Novel Estrogen Response Elements Identified by Genetic Selection in Yeast Are Differentially Responsive to Estrogens and Antiestrogens in Mammalian Cells

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A powerful and versatile system for the identification of novel response elements for members of the intracellular receptor family is presented as applied to the human estrogen receptor. In the past, a limited number of estrogen response elements (EREs) have been functionally identified in the promoter regions of estrogen-regulated genes. From these a consensus ERE has been defined that is identical to the ERE of the *Xenopus laevis* vitellogenin gene, *i.e.*, 5′-GGTCA NNN TGACC-3′. In order to investigate without bias the range of sequences that could function as EREs *in vivo*, we have developed a genetic selection in yeast expressing the human estrogen receptor (hER) and transformed with a random oligonucleotide library in a vector where expression of a selectable marker requires insertion of an upstream activating sequence. More than 1,000,000 transformants were screened and of 726 clones that contained activating sequences, 65 were found to be hormone-dependent. Sequencing revealed that the majority contained at least one 4/5 match to a canonical ERE half-site, but only one contained a full consensus ERE as previously defined. Some contained half-sites arranged as direct repeats. Twelve elements were further characterized to compare estrogen activation in yeast and mammalian cells and *in vitro* binding to hER. The results of these studies reveal that sequences that bind weakly to hER *in vitro* are fully functional as EREs in yeast and are conditionally responsive to estrogen in mammalian cells. In addition, an element was identified that is more sensitive to the partial agonist activities of tamoxifen and nafoxidine than is the consensus ERE, indicating that not only promoter context but the sequence of the binding site itself can allow distinction between receptor activated by agonist and that activated by antagonist. (Molecular Endocrinology 8: 1193–1207, 1994)

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INTRODUCTION

Members of the steroid/thyroid hormone family of intracellular receptors control a wide variety of physiological and developmental processes by directly modulating the transcriptional activity of specific target genes in response to hormone. Although the mechanistic details vary for the individual receptors, clearly all act by binding to specific target sequences on DNA (1–4). The highly conserved DNA binding domain of these receptors dictates some degree of similarity in binding site sequences as well. To date, all but one intracellular receptor have been found to bind to DNA as dimers on elements composed of two recognition half-sites arranged as direct repeats, palindromes, or inverted palindromes (5–8). Thus, glucocorticoid receptor, mineralocorticoid receptor, androgen receptor, and progesterone receptor bind to a common sequence, the palindrome 5′-AGAACA NNN TGTTCT-3′ being optimal. Hormone-specific activation of target genes containing this consensus binding site or a related sequence is likely to be mediated by interactions between the bound receptor and proteins bound at other *cis* elements on the same promoter. Availability of hormone and receptor in a given tissue may also play a role. The majority of the remaining intracellular receptors, including thyroid hormone receptor (TR), retinoic acid receptor, vitamin D receptor, peroxisome proliferator activated receptor, retinoic X receptor, and estrogen receptor (ER), recognize the same half-site, 5′-GGTCA-3′. Specificity is

determined by the orientation and spacing of two half-sites to form a complete element (9–12). The orphan receptor NGFI-B is distinct from other intracellular receptors in that it can activate by binding to the site 5′-AGGTCA-3′ as a monomer (13, 14).

Because of an extensive literature describing the biological actions of steroid and thyroid hormones and subsequent cloning and analysis of promoters of corresponding target genes, response elements for these receptors were rapidly identified by deletion strategies and confirmed by demonstrating the ability to confer hormone response to a heterologous promoter. However, for any given receptor, only a few target genes have been identified so that a good representation of the range of functional response elements has been lacking. Systematic approaches have been taken to survey the effects of single base substitutions of the glucocorticoid response element of the mouse mammary tumor virus (MMTV) promoter for recognition by either glucocorticoid receptor or progesterone receptor (15, 16). Thus, the derivation of consensus binding site sequences has evolved from molecular dissections of known response elements whereas more divergent sequences may be equally important physiologically.

We wished to develop a genetic screen for novel response elements in yeast that would rely on functional activation and could be easily applied to identify binding sites for any of the intracellular receptors. In the past, *in vitro* methods have been developed to enrich for high affinity target sequences for DNA or RNA binding proteins from pools of random oligonucleotides (17–20). This approach was first successful for the yeast activator, GCN4, and related techniques have been applied to a number of mammalian transcription factors. As for intracellular receptors, random sequence *in vitro* approaches have been taken to identify optimal recognition sequences for androgen receptor (21), vitamin D receptor (22), and TRα (23). The limitations of *in vitro* selection methods are the need for purified protein, highly efficient heterologous expression systems, or very specific antibodies. The stringency of *in vitro* selection protocols introduces a bias toward high affinity binding sites while promoters of hormone-responsive genes often utilize lower affinity binding sites that are functional only in combination with other *cis*-acting sequences. Furthermore, *in vitro* approaches do not consider the roles of chromatin or components of the general transcription apparatus in recognition of binding site by receptor. We were interested, therefore, in developing an alternative and complementary system in which a large number of random oligonucleotide sequences could be screened for function as hormone response elements *in vivo*.

A previous attempt to screen for functional estrogen response elements (EREs) using an enhancer trap protocol in yeast successfully identified novel EREs, including some sequences arranged as direct repeats, and a novel estrogen-responsive gene, complement C3, was identified by searching GENBANK with one of the sequences (24). However the number of oligonucleotide sequences that could be screened was limited since a colorimetric reporter was used. Another response element screen in yeast based on a nutritional selection identified a binding site for the orphan receptor, NGFI-B, by screening a library of genomic fragments in an appropriate reporter vector (13). Again, for technical reasons, the number of sequences that could be screened by this protocol was limited.

We report here the development and implementation of a functional screen for novel estrogen-responsive elements from a library of random oligonucleotides. More than 1,000,000 transformants were screened using intact human estrogen receptor, and 65 novel estrogen-responsive sequences were identified. We have evaluated some of these sequences for ability to bind estrogen receptor *in vitro* and compared estrogen activation of these elements in yeast and in mammalian cells. Some sequences have also been tested for the ability to distinguish between agonist-liganded and antagonist-liganded receptor. We chose estrogen receptor for developing this system because among the intracellular receptors, it has been most extensively studied in yeast. However, this system could be readily adapted to identify response elements for any of the intracellular receptors with known ligands and could be modified to identify binding sites for orphan receptors.

RESULTS

Development of Screen

We were interested in developing a functional screen for novel hormone response elements that would allow rapid screening of large numbers of random oligonucleotides. In a previous attempt to identify novel EREs, technical constraints limited the number of recombinants that could be analyzed in yeast using a β-galactosidase reporter (24). In contrast, with a selection for nutritional prototrophy, hundreds of thousands of transformants can be easily screened because only plasmids containing activating sequences generate colonies. Such a selection was used to identify a binding site (NBRE) for the orphan receptor NGFI-B (13). However, its application was limited because the transcriptional activity of the chimeric activator used was constitutive making it difficult to distinguish NBREs from binding sites recognized by endogenous yeast activators. We have developed a system where full-length human estrogen receptor (hER) expressed in yeast is used to select novel EREs from a random oligonucleotide library in a hormone-dependent manner.

