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Genome-Wide Identification of High-Affinity Estrogen Response Elements in Human and Mouse

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Although estrogen receptors (ERs) recognize 15-bp palindromic estrogen response elements (EREs) with maximal affinity *in vitro*, few near-consensus sequences have been characterized in estrogen target genes. Here we report the design of a genome-wide screen for high-affinity EREs and the identification of approximately 70,000 motifs in the human and mouse genomes. EREs are enriched in regions proximal to the transcriptional start sites, and approximately 1% of elements appear conserved in the flanking regions (−10 kb to +5 kb) of orthologous human and mouse genes. Conserved and nonconserved elements were also found, often in multiple occurrences, in more than 230 estrogen-stimulated human genes previously identified from expression studies. In genes containing known EREs, we also identified additional distal elements, sometimes with higher *in vitro* binding affinity and/or better conservation between the species considered. Chromatin immunoprecipitation experiments in breast cancer cell lines indicate that most novel elements present in responsive genes bind ERα *in vivo*, including some EREs located up to approximately 10 kb from transcriptional start sites. Our results demonstrate that near-consensus EREs occur frequently in both genomes and that whereas chromatin structure likely modulates access to binding sites, far upstream elements can be evolutionarily conserved and bind ERs *in vivo*. (*Molecular Endocrinology* 18: 1411–1427, 2004)

ESTROGENS, SUCH AS 17β-estradiol (E2), are steroid hormones produced mainly by the ovary and act by endocrine, paracrine, and possibly autocrine mechanisms on a number of target tissues. Their actions on the reproductive, cardiovascular, and central nervous systems and on bone (1–4) are mediated by two estrogen receptors, ERα and ERβ (4, 5), members of the nuclear receptor superfamily of ligand-inducible transcription factors (6–9). A central DNA binding domain, which corresponds to the conserved C region (10, 11), is responsible for primary sequence recognition and cooperative binding by receptor dimers on elements containing two appropriately spaced copies of specific recognition motifs. The ligand binding domain contains a strong dimerization interface that stabilizes receptor homodimers or heterodimers (12–14) and enhances binding to imperfect motifs and even to single copies of recognition motifs (11, 15–17). However, ERs bind with highest affinity to 15-bp palindromes composed of PuGGTCA motifs separated by three variable bp (17–19). These estrogen response elements (EREs) are recognized by ERs with high specificity, because other nuclear receptors bind either different motifs or similar motifs with different spacing and/or orientation (7, 19–21). Studies using chromatin immunoprecipitation approaches have demonstrated that ER binding to EREs is hormone inducible *in vivo* (22–25) and results in the ordered recruitment of a series of coactivator complexes, leading to histone acetylation, chromatin

Abbreviations: ADORA1, Adenosine A1 receptor; C3, complement component 3; CASP7, caspase7, apoptosis-related cysteine protease; CDS, coding sequence; ChIP; chromatin immunoprecipitation; COX7A2L, cytochrome c oxidase subunit VIIa polypeptide 2 like; CTSD, cathepsin D; E2, 17β-estradiol; EBAG9, estrogen receptor binding site associated, antigen 9; EFP, estrogen-responsive finger protein; ER, estrogen receptor; ERE, estrogen response element; FGF, fibroblast growth factor; GAD2, glutamate decarboxylase 2; GAPD, glyceraldehydes-3-phosphate dehydrogenase; GREB1, gene regulated by estrogen in breast cancer protein; IGFBP4, IGF binding protein 4; KHDRBS3 (T-STAR/ETOILE), KH domain containing, RNA binding, signal transduction associated 3; LY6E, lymphocyte antigen 6 complex, locus E; NRIP1 (RIP140), nuclear receptor interacting protein 1; OVGP1, oviductal glycoprotein 1; P-PolII, phosphorylated RNA polymerase II; RBM, RNA-binding motif; RNF14, ring finger protein 14; SCNN1A, sodium channel, nonvoltage-gated 1α; TBP, TATA box binding protein; TERT, telomerase reverse transcriptase; TESK1, testis-specific kinase 1; TFF1/pS2, trefoil factor 1; TSHB, TSHβ; VitA2, *Xenopus* vitellogenin A2; ZNF147, zinc finger protein 147.

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remodeling, and enhanced recruitment of the basal transcription machinery (26–33).

Surprisingly, only a few near-consensus EREs have been characterized to date in the promoters of E2-regulated human or mouse genes. This probably reflects, in part, the fact that ERs can mediate estrogenic regulation through multiple mechanisms, including tethering to DNA via protein-protein interactions with other transcription factors (19, 34, 35), or so-called "nongenomic" effects, which affect gene expression by modulating the activity of upstream components of various intracellular signaling pathways (36–38). The paucity of known near-consensus EREs may also result from the fact that their presence is usually investigated only in relatively proximal sequences of E2-responsive genes. However, with sequences of the human and mouse genomes now available, genome-wide screening strategies can be used to expand the repertoire of potential high-affinity ER-binding sites.

In this study, we have sought to identify high-affinity EREs in the human and mouse genomes and to determine whether these elements represent *bona fide* ER binding sites *in vivo*. Indeed, little is known about the effect of chromatin structure or promoter context on ER binding *in vivo*. In addition, it is not clear how far from transcription start sites EREs can be positioned to recruit ERs and play a role in regulating gene expression. Our characterization of high-affinity EREs in the human and mouse genomes will greatly facilitate the study of the mechanisms modulating accessibility of ERs to their binding sites and of the subsequent events leading to transcriptional regulation by estrogen. This analysis will also provide a powerful resource for researchers analyzing the molecular events underlying the broad-ranging physiological actions of estrogens.

