other classes of Alu elements which possess enhancer sequences which would respond to other nuclear hormone receptors. Specifically, mutations within the putative AluERE could give rise to vitamin D or retinoic acid receptor-dependent enhancers which can operate through direct repeats of the GGTCA sequence (28, 29). It is important to note that both the ERF-3 and BCER-1 Alu sequences are from the oldest subfamilies of Alu sequences and therefore are likely fixed in the human genome. Only the most recent members representing the HS and CS subclass are considered competent to move about the genome by recombination (22). Therefore, estrogen-inducible enhancers represented by Alu sequences of the class we have identified are not likely to move around the genome except by recombination. Interestingly, Alu-mediated recombination within the low density lipoprotein receptor gene resulting in a form of familial hypercholesterolemia has been found (30). Other events of recombination leading to insulin-dependent diabetes (31) and adenosine deaminase negative severe combined immunodeficiency syndrome (32) have also been reported. We have performed searches of the available sequences in the GenBank and have found a number of perfect matches to this class of Alu elements within the regulatory regions of known genes. One of the most interesting is within the promoter of the hepatic lipase gene (GenBank accession number M35426), a suspected target of estrogen action (33). The finding that Alu sequences can function as ER-dependent enhancers will surely impact the way we think about what is often considered functionally inactive DNA.

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