Pilot experiments were performed to establish the feasibility of this approach. The vector pBM2389 is conditional for *HIS3* expression because it requires insertion of an activator sequence upstream of a transcriptionally inert *GAL1* promoter (Fig. 1). The vector is a low copy number plasmid based on the centromeric YCp50 and also contains a constitutively expressed *TRP1* gene for primary selection. Yeast strain BJ5409/

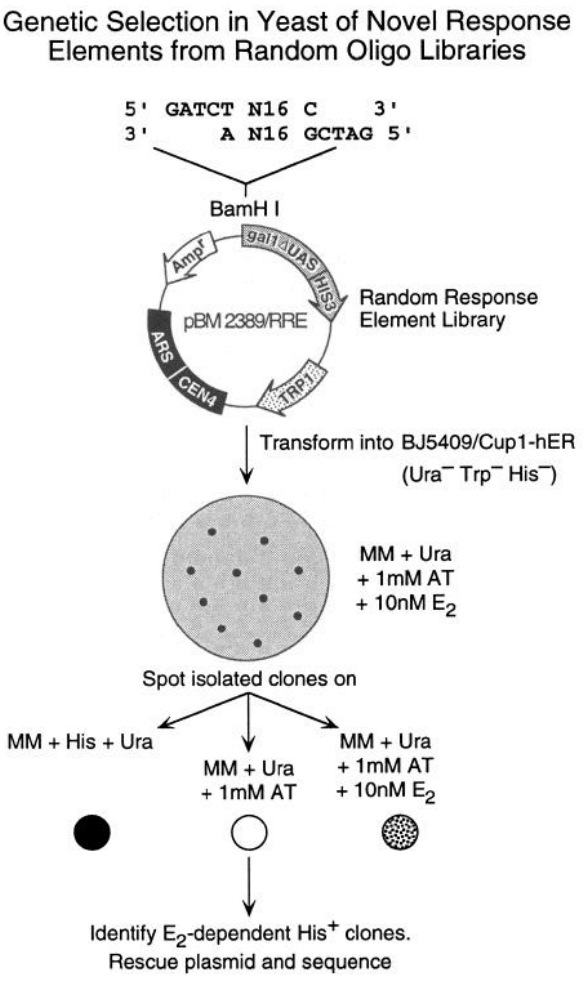


Fig. 1. Selection Protocol

pBM2389, the vector for the RREL, contains sequences for transformation of bacteria (*Amp*^{r}) and yeast (*Trp1*) and elements for maintenance as low copy autonomously replicating plasmids in yeast (*ARS1*, *CEN4*). In addition, expression of the *His3* locus is conditional upon the insertion of an upstream activator sequence in the *Bam*HI site adjacent to the inert *Gal1* promoter. The primary selection after transformation of the host strain with the random oligonucleotide library is for Trp and His prototrophy in the presence of E_2. Surviving colonies are then subjected to a secondary screen for E_2-dependence of the His$^+$ phenotype.

YEPE22 expressing hER was transformed with pBM2389 containing the vitellogenin ERE (2389-vit) or no insert (vector). BJ5409/YEPE22 requires nutritional supplementation of histidine, tryptophan, and uridine. Under growth conditions similar to those used here, BJ strains carrying YEPE22 express hER at 1–2 pmol/mg protein, which is within mammalian physiological ranges (25, 26). Transformants were selected by plating on minimal media plus histidine and uridine (minus tryptophan) then aliquots from pools of transformants were plated on varying nutritional conditions. When exogenous histidine was withdrawn, less than 10% of transformants with either plasmid grew. Addition of 17β-estradiol (E_2) increased the survival rate to nearly 100%, but colonies carrying 2389-vit were approximately 10 times larger than colonies carrying vector.

In order to eliminate background growth due to weak activation of vector sequences by E_2 in yeast expressing hER, varying concentrations of 3-aminotriazole (AT) were added to the selection plates. AT is a competitive inhibitor of the *HIS3* gene product, imidazole-glycerol phosphate dehydratase, and ability to grow in the presence of AT is proportional to the transcriptional activity of this locus (27). By comparing growth at various concentrations of AT from 0.1 to 40 mM, it was found that 1 mM AT in the presence of 10 nM E_2 under histidine deprivation allowed near wild type growth of yeast carrying 2389-vit while vector alone was insufficient to support growth. In other words, the selection protocol has been optimized with wild type hER and the vitellogenin ERE to ensure that selected sequences would have activity comparable to a natural ERE. Populations of yeast transformed with the random oligonucleotide library described below generated less than 0.5% His$^+$Trp$^+$ colonies (of varying size) relative to the total number of Trp$^+$ transformants.

Generation of RREL

Double-stranded oligonucleotides containing 16 contiguous fully degenerate nucleotide pairs and flanked by *Bam*HI and *Bgl*II recognition sites were generated by mutually primed synthesis (28). A library of greater than 800,000 independent recombinants was generated by inserting the random oligonucleotides into the *Bam*HI site of pBM2389. The library was amplified in bacteria to a limited extent, in order to keep it representative of the original population of degenerate oligonucleotides. A sample of the amplified DNA was sequenced by chain termination methods, and the inserted sequence was found be completely random for 16 nucleotides (results not shown). The majority of clones appeared to contain one oligonucleotide insert while a minority contained two or more inserts. This random response element library (RREL) was then used to transform yeast expressing hER.

Transformation of Yeast and Selection of E_2-Responsive Clones

The yeast strain BJ5409 (*his3, leu2, trp1, ura3*) was transformed with the 2 μm plasmid YEPE22 expressing the hER as a ubiquitin fusion under the control of the copper-inducible *CUP1* promoter (25). Competent BJ5409/YEPE22 cells were prepared and transformed with RREL by an optimized lithium acetate protocol and selected directly for expression of the conditional marker (*His3*) as well as the primary marker (*Trp1*). More than 1,000,000 Trp$^+$ primary transformants were selected for histidine prototrophy in the presence of 10 nM E_2 and 1 mM AT. After 4 days growth at 30 C, colonies of varying size were apparent at a frequency of less than 1 His$^+$ transformant for every 300 Trp$^+$ transformants. Figure 1 summarizes the overall screening process.

It was anticipated that the majority of selected sequences would be activated by endogenous yeast DNA binding proteins and therefore would not be dependent on the presence of E_2-activated hER. A secondary screen was developed to identify E_2-dependent clones. Dilute suspensions of cells grown overnight in liquid culture under nonselective conditions (plus histidine and uridine), were spotted on selective plates (lacking histidine) plus or minus 10 nM E_2. The primary selection was maintained (lacking tryptophan), and 1 mM AT was included. Those clones that grow in medium lacking histidine and including E_2 but minimally or not at all when both are excluded are therefore E_2-dependent. A total of 726 clones were subjected to two rounds of screening for E_2 dependence, and 65 E_2-responsive clones were further analyzed. This represents approximately 2% of all activated (His^+) clones.

Plasmid Rescue and Sequencing of E_2-Responsive Elements

Plasmids containing E_2-responsive sequences were rescued as detailed in *Materials and Methods*, and the oligonucleotide inserts were sequenced. The sequences of all single inserts are presented in Table 1. Of the 65 clones sequenced, 38 contained single oligonucleotide inserts, 17 had two inserts, and the remainder contained three to five oligonucleotides. A majority contained sequences with a 4/5 or 5/5 match to a classical consensus ERE half-site (5′-GGTCA-3′ or 5′-TGACC-3′). A few elements resembled direct repeats of half-sites rather than inverted repeats, E-230 being the best example. Some contained a half-site in combination with an AT-rich sequence, *e.g.* E-179 and E-196. One of the triple insert clones, E-160 (not shown), contained a perfect consensus ERE, *i.e.* palindromic half-sites separated by a 3 base pair (bp) spacer (5′-GGTCA AAT TGACC-3′). Two sequences were selected twice each. E-12 and E-31 were derived from two different selection plates but the same transformation reaction. E-156 and E-404 were derived from separate transformation reactions. E-31 and E-404 were therefore not included in Table 1.

The sequences of single insert clones were analyzed by computer to objectively identify consensus patterns of unspecified length in the unaligned sequence set, as described in *Materials and Methods*. The summary matrix describing the most significant pattern is presented in the legend to Table 1 and yields the consensus septamer 5′-**GGTCAMV**-3′, where M = A or C and V = not T. This is related to the consensus ERE derived from estrogen-regulated genes, 5′-GGTCA NNN TGACC-3′, but differs in that only a half-site was derived and the two 3′-positions in the yeast-selected consensus are constrained, *i.e.* M and V, whereas in the classical ERE they are thought to be unconstrained "spacer" positions. Of the 36 sequences, 19 contain a perfect match to the first five residues of the consensus. *Boldface type* in Table 1 indicates the best match to the consensus for each element. Individual matching scores to the consensus ranged from 2.42 to 10.25 (perfect).