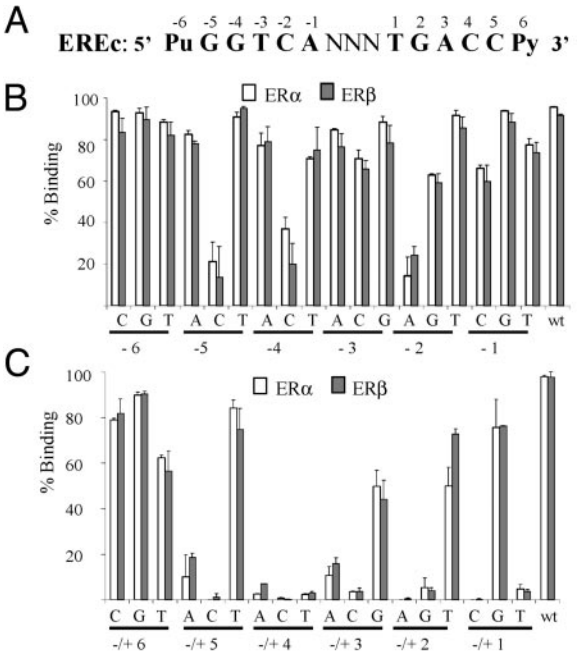

Fig. 1. *In vitro* Binding of ERs to Consensus and Near-Consensus EREs

A, Consensus ERE with base numbering used in this study. B, Binding of human ERα or ERβ to response elements derived from the vitellogenin A2 (VitA2) ERE carrying a single replacement in one of the two arms of the palindrome. C, Binding of human ERα or ERβ to response elements derived from the VitA2 ERE carrying two symmetrical replacements. The percentage of probe found in a specific complex with human ERα or ERβ was quantified by phosphor imager and is shown with the corresponding SEM values.

RESULTS

Identification of Sequences Corresponding to High-Affinity ER Binding Sites *in Vitro*

Palindromic response elements composed of two PuGGTCA motifs separated by 3 bp, such as the one found in the *Xenopus* vitellogenin A2 (VitA2) promoter, represent the highest affinity binding sites for ERs *in vitro* (Fig. 1A) (17–19). Surprisingly, only three perfect palindromic EREs have been identified to date in the vicinity of E2-regulated genes in the human genome. These genes, EBAG9, COX7A2L, and EFP/ZNF147, were cloned by screening of a CpG island genomic library for binding to ERs (39–41). In addition, a few near-consensus EREs have been characterized in human E2-responsive genes (see Table 1 for EREs diverging from consensus at one or two positions). A systematic analysis of single nucleotide substitutions in gel shift assays indicates that all elements with one variation still bind ERs *in vitro* (Fig. 1B). However, some replacements were more detrimental than others (−5C, −4C, −2A), reflecting steric or charge clashes between specific ER amino acids and bases of the variant EREs (Nguyen, D., G. Pesant, S. Cheinberg, and S. Mader, in preparation). Note that, as expected from the high degree of homology between the DNA binding domains of ERα and ERβ (90% in region C as defined in Ref. 10), all mutations had a similar impact on complex formation by the two receptors. Introduction of symmetrical replacements in both arms of the palindrome generally affected ER binding much more drastically than when a unilateral change was introduced, although a few replacements were well tolerated even when present in both arms (Fig. 1C). Interestingly, examination of the known natural elements containing two replacements indicates that they all contain one change with mild effect on affinity (−6Py/+6Pu or −1G/+1C; Fig. 1, B and C and Table 1) combined with another replacement.

Screening the Human And Mouse Genomes for High-Affinity EREs

Based on the above-described observations, we decided to identify all occurrences of EREs conforming to consensus, or with one variation, or with two vari-

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Table 1. EREs in the Human Genome and Corresponding Elements in the Mouse Genome