A second best match was also determined for each element. Scores for these sequences ranged from <0 to 7.34. Those that scored better than 2.42 are indicated by *underline* in Table 1. In five cases the second match was such that the two septamers formed opposing 5 bp half-sites with 3 bp spacing, as in the accepted concept of an ER binding site: E-79, E-261, E-296, E-322, and E-538. In the case of E-459, which by visual inspection appears most like a classical ERE, a septamer that opposes the best match has a very low score reflecting the importance of the 5′-G and the 3′-not T. That is, the septamer 5′-AGTCATT-3′ has a matching score of only −0.59 but creates the appropriate spacing with the best match, 5′-TCTGACC-3′, which has a matching score (for the inverse complement) of 7.65.

More often the two highest scoring septamers were oriented as direct repeats with spacings that varied from 8 bp (E-9) to −7 bp (overlapping, E-708). For E-12 and E-230, the direct repeats both had scores >7 indicating the presence of two strongly matching half-sites in each of those inserts.

In those cases where M and V were both equal to C, the septamer was actually a palindrome in itself, *e.g.* E-196 and E-635. Several clones contained only one apparent matching septamer: E-60, E-161, E-179, E-405, and E-705. Overall, it seemed that activation by hER in yeast could be accomplished through a variety of *cis* elements composed of apparent half-sites arranged in various spacings and orientations.

Analysis of Activation in Yeast

We wished to compare activation of the selected oligonucleotides by E_2-liganded hER in yeast and in mammalian cells and to correlate *in vivo* activity to the ability to bind hER *in vitro.* In order to simplify this process, only single insert sequences were further studied. All single insert plasmids were reintroduced into BJ5409/YEPE22 in order to confirm that the selected sequence was directly responsible for the phenotype observed.

Resistance to the competitive inhibitor, AT, was chosen as an index for the transactivation potentials of the individual elements in yeast. As mentioned above, AT starves the cells for histidine by blocking biosynthesis, and resistance to this compound is directly related to the transcriptional activity of the *HIS3* gene (27). BJ5409/YEPE22 cells transformed with the rescued plasmids were plated on increasing concentrations of AT in the presence of 10 nM E_2 in media lacking histidine and scored for growth after 4 days at 30 C. The individual elements were then ranked for resistance to AT relative to the vitellogenin ERE, and the results are summarized in Table 1. In this screen, ERE_{vit} allowed growth to 4 mM AT. Two elements, E-28 and E-459, were judged to be more effective targets for hER in yeast than ERE_{vit}, several were as effective as ERE_{vit}, and the majority were weaker. Note that more than half the selected elements were comparable in AT resist-

Table 1. Sequences of Novel EREs Selected in Yeast

Clone	AT mM	Sequence			Matching Score
E-28[a]	8.0	GGATCCG	TGACGCGGTGACGTA	AGATCC	8.01 >
E-459[a]	8.0	GGATCT	CCTAGTCATTCTGAC	CGGATCC	7.65
E-12[a]	4.0	GGATCCG	GTCAAAAATGTCACGG	AGATCC	10.15 >
E-296	4.0	GGATCT	TGCGTTCAACTTGAC	CGGATCC	10.25
E-538[a]	4.0	GGATCT	GTCAAGCCGACCTTTT	CGGATCC	7.52 >
E-572[a]	4.0	GGATCT	TGGCAGGCCACCTTGC	CGGATCC	5.52
E-634	4.0	GGATCCG	TTGTGGGGTCAAGGT	AGATCC	10.25 >
E-635[a]	4.0	GGATCCG	GGTTCGGGGTCACCTT	AGATCC	9.07 >
E-705[a]	4.0	GGATCCG	GCGGGCGCGGAATTAA	AGATCC	5.17 >
E-708	4.0	GGATCT	TTTTTAACTACAA	CGGATCC	4.29
E-54	2.0	GGATCCG	GAGTTTGGGTCACGG	AGATCC	10.07 >
E-60	2.0	GGATCCG	GGGAGCTGGTAAAATT	AGATCC	7.83 >
E-79	2.0	GGATCCG	AGGGCAGGGCTACCAA	AGATCC	5.26
E-116	2.0	GGATCCG	GAGATTGGGGTGACCG	AGATCC	9.07
E-138	2.0	GGATCCG	CGTGTTACGTGATGTA	AGATCC	4.70 >
E-156	2.0	GGATCT	CCGGGTCATAACAACA	CGGATCC	8.39 >
E-179[a]	2.0	GGATCCG	AAAAAAGATATGACCT	AGATCC	8.39
E-196[a]	2.0	GGATCCG	GAAATTTTTGTGACCG	AGATCC	9.97
E-197	2.0	GGATCCG	GGGGCGTTCTGATCTG	AGATCC	3.11 >
E-405	2.0	GGATCT	CCGTGACCCCAAACCT	CGGATCC	10.07
E-154	1.0	GGATCT	TACATTCAGGACGCCA	CGGATCC	4.58 >
E-161	1.0	GGATCCG	AAGGGAGGTCAAGGCG	AGATCC	10.25 >
E-235	1.0	GGATCCG	ATGACCAGAAAATGTC	AGATCC	7.48
E-261	1.0	GGATCCG	GGTTATTGCAACCGAG	AGATCC	5.84
E-276	1.0	GGATCT	TTTCACCCCATGTGAA	CGGATCC	8.09
E-9	0.5	GGATCCG	GTTGAGTTTAGCAAGG	AGATCC	6.12 >
E-167	0.5	GGATCCG	GGTCAAGGAAACATAA	AGATCC	10.25 >
E-187[a]	0.5	GGATCCG	GGTAGGGGGTCAAGTT	AGATCC	10.25 >
E-225	0.5	GGATCCG	ACCTCAGGTCGAAATG	AGATCC	8.67 >
E-324	0.5	GGATCT	ATCACGACCTCAAATC	CGGATCC	7.00
E-444	0.5	GGATCCG	TAGAAATAAAAAAGGG	AGATCC	2.42 >
E-500	0.5	GGATCT	CTTCAGCTCCTTTGAC	CGGATCC	10.15
E-172	nt	GGATCT	GCTGACAGGCTTCAT	CGGATCC	4.02
E-189	nt	GGATCCG	GAAAAACCGGGAAGTC	AGATCC	4.83 >
E-230[a]	nt	GGATCT	GACCCTGTGACCTGCT	CGGATCC	9.97
E-322[a]	nt	GGATCCG	TAGGTTGCCTTGACAT	AGATCC	7.52
ERE_{vit}	4.0	GGATCT	AGGTCACAGTGACCTG	CGGATCC	9.97 >

The sequences of all single inserts selected in yeast are shown, oriented with the *Gal*1 promoter to the right. Due to an asymmetry in the construction of the insert, the *Bam*H1 side of the cloning site contributes seven bases to the insert fragment while the *Bgl*II side contributes six bases. The sequences are sorted in descending order of 3-aminotriazole (AT) resistance. The summary matrix found in these sequences by *wconsensus* is:

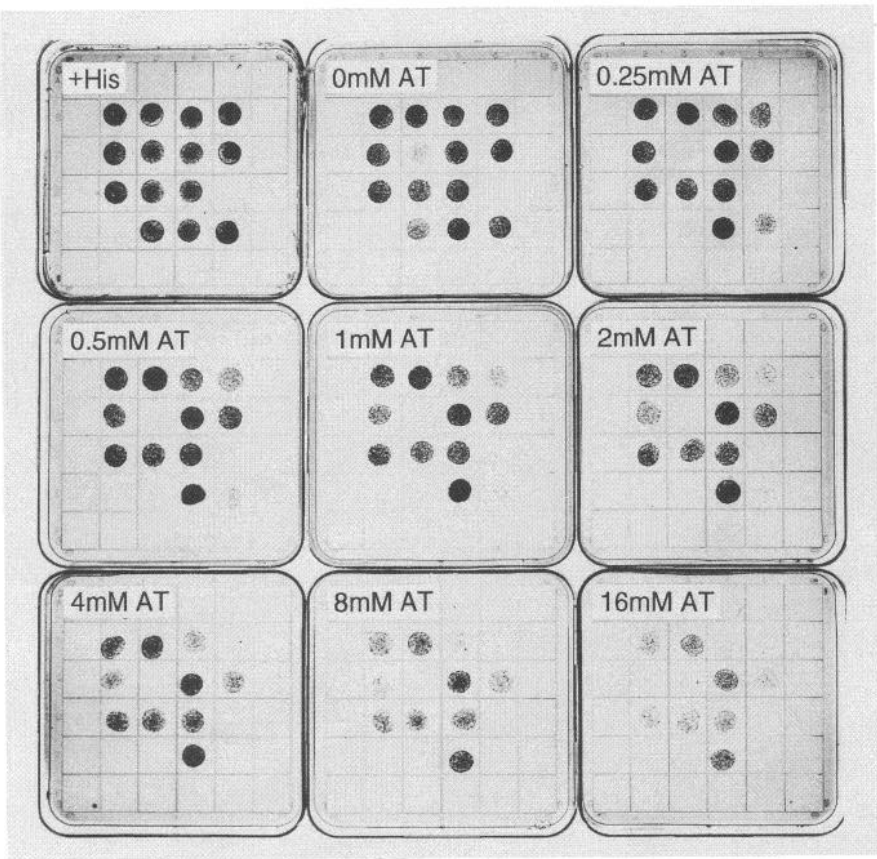
5′							3′
A	0	0	3	4	27	16	14
G	32	34	2	5	9	2	15
C	0	0	0	24	0	14	7
T	4	2	31	3	0	4	0

Boldface type indicates the septamer which best fits the summary matrix; the score for the match is to the *right* of the sequence. The second best fit is *underlined*. Septamers are found in both orientations; > next to the score indicates the site is oriented as given by the matrix. The naturally occurring vitellogenin ERE (ERE_{vit}) is at the *bottom*.

[a] Twelve elements chosen for further study.

ance to ERE_{vit} within a factor of 2. Cells carrying vector with no insert or an unrelated element were not viable at 0.25 mM AT (see Fig. 2) while all the elements identified as EREs in yeast and subjected to this screen were resistant to at least 0.5 mM AT. Once again, these observations indicate that the conditions of the selection were appropriate for identification of sequences that could function similarly to a natural ERE.

Yeast possess the transcriptional machinery necessary to activate an ERE by hER expressed under the control of yeast promoters in response to hormone added extracellularly. We were concerned, however, that elements identified as positive EREs in yeast might behave differently in mammalian cells. Twelve elements selected as hER targets in yeast were chosen for analysis in mammalian cells. These are indicated by



E-12	E-28	E-179	E-187
E-196	E-322	E-459	E-538
E-572	E-635	E-705	
	Vector	ERE	NRE

Fig. 2. Activation in Yeast

Competent BJ5409/YEPE22 cells were transformed with rescued plasmids conferring E_2 response and analyzed for resistance to AT as detailed in *Materials and Methods*. Plates were photographed after 4 days growth at 30 C.

footnote a in Table 1. A broad representation of elements was chosen with consideration for 1) homology to the classical ERE; 2) presence of direct or inverted repeats, or half-sites only; 3) high matching scores to the computer-generated consensus pattern; and 4) strength of activation in yeast. A comparison of the AT resistance of these 12 clones in yeast is shown in Fig. 2. Cells carrying vector die at 0.25 mM AT while cells carrying a plasmid with a nonresponsive oligonucleotide insert (NRE) do not survive the conditions used in the screen, *i.e.* 1 mM AT. As noted before, E-28 and E-459 are the most potent EREs in yeast, although in this particular experiment they performed very similarly to ERE_{vit}. Plasmid clones E-230 and E-322 had undergone DNA rearrangements since their original isolation and were not able to be analyzed in this particular experiment.

Binding of hER to Novel Elements *in Vitro*

To determine whether relative ability to function as an ERE in yeast correlates with the affinity of an individual element for hER, we examined the binding characteristics by mobility shift assay. Each of the 12 elements was prepared as a double-stranded oligonucleotide and labeled by fill-in reaction to generate a 26 or 27 bp probe. When incubated with extracts prepared from yeast expressing hER, none of these probes was retarded by hER under conditions where up to 6% of ERE_{vit} is shifted to a specific band (Fig. 3a). Two inde-

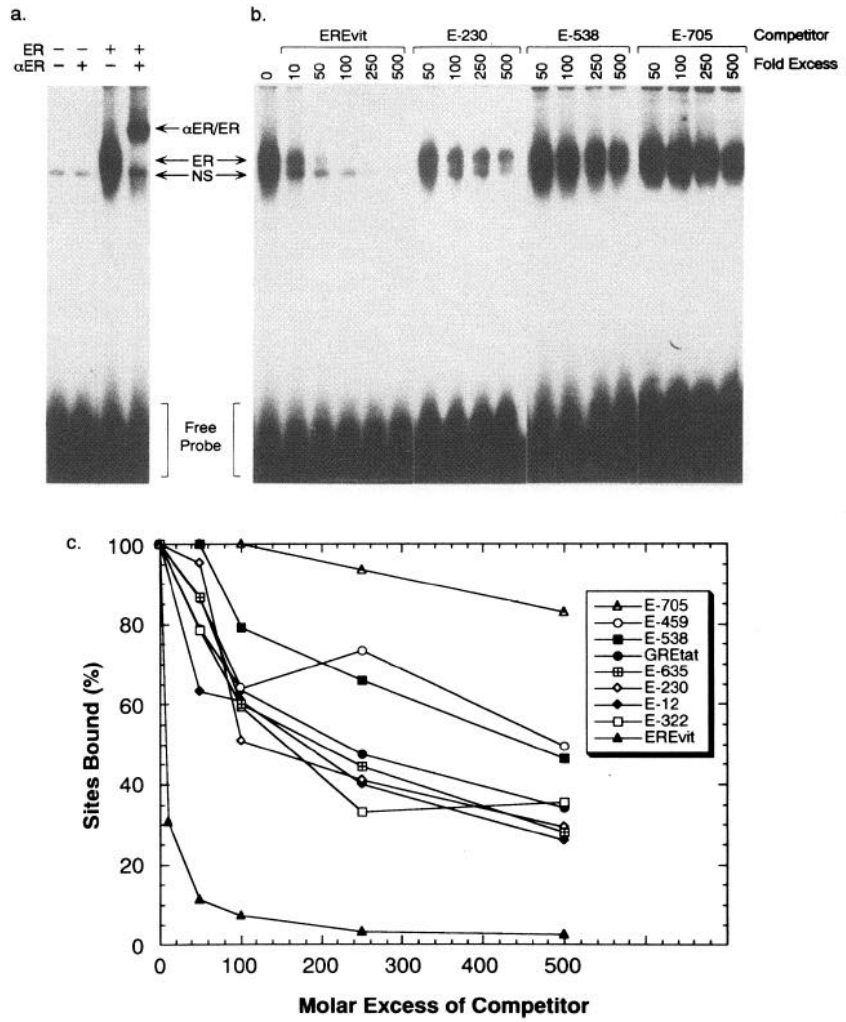


Fig. 3. *In Vitro* Binding to hER

Selected oligonucleotides were analyzed by mobility shift assay as detailed in *Materials and Methods*. a, Control experiments showing specific binding of ERE_{vit} by hER. Complexes representing ER/ERE and ER/ERE supershifted by antibody (αER) are indicated by *arrows*. Nonspecific bands (NS) and free probe are also indicated. b, Binding of ERE_{vit} by hER was competed by excess cold oligonucleotide. The molar excess of competitor is indicated *above* each lane. c, Competition experiments with 12 different cold oligonucleotides were performed as in panel b, and retarded bands and free probe from each lane were excised and radioactivity was quantitated by liquid scintillation counting. Sites Bound (%) was determined by dividing the counts per min in the ER-specific retarded complex by the mean counts per min of the same complex in several noncompeted reactions.

pendent experiments verify that this band is due to binding by authentic hER. First, all of this band can be supershifted by a monoclonal antibody to the amino terminus of hER. Second, this band is not seen when ERE_{vit} is incubated with extracts from the same yeast strain not transformed with the ER expression plasmid. Increasing the amount of probe for the test oligonucleotides up to 5 times that used for ERE_{vit} or including antibody in the binding reaction also failed to generate an ER-specific band with any of the novel sequences (not shown). Kato *et al.* (29) have reported stabilization of weak ERE/ER complexes by antibody. Nonspecific bands of varying mobility are detected in both hER-expressing and nonexpressing extracts incubated with the individual probes and are not supershifted by antibody to hER.