HUMAN GENOME					MOUSE GENOME				
Symbol	UniGene #	ERE sequence	Pos.	Ref	Symbol	UniGene #	ERE sequence	Pos.	Ref
PREVIOUSLY CHARACTERIZED EREs									
Perfect elements									
EFP[a]	Hs.1579	GGGTCATGGTGACCC	+23,088	39	Trim25[b]	Mm.248445	AGGgCAGGGTGACCT	+17,499	
EBAG9	Hs.9222	GGGTCAGGGTGACCT	+6 (-64)	40	Ebag9	Mm.287896	GGGTCAGGGTGACCT	-4886	
COX7A2L	Hs.423404	GGGTCAAGGTGACCC	+383 (+443)	40	Cox7A2l	Mm.30072	GGGTCACCTTGACCC	+336	
1 mismatch									
OVGP1	Hs.1154	GGGTCACTGTGACtC	-156 (-170)	74	Ovgp1	Mm.5110	cGGTCATTGTGACtC	-110 (-110)	75
AGT	Hs.19383	AGGgCATCGTGACCC	-10 (-25)	76	Agt	Mm.285467	AGGTCACCTTGACCC	-2379 (-2742)	77
2 mismatches									
pS2 / TFF1	Hs.350470	AGGTCACGGTGgCCa	-392 (-406)	78	Tff1	Mm.2854	ctGTCATCTTGtCCa	-462*	
OXT	Hs.113216	cGGTgACCTTGACCC	-166 (-168)	79	Oxt	Mm.16745	cGaTgACCTTGACCC	-210*	
BCL2	Hs.79241	tGGTCcACCTGACCC	+319 (+276)	80	Bcl2	Mm.233518	tGGTCcATCTGACCC	+279	
F12	Hs.1321	AGGgCAGCTTGACCa	-30 (-47)	81	F12	Mm.42224	GaGcaAGCTTGACCa	-75*	
TERT	Hs.439911	tGGTCAGGCTGAtCT	-2687 (-2677)	82	Tert	Mm.10109			
C3	Hs.284394	AGGTggCCCTGACCC	-226 (-237)	83	C3	Mm.19131	AtcTggCCTTGACCC	-208*	
CTSD	Hs.343475	GGGcCgGGCTGACCC	-125 (-270)	84	Ctsd	Mm.231395			
LTF	Hs.437457	AGGTCAAGGcGAtCT	-81 (-358)	85	Ltf	Mm.282359	AGGTCAAGGTaACCC	-340 (-342)	86
OTHER ELEMENTS FOUND IN ESTROGEN TARGET GENES WITH KNOWN EREs									
C3	Hs.284394	GGGTCtTTGTGACCg	-9210						
CTSD	Hs.343475	GGGcCACCATGACCC	-8763		Ctsd	Mm.231395	AGGcCAATCTGACCT	-8157	
TERT	Hs.439911	GGGaCAGAGTGACCC	-5663						
OXT	Hs.113216	AGGTCAGCCTGACCg	-2465		Oxt	Mm.16745	tGGTCACCGTGAtCC	-5424	
		AGGTCATCGTGcCCa	-2596						
LTF	Hs.437457	AGGTgAGTCTGACCa	-1166						
OVGP1	Hs.1154	GGGTCcCTCTGACCT	+200		Ovgp1	Mm.5110	AGGTCAGGATGtCtg	+866*	
COX7A2L	Hs.423404	AGGTCAGGATGtCCa	-5246						
EFP[a]	Hs.1579	AGGgCAGGGTGACCT	+22,583		Trim25[b]	Mm.248445	AGGgCAGGGTGACCT	+17,499	
SELECTED NEW ELEMENTS (used for gel shift and/or ChIP experiments)									
ADORA1	Hs.77867	AGGTtAGGGTGACCT	-1171	54					
		GGGTCAGGGTGAaCT	-486						
		GGGTCgGGGTGAaCT	-392						
		GGGTCAGGGTGAaCT	-312						
CASP7	Hs.9216	GGGTCAGGGTGAaCT	-272	87	Casp7	Mm.298737	GtGTCAACTTGACCa	+1458	
		GGGTCAGGGTGAaCg	-232						
		tGGTCAGGGTGAaCT	-195						
		GGGTCAGGGTGAaCT	-141						
GAPD	Hs.169476	AGGaCATCGTGACCT	-738	88	Gapd	Mm.288146	AGGTCAGGATGcCCT	-745	
		AGGTCAAAATGACCT	-21,207						
GREB1	Hs.438037	AGGTCATCATGACCT	-9517	89	Greb1[c]	Mm.218957	AGGTCgCGCTGACCC	-7666	
		GGGTCATTCTGACCT	-1583				AGGTCAGGATGACCC	-3289	
IGFBP4	Hs.1516	AGGTCATTGTGACaC	-4124	54	Igfbp4	Mm.233799	AGaTCACGCTGACCT	-6697	
LY6E	Hs.77667	GGGaCAAGATGACCT	-499	54					
NRIP1	Hs.155017	GGGTCACTTTGACCC	-706	54	Nrip1	Mm.20895	AGGTCATTTTGACCC	-636	
SCNN1A	Hs.446415	AGGTCAGCCTcACCC	-559	54					
GAD2	Hs.231829	AGGTCgCAGTGACCT	-564		Gad2	Mm.4784	AGGTCACAGcGACCT	-349	
TSHB	Hs.406687	AGGTCAGCTTGACaT	-4099	59	Tshb	Mm.110730	AtGTCAAACTGACCT	-535	

The sequence of previously characterized consensus EREs or elements with one or two mismatches in the human genome are shown with the corresponding references. Positions of the EREs with respect to the 5′-ends of mRNAs are as indicated in genomic data files, and may differ slightly from the positions (*in brackets*) indicated in the references. All other EREs in this table (selected examples from Table 3) were identified through our genomic screens (except for a few elements in mouse orthologs, identified by *asterisks,* which due to a higher number of mismatches were identified manually). References provided for novel EREs describe estrogenic regulation of the corresponding gene.

[a] EFP is now called ZNF147 in the human genome database.

[b] Trim25, the EFP/ZNF147 mouse homolog, corresponds to LOC217069.

[c] Greb1, also referenced as Greb1-pending, corresponds to 5730583K22Rik.

ations including either −6Py/+6Pu or −1G/+1C. This defines 588 different sets of 15-bp sequences with random 3-bp spacers; 71,119 elements were identified in the human genome and 65,012 were identified in mouse. Note that the elements containing CpG dinucleotides were found to be largely underrepresented in both genomes (data not shown), consistent with the lower genomic frequency of this dinucleotide due to deamination of the methylated C (42). In particular, symmetrical palindromes containing PuGGTCG motifs (two CpGs), which represent good ER-binding sites (Fig. 1C), were found only 14 times in the human genome, whereas consensus palindromes were represented 891 times. The distribution of consensus EREs among the chromosomes is generally consistent with chromosomal size in both genomes (Fig. 2, A and B). Whereas perfect EREs were not clustered together, several imperfect palindromes were found to be part of tandem repeat elements at different chromosomal locations in the human and mouse genomes, increasing the frequency of these elements (Bourdeau, V., Y. Nagai, and S. Mader, in preparation).

The numbers of near-consensus EREs found in the human and mouse genomes suggest that functional EREs should be found in the vicinity of genes more frequently than reported to date. Note that most previously identified EREs are located within 0.5 kb of transcriptional start sites (see Previously Characterized EREs in Table 1, with the exception of the TERT and the EFP/ZNF147 EREs). It is not clear whether this bias reflects a functional requirement for proximal

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EREs or the fact that searches for EREs in E2 target genes usually focus on promoter sequences immediately upstream of the transcriptional start site. Using a cut-off distance of −10 to +5 kb from transcriptional start sites, we found EREs in the vicinity of 17,353 transcriptional start sites in human, and 15,284 in mouse (see web site at URL: http://mapageweb.umontreal.ca/maders/eredatabase/) Note that the number of genes containing EREs is slightly smaller (12,515 in human and 11,810 in mouse), as there can be several mRNAs and more than one ERE per gene. We further examined the distribution of EREs in the −10 to +5-kb region with respect to the 5′-end of mRNAs (whenever several mRNAs are mapped, distances were calculated to the most 5′-initiation site). Note that EREs found close to more than one gene were counted several times for characterization of the distribution with respect to transcriptional start sites. Surprisingly, EREs were more abundant in the 0 to +1-kb region around transcriptional start sites in both genomes (excess of ~25%), whereas the number of elements found upstream of −1 kb or downstream of +2 kb were usually near or below the average number of near-consensus EREs found per kb between −10 and +5 kb (Fig. 2C). Compared with the frequency expected from random distribution of all EREs found in the genome, the number of individual EREs in the −1 to +2-kb region of 5′-mRNAs was higher than expected by 64% in human and 29% in mouse (data not shown).

These data suggest that the distribution of near-consensus EREs reflects mostly a random distribution of these sequences in the different chromosomes, with enrichment in the close vicinity of transcriptional start sites. The large total number of EREs located in the vicinity (−10 to +5 kb) of transcriptional start sites may suggest that these elements can mediate regulation of a much larger fraction of the genome in different estrogen target tissues than previously recognized. However, some of these elements may not represent binding sites *in vivo*, possibly because of chromatin accessibility, and/or may not participate in transcriptional regulation.

Identification of EREs Conserved in Human and Mouse

Near-consensus EREs previously identified in human genes often have counterparts at similar positions in their mouse orthologs (Table 1). Because conservation of newly identified EREs between the two species may indicate a functional role of these elements, we searched for the presence of EREs located in known human and mouse orthologs with less than 2 kb difference in distance from their respective transcriptional start sites (see *Materials and Methods*). EREs in 660 different pairs of orthologs were thus identified (supplemental Table 1; published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). As expected from the distribution of EREs in the vicinity of genes, conserved EREs were also more abundant in the 0 to +1-kb region after the transcription start site (Fig. 2C; note that this analysis was intentionally limited to the −8 to +3-kb region. The numbers of conserved EREs found between −10 and −8 kb or +3 and +5 kb would appear artificially low, because a conserved ERE found at a distance of 2 kb or less in the other species may not be included in the −10 to +5-kb window from the transcriptional start site). However, conserved EREs were proportionately more represented in the 0 to +2-kb region than bulk EREs, whereas elements in the −5 to −8-kb region were markedly less represented (Fig. 2C). Nevertheless, we note that a significant number of conserved EREs mapped between −5 and −10 kb of the transcriptional start sites (24.6%). It remains possible that some of these distal EREs may be located closer to another gene, or even within an open reading frame, accounting for their conservation.

To investigate whether location of EREs in coding sequences (CDS) may account for their conservation, we characterized the position of these elements for a subset of conserved EREs, *i.e*. those that were identical in sequence between the two orthologs (Table 2). A total of 41 elements were found in the vicinity of 47 genes. Of these elements, 14 were located fully and four were found partly in a CDS (Table 2 *bottom* and *middle*, respectively), whereas 23 were not found in any annotated open reading frames (Table 2, *top*). More than half of these (12 EREs) were located between −1 to +1, another seven between −1 and −10 kb, and another four between +1 and +5 kb. Thus these results indicate that EREs that are conserved independently of their presence in a CDS are more likely to be found close to the 5′-end of mRNAs, although some elements are still located at several kb kilobase pairs from the start sites. Furthermore, the strong conservation of these elements, which is unlikely to result from chance alone, may indicate a role in the regulation of the neighboring genes.

Three known E2 target genes were present in the set of genes containing perfectly conserved EREs. The EBAG9 and COX7A2L genes contain previously identified EREs (Table 1). The fibroblast growth factor (FGF) 9 gene is also a known E2 target (43), although the presence of a near-consensus ERE in the proximal promoter was not previously reported. FGF9 mediates the proliferative action of estrogen on stromal endometrial cells (43). It also plays a role in glial cell development (44) and testicular embryogenesis (45). Its functional inactivation in mouse leads to male-to-female sex reversal (45). Interestingly, several other genes containing fully conserved EREs in their flanking sequences (Table 2) are linked to testicular function. TESK1 is expressed in testicular germ cells in a developmentally regulated manner and is thought to play a role in spermatogenesis around the stages of meiosis and/or early spermiogenesis (46, 47). KHDRBS3 (T-STAR/ETOILE) is a SAM68 homolog that is ex-

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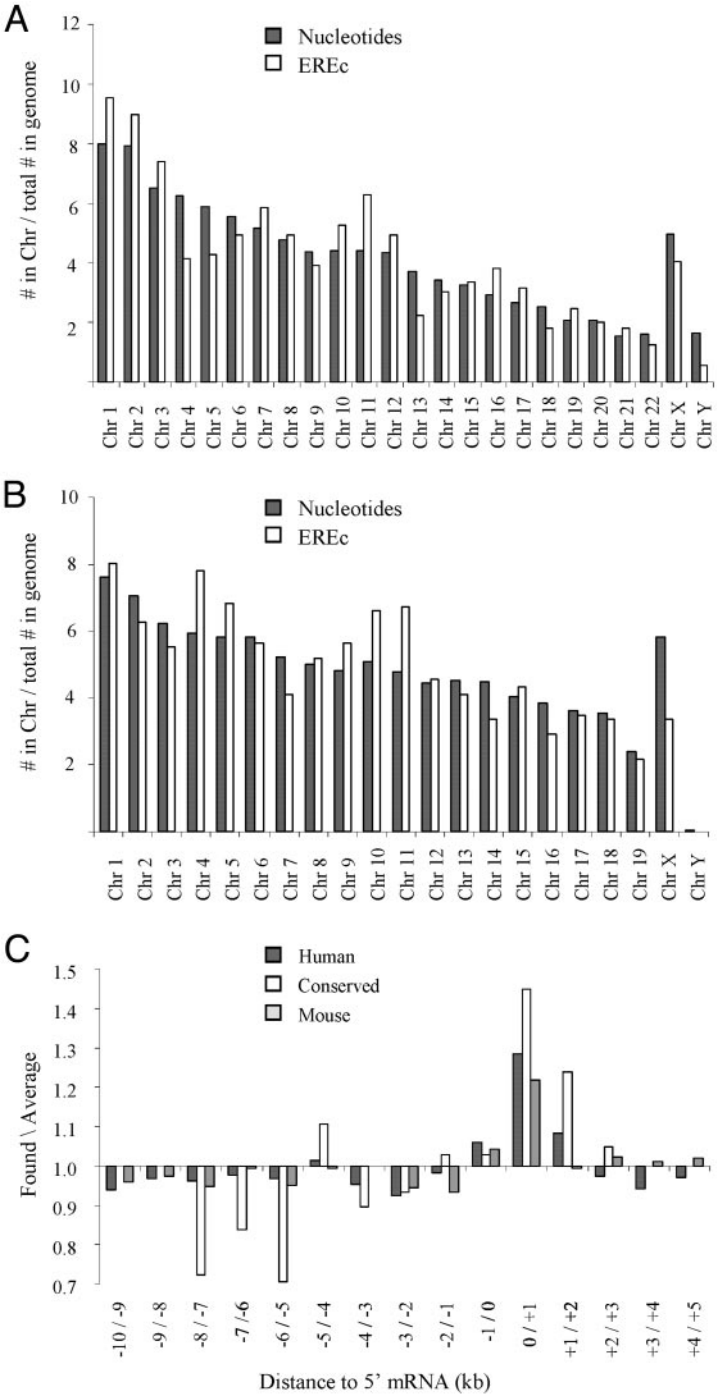