Next we examined the possibility that some of these oligonucleotides could compete for binding of ERE_{vit} by hER if present at high enough concentration. The same yeast extract was preincubated for 5 min with 10- to 500-fold excess cold double-stranded oligonucleotide before addition of labeled ERE_{vit} of the same length. Representative competitions are shown in Fig. 3b, and quantification with nine different competing oligonucleotides is shown in Fig. 3c. Cold ERE_{vit} is able to reduce binding to 10% at a 10-fold excess. Oligonucleotides representing novel elements E-12, E-230, E-322, and E-635 reduce binding to 50% at 200- to 250-fold excess. E-459 and E-538 compete less effectively and E-705 was very ineffective. E-28, E-179, E-187, E196, and E-572 were also ineffective competitors (not

shown). These results indicate that even though some elements are functional EREs in yeast, as isolated sequences they bind hER with an affinity less than 1/20 that of ERE_{vit}.

Analysis of Activation in Mammalian Cells

Since these 12 elements had low affinity for hER as isolated sequences but were effective in conferring estrogen response to a heterologous yeast promoter, it was imperative to determine whether they could impart estrogen sensitivity to a mammalian reporter. Oligonucleotides corresponding to these 12 elements selected in yeast were inserted into the *Bam*HI site upstream of the minimal Herpes simplex virus thymidine kinase promoter driving firefly luciferase expression (pBL-TK-Luc). The resulting reporters were cotransfected with hER (expressed constitutively from the Rous sarcoma virus promoter) into HepG2 cells and assayed for response to increasing concentrations of E_2. We have found that HepG2 cells cotransfected with hER provide a good model system in which to study the activity of reporters containing a single copy ERE. Although it is not possible to determine the exact concentration of hER in these cells, we have chosen the minimal amount of pRShER plasmid required to generate a maximal response with a single copy of the vitellogenin ERE upstream of the TK promoter. When analyzed in this setting, ERE_{vit} was induced 29-fold in response to hormone (Fig. 4). E-635 was the best of the novel response elements examined, exhibiting a 13-fold induction. This element contains several overlapping five-nucleotide stretches with three to five base matches to the classical half-site 5′-TGACC-3′. Within this element, the best agreement with the vitellogenin ERE is 5′-GGTgA Ccc cGAaC-3′. Another element, E-459, which contains a strong homology to ERE_{vit}, 5′-aGTCA tTc TGACC-3′, and which was strongly induced by E_2 in yeast, induced less than 5-fold as a single element in pBL-TK-Luc. E-12 and E-322 each induced 8- to 9-fold. There was minimal E_2 response when the remaining eight elements were analyzed as single copies.

Hormone response elements generally work cooperatively with other *cis* elements in natural promoters (8). We reasoned, therefore, that the difference between performance of these sequences in yeast *vs.* mammalian cells could be due to a number of factors but most notably difference between context of flanking sequences in the yeast pBM2389 vector and the mammalian TK-Luc reporter. Often in synthetic constructs, multimerizing a binding site for a transactivator can overcome the loss of activity when it is removed from the context of a natural promoter. A series of TK-Luc reporters containing multiple inserts of the 12 novel response elements was created and analyzed for transcriptional activation by hER in HepG2 cells as before. When tested as multicopy response elements, six of 12 elements were induced in a hormone-dependent manner by hER, as shown in Fig. 4. In general, the maximum stimulated value of luciferase was proportional to the number of inserts for a given element. In some cases the basal level of expression, *i.e.* no hormone added, also increased so that the fold-induction values did not increase accordingly. Two elements, E-12 and E-230, contain apparent direct repeats of half-sites and induce similarly to the ERE_{vit} inverted repeat when multimerized. The best fold-induction was seen with three copies of E-635, while the maximum stimulated level of luciferase was achieved with five copies of E-322. E-459, which most closely matches ERE_{vit}, was stimulated by E_2 but not nearly as effectively as the more divergent elements identified in this screen.

Since evaluation of the sequences in both yeast and HepG2 cells involves overexpression of hER, it could be argued that the responses generated are simply the result of mass action effects on nonspecific interactions. We have transfected the same series of TK-Luc reporters into the human breast cancer cell line, MCF-7, and treated with E_2, relying on the endogenous ER. We were able to detect induction of ERE_{vit}, E-322, and E-635 TK-Luc reporters, but for all these the induction profiles were much less robust than in HepG2. Since MCF-7 is known to contain mutant and variant forms of ER, we are unsure of the physiological relevance of these results. Nevertheless, the responses generated in HepG2 expressing transfected hER are clearly ligand-dependent and therefore not merely the result of excessive levels of receptor overwhelming normal control mechanisms.

The same series of mammalian reporters have been tested for function as thyroid response elements (J. Bilakovics and S. Dana, unpublished results). Since estrogen and thyroid hormone receptors recognize the same half-site (5′-GGTCA-3′), HepG2 cells were cotransfected with each novel response element reporter and the human thyroid hormone β-receptor (hTRβ) in the same RSV expression vector and treated with thyroid hormone (L-T_3). None of the 12 elements, whether as single or multiple inserts, were activated by hTRβ in the presence of L-T_3 (results not shown). This supports the specificity of the genetic screen and further discredits the idea that excessive levels of ER are activating through nonspecific interactions. Under the same conditions, a single copy of TREp, which consists of two half-sites composed as an inverted repeat with 0 bp spacer, induces 3- to 4-fold while four copies induce more than 1000-fold in response to L-T_3.

Agonist Response to Antiestrogens

Other investigators have noted tissue- and promoter-specific patterns of response to tamoxifen as a partial agonist, which can be explained by the ability of the TAF1 function of hER to activate in a particular setting (30, 31). In addition, differential electrophoretic mobility of tamoxifen-liganded ER bound to an ERE, as compared to E_2-liganded ER, indicates different conformations of the receptor (32). In order to determine whether ligand-induced conformational changes detected *in vi-*