Fig. 2. Distribution of EREs in the Human and Mouse Genomes

A, Distribution of consensus EREs on human chromosomes. The percentage of nucleotides in individual chromosomes is plotted, along with the percentage of total response elements found per chromosome. B, Distribution of consensus EREs on mouse chromosomes. C, Distribution of EREs found between −10 and +5 kb of mRNA 5′-ends. The ratio between the number of EREs found and the expected number assuming a random distribution of these elements in the −10 to +5 interval was calculated for consecutive 1-kb segments of flanking sequences in the human (*dark bars*) and mouse (*gray bars*) genomes. For the elements conserved between the two genomes (*white bars*), the distribution was characterized only between −8 to +3 kb in order not to introduce a bias in the 2-kb constraint in relative distances between the two species.

pressed primarily in the testis and interacts with RBM, an RNA-binding protein implicated in spermatogenesis (48). FGF8 is an androgen-induced gene expressed in testes specifically in prespermatogonia during fetal d 16–17 (49). Finally, RNF14 (ARA54) is an androgen receptor coactivator expressed in testis and prostate

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Table 2. EREs with Identical Sequences in Human and Mouse Orthologs

Human Gene Name*	UniGene #	ERE Sequence	Hs Dist. ERE-mRNA	Mm Dist. ERE-mRNA	Diff. Dist. Mm-Hs ERE-Gene	Diff. Dist. Mm-Hs ERE-mRNA	Diff. Dist. Mm-Hs ERE-CDS
EREs located in a non-coding sequence (CDS)							
HOXD13	Hs.152414	tGGTCAAAATGACCC	-8865	-8719	146	146	257
KHDRBS3	Hs.13565	AGGTCATGATGcCCA	-7491	-6925	552	566	724
LDB1	Hs.26002	GGGTCACTTTGACaC	-5679	-5682	5869	3	520
RNF14[1]	Hs.170926	AGcTCAAGTTGACCC	-5410	-6671	3522	1261	532
HOXC12	Hs.381267	AGGTCtTTCTGACCT	-5201	-5113	88	88	88
PCDH12[1]	Hs.115897	GGGTCAACTTGAgCT	-4640	-5554	872	914	10
KIF24[2]	Hs.201179	tGGTCAGACTGACaT	-2923	-3246	323	323	7425
PDE8A	Hs.78746	ctGTCAGTGTGACCC	-2583	-1914	799	669	674
NDRG2	Hs.243960	GaGTCATCCTGACCC	-1347	-1900	178	553	21
TESK1	Hs.79358	GGGTCACAATGACta	-991	-981	1457	10	260
NKX6-1	Hs.347185	GGGTCAGTTTGgCCa	-963	-410	353	553	82
PHF5A[3]	Hs.273234	GGGaCgCCTTGACCT	-659	-807	148	148	153
SLC10A2	Hs.194783	ctGTCACTTTGACCT	-382	-753	542	371	177
FGF9	Hs.111	tGGTCATTCTGAtCT	-204	-237	2338	33	6
EBAG9	Hs.9222	**GGGTCAGGGTGACCT**	6	-4886	226	4892	5816
FGF8	Hs.57710	GGGaCgGCGTGACCC	67	153	128	86	67
CNNM4	Hs.175043	tGGTCAACGTGAgCC	215	57	158	158	42
ACO2[3]	Hs.300463	AGGTCAAGGcGtCCC	219	203	122	16	2
COL4A2	Hs.407912	AGGTgAGAGcGACCC	243	284	41	41	26
EDG2	Hs.75794	AGGTCAGTGcGcCCC	257	432	518	175	16676
COX7A2L	Hs.423404	**GGGTCAAGGTGACCC**	381	336	7812	45	16
UBE2R2	Hs.11184	AGGgCAGGGTGACCT	755	715	279	40	20
NFKBIA	Hs.81328	AGGTCtTTATGACCC	1233	1107	29	126	89
NR1D1	Hs.276916	GGtTCACCCTGACCa	2314	1673	88	641	89
NUDT2[2]	Hs.429142	AtGTCAGTCTGACCa	2617	2981	364	364	2470
HNRPDL	Hs.372673	GtGTCACCCTGACCa	4639	3226	518	1413	957
EREs located partially in a coding sequence (CDS)							
B4GALT7	Hs.54702	AGGTCATCCTGgCCC	-6523	-5409	1105	1114	1122
SIRT7[4]	Hs.184447	GGGTtATGAcGACCC	-5012	-4845	378	167	2786
SLC25A12	Hs.353282	cGGTCAAGGTGACtC	27	77	216	50	0
CD7	Hs.36972	AGGTCAGTGTGAgCC	1313	1751	357	438	385
MAFG[4]	Hs.252229	GGGTtATGAcGACCC	4516	9	969	4507	0
EREs located in a coding sequence							
DEF6	Hs.15476	AGGTCAGCCTGtCCC	-4108	-4746	652	638	651
ARHGAP4[5]	Hs.3109	AGGTCAGCGaGACCa	-3795	-5798	1885	2003	1961
ZNF142[6]	Hs.80526	cGGTCAGCCaGACCC	-2345	-2383	5	38	728
GPR73L1	Hs.375029	tGGTgATTATGACCT	103	94	3818	9	9
ENDOG	Hs.420106	GGGcCgGCCTGACCC	170	64	473	34	140
EDN3	Hs.1408	GGcTCACAGTGACCT	220	376	333	106	0
SFRS2	Hs.73965	tGGaCAACCTGACCT	221	219	595	2	0
RARG	Hs.1497	GGGcCAGCCTGACCT	288	4880	232	4592	0
TPBG	Hs.82128	tGGTgAGCCTGACCT	820	2324	448	1504	18
FZD6	Hs.114218	GGGTCATTATGACCa	1355	270	176	1085	0
PAX9	Hs.132576	GGGTCATTAcGACtC	1603	1309	267	294	94
AMBP	Hs.76177	AGaTCAGCATGACCa	1692	1545	147	147	140
BCS1L[6]	Hs.150922	GGGTCtGGCTGACCg	1847	2020	448	173	65
PRSS8	Hs.75799	AGGTCAGCATcACCT	2528	2209	268	319	199
AP1G2	Hs.343244	tGGcCAACCTGACCC	4610	3756	474	854	98
ARD1[5]	Hs.433291	AGGTCAGCGaGACCa	4961	4916	122	45	100

Human and mouse EREs with identical sequences were selected from the compilation of EREs located in known human (Hs) and mouse (Mm) orthologs with less than 2 kb difference in either their respective distances to the beginning of genes, mRNA, or coding sequences (see supplemental data for a full list). Elements that are fully conserved in sequence in the two orthologs were further analyzed to determine whether they are part of an annotated CDS (*bottom*) or not (*top*). Four additional elements were overlapping a CDS in at least one species (*middle*). Note that some elements are listed twice, as they are found in the vicinity of two genes and are shown in the orientation corresponding to each gene. Number pairs identify identical EREs. Elements in *bold* are known EREs.

*, Mouse gene names are similar to the human names except for NDRG2 and ZNF142, whose counterparts are Ndr2 and Zfp142, respectively.

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(50). Although estrogenic regulation of these genes remains to be demonstrated, these observations are noteworthy in view of the fact that estrogen deficiency in aromatase knockout models or genetic ablation of the ERα gene leads to defects in spermatogenesis and male sterility (51–53).

Proximal and Distal EREs in Known Estrogen-Responsive Genes

To assess the potential of EREs found in this screen to bind ERs, we first compared our list of elements lying between −10 and +5 kb of the 5′-ends of known genes to published data on E2-up-regulated genes identified through expression studies in different human target tissues. This generated a list of 345 elements close to 236 genes (Table 3). Several of these genes were identified from a gene array experiment performed in the presence of cycloheximide, suggesting direct regulation (54). Interestingly, several promoters already shown to contain functional EREs were found to include additional elements, sometimes located more distally (Table 3; see also selected elements in Table 1). When assayed for binding to ERα in gel shift assays, several of these novel elements bound similarly or even better than those previously characterized (Fig. 3A, OVGP1, CTSD, and TERT). Of interest, the mouse ortholog of the CTSD gene did not contain a near-consensus element corresponding to the known human proximal element, but our search identified a new element located 8 kb upstream that is conserved between human and mouse. Another example of a gene containing conserved distal EREs is the EFP/ZNF147 gene. Our screen (performed in this case with a larger cutoff) identified a conserved imperfect element in the 3′-untranslated region of the mouse and the human orthologs, different from the consensus ERE previously reported in the human gene (Table 1). The presence of distal elements in E2-regulated genes suggests that they may participate in the estrogenic regulation of these genes.

Some estrogen-inducible genes for which EREs had not been characterized were found to contain several high-affinity elements. For instance, the GREB1 (gene regulated by estrogen in breast cancer) gene contains three consensus EREs located at −1.5 kb, −9.5 kb, and −21 kb (Table 1). In addition, the human CASP7 gene was found to contain seven tandem repeats of a near-consensus ERE within the proximal 500 bp (Table 1). The presence of multiple elements raises the possibility of synergism at the level of either DNA binding or transcriptional activation in the regulation of these genes by estrogen and may suggest propagation of chromatin changes induced by recruitment of ER coactivators over a large distance.

Functional Analysis of Newly Identified EREs

To assess whether EREs selected among those found in the promoters of E2-responsive genes (Table 1, *bottom*) bind ERα *in vitro* with the predicted high affinity, gel shift assays were performed with elements in their natural context (15 bp core with 6 bp of flanking sequences; Fig. 3B). Although flanking sequences slightly modulated binding compared with the VitA2-derived elements carrying the same core ERE sequence, all elements were found to bind ERα efficiently. Chromatin immunoprecipitation (ChIP) experiments were performed next to monitor *in vivo* recruitment of hERα on the newly identified EREs. These experiments were conducted in ERα-positive MCF7 cells, ERα-negative MDA-MB231 cells, or MDAMB231 cells stably transfected with hERα (MDA::hERα). As positive controls for ERα recruitment, both the pS2/TFF1 and complement component 3 (C3) elements (Table 1) were used and, as expected, demonstrated E2-dependent recruitment of ERα on their proximal promoters (Fig. 4). We investigated binding of ERα to 14 EREs found in 10 genes known to be regulated by estrogen in breast cancer cells (ADORA1, CASP7, CTSD, GAPD, GREB1, IGFBP4, LY6E, NRIP1, SCNN1A, TERT; Table 1 and references within). In the two ERα-expressing cell lines, E2-induced ERα binding was observed on 13 of 14 of the elements (which include both conserved and nonconserved EREs; see Table 1), except for the GAPD ERE where binding was restricted to MCF7 cells (Fig. 4). No binding was observed in the parental MDA-MB231 cells, or with preimmune rabbit IgG, on any of the promoters tested. Consistent with the published regulation of these genes by estradiol in breast cancer cells, association of the fragments of genomic DNA around the transcriptional start sites with the TATA box binding protein (TBP) and the phosphorylated polymerase II (P-PolII) was induced in the presence of E2 (Fig. 4). Notably, in addition to the proximal elements (ERE2) found in the CTSD and TERT genes, both upstream elements (ERE1) bound ERα. Binding of both the GREB1 EREs at −9.5 kb (ERE2) and at −1.5 kb (ERE3) was also observed, but the most upstream ERE (−21 kb, ERE1) did not bind despite its consensus ERE sequence. This may suggest that this element was not accessible for ER binding, possibly because of chromatin organization.