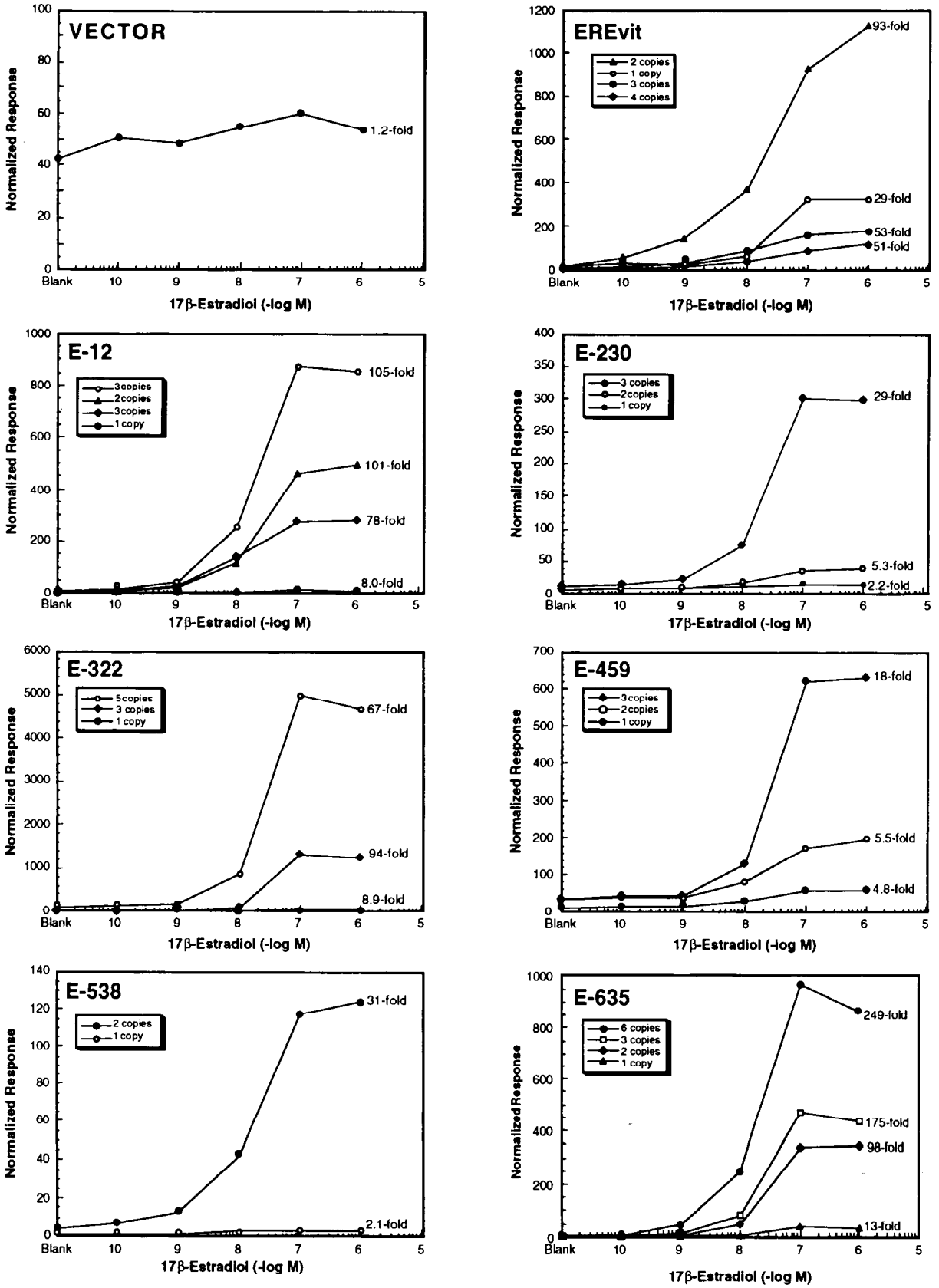


Fig. 4. Activation in Mammalian Cells

HepG2 cells were cotransfected with pRShER and luciferase reporters and assayed for dose response to E_2 as detailed in *Materials and Methods*. For each element several reporters were analyzed with one to six copies of the corresponding oligonucleotide inserted upstream of the TK promoter of pBL-TK-Luc. For E-12, two different three-copy reporters were derived which differed in the relative orientations of the individual oligonucleotides. Fold-induction values are included to the *right* of each plot and were derived by dividing the normalized response at 10^{-7} M E_2 by that in the absence of hormone.

tro might be reflected in differential response element specifities, we compared activation of ERE_{vit} and two of the more responsive elements (E-322 and E-635) by four different ligands. The same series of multiple response element luciferase reporters were cotransfected with hER into HepG2 cells, and dose-response curves were obtained for E_2 and the estrogen antagonists, nafoxidine, 4-hydroxytamoxifen, and keoxifene.

The results for ERE_{vit} and E-635 are presented in Fig. 5. Both nafoxidene and 4-hydroxytamoxifen behaved as partial agonists on ERE_{vit}, as has been seen before in HepG2 cells, with the maximal responses being less than 5% that obtained with E_2. These compounds were more active with the E-635 series of reporters, such that the maximal response to either compound was approximately 10% that with E_2. Potency was not affected by the response element. E-322 reporters were also activated by 4-hydroxytamoxifen and nafoxidene. The response was intermediate to that of ERE_{vit} and E-635 (results not shown). Keoxifene did not generate significant partial agonist activity with any of the reporters tested. These data indicate that even *in vivo*, response element sequence can discriminate between different conformations of receptor induced by binding different ligands.

DISCUSSION

We have used a genetic selection in yeast to identify sequences from a random oligonucleotide library that can function as EREs. Using a single step, nutritional selection allowed us to screen 1,000,000 yeast transformants easily, and development of a simple secondary screen for hormone dependence resulted in the identification of 65 novel functional EREs. A consensus pattern of seven nucleotides was identified when the 36 single insert sequences were compared: 5′-**GGTCAMV**-3′, where M = A or C and V = not T. This consensus includes the classical ERE half-site, 5′-GGTCA-3′, which is found in the vitellogenin promoter as the 5′-half of a perfect palindrome separated by three nucleotides. In fact, 19 of these 36 sequences contained at least one perfect half-site. It is generally thought that the three spacer nucleotides are unconstrained but in the current study a preference for the two bases immediately 3′ to GGTCA is noted.

A survey of 12 of these elements in a mammalian system indicated that six could function as EREs when multimerized and placed upstream of the minimal HSV TK promoter. It is likely that for any one of these elements, the context of surrounding sequences in combination with the particular host cell transcription factor complement will dictate the efficiency of their activation. Those six that did not activate when multimerized and introduced into HepG2 may rely on interaction with yeast-specific factors, and some might function in mammalian cells as well given the appropriate promoter context in a suitable cell line. In general, we found yeast to be less discriminating and therefore were able to identify elements that would have escaped *in vitro* methods of binding site selection, resulting in a range of functional EREs rather than a tightly conforming optimal consensus sequence. Perhaps if a higher concentration of AT were used in the primary screen more high affinity sites would have been selected. However, it is also possible that more multiple insert clones would have been isolated, as was the case when testosterone-activated androgen receptor was used in this system (our unpublished results). We are currently applying this approach to select estrogen-responsive sequences from genomic DNA. The random oligonucleotide library should be useful in identifying functional response elements for orphan receptors and thereby facilitate identification of ligands, while the genomic DNA library may allow identification of target genes for any of the intracellular receptors.

These same elements, although most contain half-sites also recognized by the thyroid receptor family, are not activated by TRβ. Interestingly, Katz and Koenig (23) have recently found that mouse TRα selects sequences *in vitro* that contain the octamer 5′-TAAGGTCA-3′ and that a single copy of this sequence can confer L-T_3 response to a heterologous promoter (23). Thus, in that case, three bases 5′ to the half-site are constrained for recognition by TRα while in our system two bases 3′ to the half-site show preferential recognition by hER. The closest match to this octamer among the elements selected here occurs within E-635, 5′-TAAGGTGA-3′, which is not activated by L-T_3 even in six copies whereas four copies of TRE_{pal} confer a 1000-fold response to the same reporter. Furthermore, six copies of a conventional TRE half-site, 5′-GGTCA-3′ (found within three copies of E-230 or six copies of E-635), are not activated by L-T_3. These observations support the idea that these selected elements are in fact EREs and reinforce the importance of contributions of half-site sequence, orientation and spacing, and surrounding sequences to the specificity of recognition.

This work supports a number of previous studies documenting synergism of EREs both *in vivo* and *in vitro*. Imperfectly palindromic EREs as well as half-sites can interact among themselves or with binding sites for other transcription factors (8, 29, 33–35). Spacing between isolated elements is important, most likely reflecting a dependence on relative placement on a DNA helix. This probably explains the differences in fold-induction values when a given response element is duplicated or triplicated before the TK promoter.