Binding of ERs to EREs located in two genes that are not known to be estrogen targets in breast cancer cells was also investigated (GAD2, TSHB, Fig. 4). In contrast to results obtained with other ERE-containing promoters, ERα binding was not detected on the GAD2 ERE. Moreover, no binding of TBP or P-PolII was observed in the absence or presence of hormone. GAD2 catalyzes the synthesis of the inhibitory neurotransmitter γ-amino butyric acid, and its expression is restricted to neural tissues and pancreatic islets (55). Its regulation by estrogen has been described in rat and goldfish brain (56, 57). However, no expression of GAD2 was detected in breast cancer cells by RT-PCR analysis (data not shown). Absence of ER binding to these sites in breast cells may reflect limited access due to chromatin conformation. On the other hand,

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Table 3. EREs Found in Up-Regulated Estrogen Target Genes

Gene	UniGene #	Pos.	ERE Sequence	REF
ADCY9	Hs.20196	-3928	tGGTCATACTgACCT	132
		1856	AaGTCACACTGACCC	132
ADFP	Hs.3416	147	AGGTCAGGTTGACCa	133
ADORA1	Hs.77867	-3824	AGGTCAAGATGtCCT	54
		-1171	AGGTtAGGGTGACCT	
AGT	Hs.19383	-10	GGGTCACGATGcCCT	133
AHSG	Hs.324746	-3185	GGaTCAAACTGACCC	90
AIM1	Hs.422550	363	GGGTCACGCcCACCC	91
AKR1C4	Hs.177687	1657	GGGTCAATTTaACCa	91
ALPI	Hs.37009	-9620	tGGTCACTCTgACCT	133
		-419	AGGTCtGGCTGACCC	
		2170	AGGaCACGCTGACCC	
ALPP	Hs.284255	-6033	GGaTCACAGcGACCC	92
AR	Hs.99915	-599	GGGTagAAATGACCT	93
ARHC	Hs.179735	-9916	AGGTCACAGcCACCT	91
		-9815	AGGcCAGAATGACCa	
		-3315	GGcTCAGACTGACCC	
ART3	Hs.24976	-9809	GGGTCACAGcaACCT	94
ASS	Hs.160786	585	GGGTgACTCTGACCT	95
ATP2B2	Hs.89512	-2600	GGGTCATGTcCACCT	94
		-358	GGGTCAGGGTGcCCC	
AXIN1	Hs.512765	-6019	tGGTCAGGAgGACCC	54
		-5940	AGGTCAGTGTGACtg	
B4GALT1	Hs.396798	-3158	AGGTgAAGCTGACCC	54
		3242	AGGTCACACTaACCC	
BDKRB2	Hs.250882	-3298	tGGTCtGTCTGACCT	133
BCL2	Hs.79241	319	GGGTCAGGTgGACCa	80
BCL2L11	Hs.84063	-6090	GGGTgATCTcGACCT	94
BICD1	Hs.412020	929	AGGTCACCCTGACta	94
BIRC3	Hs.127799	-6658	AGGgCATATTGACCT	133
BIRC5	Hs.1578	-6502	tGGTgACAGTGACCT	132
		4986	GGcTCACTGcGACCT	132
BTEB1	Hs.150557	-9843	AGGTCACTGcaACCT	133
BUB1	Hs.287472	-4500	AGGTCATGCgGACCT	132
		-3360	AGGcCAGCCTGACCa	132
C11orf8	Hs.432000	4209	AGGTgATATTGACCa	133
C3	Hs.284394	-9210	GGGTCtTTGTGACCg	133
		-226	GGGTCAGGGccACCT	133
CALCR	Hs.640	927	GGGTCAGTATGACag	132
CALM2	Hs.425808	-8603	cGGTCACTGTaACCT	95
		1287	AcGTCAAGAcGACCT	
CASP7	Hs.9216	-486	GGGTCAGGGTGAaCT	87
		-392	GGGTCgGGGTGAaCT	
		-312	GGGTCAGGGTGAaCT	
		-272	GGGTCAGGGTGAaCT	
		-232	GGGTCAGGGTGAaCg	
		-195	tGGTCAGGGTGAaCT	
		-141	GGGTCAGGGTGAaCT	
CASP8	Hs.243491	1374	AGGcCATCTTGACCT	133
CAV1	Hs.74034	-6690	tGGcCAGGCTGACCT	95
		2616	GGGTCAGCCTGcCCT	
CBFA2T3	Hs.110099	-2378	GGGTCAGCCTGACgC	132
CCBP2	Hs.24286	-1509	AGGTCtTTATGACCT	132
CD33	Hs.83731	82	GGGtCaAGCTGACCC	133
CD34	Hs.374990	-8306	AGGTCAAATTcACCC	54
CD69	Hs.82401	-9834	GGGTCAACATGACtg	94
CD86	Hs.27954	-7718	GGGTggGAGTGACCC	94