None of the selected sequences bind hER effectively *in vitro*, although differences in ability to compete with ERE_{vit} binding can be quantitated. However, a glucocorticoid response element from the rat tyrosine aminotransferase gene (GRE_{tat}) competes as effectively as elements that are strong EREs in yeast. While disconcerting, this fact underscores the probability that these functional response elements would not have been selected by *in vitro* methods. However, a naturally occurring estrogen responsive region of the brain crea-

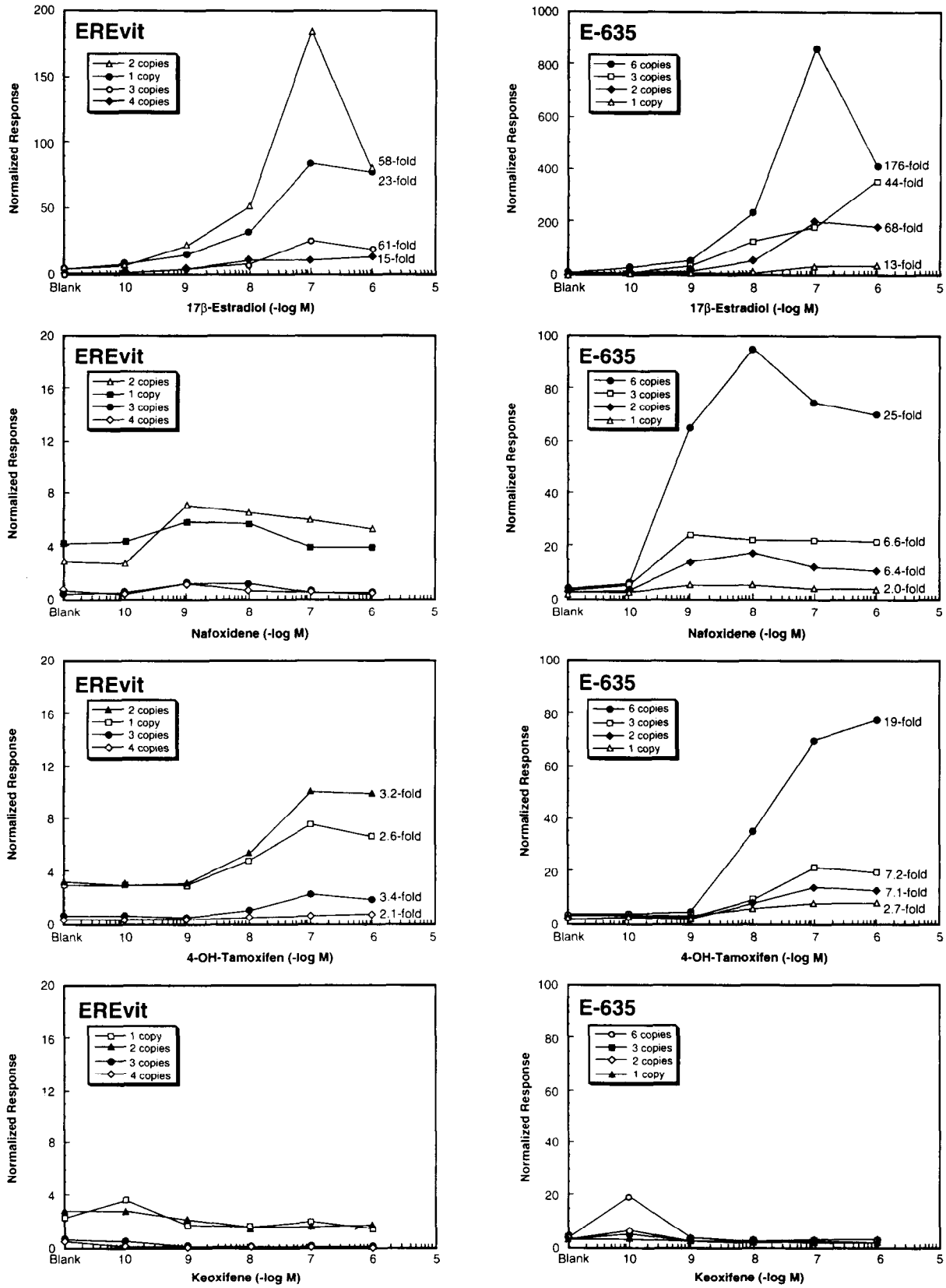


Fig. 5. Activation by Antiestrogens

The same ERE_{vit} and E-635 luciferase reporters as in Fig. 4 were cotransfected with pRShER into HepG2 cells and tested for dose response to E_2 or each of the three antiestrogens as indicated. Fold-induction values refer to the maximum response divided by the response in the absence of hormone for each curve. Induction values less than 2 are not indicated.

tine kinase promoter does not bind ER *in vitro* but can confer estrogen response to a heterologous minimal promoter in transfection indicating that *in vitro* binding capability does not always parallel biological activity (R. Mukherjee, personal communication).

Although the complex promoters of estrogen-regulated genes can be differentially regulated by anti-estrogens, reflecting the ability of the TAF1 domain to function, the sequence of the response element itself has not been reported to alter ligand specificity (30, 31). In the experiments presented here, tamoxifen and nafoxidene, which are both triphenylethylene structures, show significant partial agonist activity on one element, while a related benzothiopene, keoxifene, though structurally related, is inactive. We have not been able to detect any distinctions in protease sensitivity of hER incubated with tamoxifen or keoxifene (D. Clemm and D. P. McDonnell, manuscript in preparation). That a response element can distinguish between these two forms of ER *in vivo* is therefore quite significant and may have pharmacological consequences. It would be interesting to perform a similar screen using tamoxifen as the selecting ligand in yeast followed by a secondary screen against a number of different ligands. This might be informative to determine whether some of tamoxifen's actions *in vivo* are through ER-activation of genes distinct from the targets of the natural ligands.

We have searched the mammalian entries of GENBANK with sequences which conferred estrogen responses in HepG2 cells. In these cases we used 15 nucleotides which included an imperfect palindrome or direct repeat plus one nucleotide on either side. For example, when we searched with the sequence 5′-AGGTCA CAG GGTCAG-3′ and its inverse complement, representing the direct repeat element E-230, we found matches to the coding sequence of rat, mouse, and human calmodulin with one mismatch at the central nucleotide (T instead of A). In addition, the inverse complement sequence with one mismatch in the spacer, 5′-CTGACC CTc TGACCT-3′, was found within 100 bases upstream from the transcriptional start of the human prothrombin gene. E-322, which generated the largest E_2 response of those elements tested in HepG2 cells, matched sequences in the mouse kallikrein gene cluster, the promoter of the mouse ribosomal protein L7 gene, and the 3′-untranslated region of the mouse primase gene transcript. Although we do not know the significance of these and other matches, we believe use of this system for identifying novel target genes for estrogen action deserves further investigation.

In conclusion, we have demonstrated the utility of an alternative binding site selection method that can serve as a prototype for genetic screens of libraries made from random oligonucleotides, genomic fragments, or sequences representing actively transcribed regions of chromatin. We believe that these approaches can contribute significantly to the identification of novel targets for regulation by members of the steroid/thyroid hormone family of intracellular receptors and may be instrumental in the discovery of both target genes and ligands for the orphan members of the family. Furthermore, the results of this study give new perspective to the concept of a consensus ERE and emphasize once again that recognition of response element by receptor relies on more than a simple bimolecular protein/DNA interaction.

MATERIALS AND METHODS

Materials

Chemicals such as nutrient supplements, 17β-estradiol, and 3-aminotriazole were purchased from Sigma (St. Louis, MO). Materials for preparing yeast media were from Difco-BRL (Bethesda, MD). Restriction endonucleases and other DNA-modifying enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN). Oligonucleotides were obtained from NBI (Plymouth, MN) and IDT (Coralville, IA). The vector for the response element library, pBM2389, was generously provided by Jingdong Liu and Mark Johnston (Washington University, St. Louis, MO). Whole cell extracts from yeast expressing hER and antibodies to hER for mobility shift assays were provided by Elizabeth Allegretto of Ligand Pharmaceuticals (San Diego, CA).

Yeast Strains and Plasmids

The yeast strain *Saccharomyces cerevisiae* BJ5409 (*MATα, ura3-52, trp1, leu2Δ1, his3Δ200*) was used for response element selection. BJ5409/YEPE22 was derived by lithium acetate/polyethylene glycol transformation and selection for leucine prototrophy. The hER expression plasmid, YEPE22, was derived from YEPE10 (25) by replacing the *TRP1* locus with *LEU2* from Pm852 (a gift of T. R. Butt, Smith, Klein, and Beecham, King of Prussia, PA). pBM2389, the vector for the response element library, is a low copy plasmid containing a CEN4 origin of replication. Its other features are described below. Positive and negative control plasmids were derived by inserting an oligonucleotide based on the ERE from the *Xenopus laevis* vitellogenin gene (ERE_{vit}) (36) or an unrelated oligonucleotide (NRE) into the *Bam*HI site of the selection plasmid pBM2389. All yeast transformations were performed according to the method of Gietz and Schiestl (37).

Preparation of RREL

The following 35-base oligonucleotide was synthesized at Ligand Pharmaceuticals using a Milligen Cyclone Synthesizer by designating a mix of all four nucleotides at each random position (N): 5′-GTGTGAGATCT$(N)_{16}$ CGGATCCG-3′

The oligonucleotides were made double-stranded by mutually primed synthesis (28), digested with *Bam*HI and *Bgl*II, and inserted into the *Bam*HI site of pBM2389. A library of greater than 800,000 transformants was amplified to a limited extent to minimize any selective bias of sequences represented and DNA was prepared by Qiagen Plasmid Maxi Kit (Qiagen, Inc., Chatsworth, CA) for transformation of yeast.

Response Element Screen, Plasmid Rescue, and Secondary Assays in Yeast

The RREL was introduced into BJ5409/YEPE22 by optimized lithium acetate transformation such that up to 30,000 Trp^+ transformants were obtained for each microgram of input DNA. Over 1,000,000 transformants were screened in three separate transformations. Transformation reactions were plated immediately on minimal media plus uridine, 1 mM 3-aminotria-

zole (AT), and 10 nM 17β-estradiol (E_2) to select simultaneously for expression of the constitutive marker, *TRP1*, and activation of the conditional marker, *HIS3*. This was found to be more efficient than stepwise selection for Trp^+ followed by selection for activation of *HIS3*, and allowed transformations to be plated at a density of 4,000 to 30,000 primary (Trp^+) transformants per 100-mm plate, depending on the efficiency of the particular transformation. The number of primary transformants was estimated by plating dilutions of the transformation reactions on minimal medium plus histidine and uridine.

Surviving clones were subjected to a secondary screen for hormone dependence. Each colony was dispersed in water and 5 μl of a dilute cell suspension were plated on three conditions: 1) minimal medium plus histidine and uridine; 2) minimal medium plus uridine and 1 mM AT; and 3) minimal medium plus uridine, 10 nM E_2, and 1 mM AT. Clones that grew under conditions 1 and 3 but not under condition 2 were judged to be activated by E_2 and assumed to contain estrogen-responsive DNA elements. Plasmid DNA was rescued by preparing DNA from oxalyticase-treated yeast using an automated nucleic acid isolation unit (AutoGen, Beverly, MA) followed by transformation of *Escherichia coli*. In order to enrich for recovery of the centromeric plasmid containing the selected element, the DNA was first digested with NotI, which cuts once in the 2 μm-based YEPE22 carrying the estrogen receptor. Sequences of the estrogen-responsive inserts were determined by dideoxy chain termination methods (Sequenase, USB, Cleveland, OH).

The strength of activation of individual elements by estrogen in yeast was estimated by assessing growth of the activated clones in increasing concentrations of AT. Dilute suspensions of yeast were spotted on minimal medium plus uridine, 10 nM E_2, and concentrations of AT ranging from 0.25 mM to 16 mM. After 4 days at 30 C, growth was rated relative to growth on plates supplemented with uridine and histidine (nonselective) as −, +/−, +, ++, +++, or ++++ independently by two individuals. This process was repeated once, and the individual elements were then ranked relative to ERE_{vit} as to their activation potential in yeast.

Computer Analysis of Sequences

Thirty-six unique sequence single inserts were analzyed by computer in two steps. Although the random portion of the synthesized oligonucleotide pool was specified at 16 bases, the insert sequences excluding fixed nucleotides ranged in length from 13–16 bases. These sequences, plus one adjacent base from each flanking restriction site used for cloning, were analyzed by the wconsensus program (38, 39) to identify consensus patterns of unspecified length in unaligned sets of functionally related sequences. The output of wconsensus is a summary matrix that gives the frequency of each base at each position. In the second step, the program patser compared each complete insert sequence, including the flanking restriction sites, to the summary matrix to assign an individual matching score. In both steps each sequence and its inverse complement were analyzed.

The flanking restriction sites were omitted from the wconsensus analysis to avoid obtaining consensus patterns based on these sites. Including them in the patser analysis enabled an examination of the possible contribution to an hER recognition site based on the consensus. Eight of the 36 clones utilized bases from the flanking restriction site in the highest scoring match to the consensus pattern; seven used only one base while one used five bases (see Table 1).

Mobility Shift Assays

The preparation of whole cell extracts from yeast expressing hER has been described (40). The same oligonucleotides used to construct mammalian reporter plasmids were ^{32}P-labeled by fill-in reaction with Klenow enzyme. Binding reactions contained 15 μg extract and 1 μg poly dI-dC in 20 μl Binding Buffer [5 mM Tris pH 7.9, 15 mM HEPES pH 7.9, 5 mM EDTA pH 8, 3.5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1% Tween 20, 10% glycerol]. After 20 min preincubation at room temperature, labeled oligonucleotides were added, and incubations were continued for 30 min at room temperature. Where indicated, 200 ng antibody to the amino terminus of hER (monoclonal antibody HER-AT4-H6A1, a gift of Elizabeth Allegretto, Ligand Pharmaceuticals) was added with the oligonucleotide. For competition experiments, complementary oligonucleotides were annealed and *Bam*HI and *Bgl*II overhangs were filled in by Klenow. Each cold competing double-stranded oligonucleotide was added to the reaction 5 min before addition of the labeled ERE_{vit} oligonucleotide. Reactions were run on 5% polyacrylamide gels for 2 h at 240 V in Tris-Glycine buffer. Gels were dried and autoradiographed. For quantification, retarded complexes and free probe were excised from the dried gels for liquid scintillation counting.

Construction and Analysis of Mammalian Reporters

Oligonucleotides identical to those selected from the RREL were synthesized, phosphorylated, annealed, and inserted into *Bam*HI-digested pBL-TK-Luc, which was derived by replacing the MMTV promoter of MTV-Luc (41) with a *Hin*dIII to *Xho*I fragment from pBLCAT2 (42) containing a polylinker upstream of the −105 to +51 Herpes simplex virus thymidine kinase (TK) minimal promoter. All clones were analyzed by restriction enzyme digests to determine number and orientations of inserts, single insert clones were verified by sequencing, and multiple insert clones that were E_2-responsive were also verified by sequencing.

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah). For transfections, they were plated in phenol red-free DMEM containing 10% fetal bovine serum at a density of 2×10^5 cells per well in gelatin-coated 12-well tissue culture plates 1 day before transfection. DNA was introduced into cells by calcium phosphate-DNA coprecipitation (43). Precipitates contained 9.5 μg reporter, 0.5 μg pRShER, 5 μg pRSV-βGal, and 5 μg pGEM4 (as carrier) per ml. After 6 h exposure to DNA, hormone, at concentrations specified in the figure legends, was added to the cells in phenol red-free DMEM containing 10% charcoal-treated serum. Forty-two hours later the cells were lysed and assayed for expression of luciferase and β-galactosidase as described (43). All values represent duplicate wells and are normalized for transfection efficiency using β-galactosidase as an internal control. Unless otherwise indicated, fold-induction values are calculated by dividing the mean normalized response at 1×10^{-7} M E_2 by that in the absence of hormone.

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