CDC27	Hs.406631	-6134	tGGTCAGGCTGgCCT	133
CDC6	Hs.405958	2208	AGGTCACTCTGAaCT	91,87
CDR1	Hs.446675	-9550	tGGTCACCATcACCT	94
CEBPB	Hs.99029	-3454	GGGTCAAATTGAaCa	91
CITED2	Hs.82071	2486	AGcTCATCATGACCT	94
CNR1	Hs.75110	-3061	GGGcCAGTGTGACCT	94
		2628	AGGTCAGGATGAgCa	
COL18A1	Hs.413175	-7884	ttGTCAAAGTGACCC	133
COMT	Hs.240013	-8302	AGGTCATCCTGcCCT	96
		-1798	tGGcCACTGTGACCT	
COX7A2L	Hs.423404	-5246	AGGTCAGGATGtCCa	40
		381	**GGGTCACCTGACCC**	
CP	Hs.282557	-3500	AGGTCACTCTGACta	94
CPM	Hs.334873	-2066	**AGGTCACTCTGACCC**	133
CRHR1	Hs.417628	-7603	AGGTCAGGGTGgCCa	94
CSPG2	Hs.434488	-8225	GGGTggGAGTGACCC	94
CST4	Hs.56319	-7706	AGGTCAAATTGtCCC	133
CTBP1	Hs.196083	2874	GGGTtACCAcGACCC	54
CTSD	Hs.343475	-8763	GGGcCACCATGACCC	54,87
		-125	GGGTCAGCCcGgCCC	
CXCL2	Hs.75765	2628	AGGTCtTTATGACCT	94
DC6	Hs.443163	-6888	AGGcCAGAGTGACCa	97
DCC	Hs.172562	-9826	GGGgCAGCCTGACCa	133
DDIT3	Hs.355867	-751	AGaTCAGCCTGACCa	94
DHCR24	Hs.75616	2615	ttGTCATTTTGACCT	91
DIM1	Hs.433683	-6657	GGGTCATTCaGACCT	97
DLG5	Hs.500245	-4324	GGGTCACGAaGACCa	87
		1303	AGGcCAATCTGACCa	
DNAH8	Hs.172101	-1457	AGGTCAGTTTGtCCT	133
DSCAM	Hs.49002	-9891	AGGcCATTTTGACCT	94
DTNA	Hs.255526	-2020	AGaTCAAGGTGACCT	133
DTR	Hs.799	-1080	GGGTCAGTCTGgCCC	94
E2F1	Hs.96055	-9101	tGGTCAGGCTGAtCT	98
		-3250	tGGTCAACATGACCT	
E2IG2	Hs.31500	-711	AGaTCAGCCTGACCa	95
E2IG4	Hs.8361	-7843	AGtTCAGCATGACCa	95
		1377	GGGcCgGCCTGACCC	
E2IG5	Hs.5243	-8234	AGaTCAGCCTGACCa	95
EBAG9	Hs.9222	6	**GGGTCAGGGTGACCT**	40
EDEM	Hs.154332	-7338	tGGTCAGTGTGAaCC	99
EDG2	Hs.75794	-7418	ctGTCACCCTGACCC	133
		257	GGGgCgCACTGACCT	
		1826	GGaTCACCTTGACCT	
EGFR	Hs.77432	3406	AaGTCACTTTGACCC	100
EGLN2	Hs.324277	-8489	GGcTCACTGcGACCT	97
EGR3	Hs.74088	2335	GGGgCgGGTTGACCC	93,94
EIF5A	Hs.310621	1913	AGGTCcTGTTGACCT	95
		3296	AGGTCAATTTGAgCC	
ELA2A	Hs.21	-7130	AGGgCAGAGTGACCa	133
EPO	Hs.2303	-1664	AGGTCATTGTcACCT	94
		2697	GGGaCAGGATGACCT	
F12	Hs.1321	-30	tGGTCAAGCTGcCCT	81
FBP1	Hs.360509	4639	AGGcCAGTTcGACCC	91
FBP2	Hs.61255	112	GGGTCAGGGTGAgCa	101
FGF2	Hs.284244	-3472	AGGTtACAGTGACCC	102
		4284	AGGTggGCCTGACCC	
FGF9	Hs.111	-204	tGGTCATTCTGAtCT	43
FHR-4	Hs.194776	85	AtGTCATTCTGACCT	94
FKBP4	Hs.848	1065	AGGgCATCTTGACCT	95,103
FOS	Hs.25647	-3411	AaGTCACCCTGACCT	93,104
FOSL2	Hs.301612	-7343	tGGgCAAGTTGACCT	91
FOXO1A	Hs.170133	391	cGGcCATGGTGACCC	133
G6PD	Hs.80206	-59	GGGTCAAGCcGgCCC	54
		4556	AGGTCATGCTGAgCT	
GAB2	Hs.30687	-3399	AGGTCAGTGTGACCa	105
GAL	Hs.278959	-8992	tGGTCAGGCTGACCT	106
		-4905	GGGTCACTGgGACCC	
GAPD	Hs.169476	-8031	AGGTggCAGTGACCC	88
		-5870	cGGTCcCCATGACCC	
		-738	AGGaCATCGTGACCT	
GAS6	Hs.437710	1780	GGGTCACCCccACCC	133
GCH1	Hs.86724	-3702	cGGgCATGGTGACCC	133
		3788	AGGTCAGGATcACCa	
GHRHR	Hs.767	-3049	tGGcCAGGCTGACCT	133
		4741	AGGTCATGTgGACCa	
		-1619	tGGTCAGGCTGgCCT	
GNRHR	Hs.73064	3727	GGGTCATCATGACCa	107
GREB1	Hs.438037	-9517	**AGGTCATCATGACCT**	89
		-1583	**GGGTCATTCTGACCT**	
GTF2I	Hs.408507	-8553	GGGTCAGTCTGAgCT	91
		-6471	AGaTCAGCCTGACCa	
		-2171	AGcTCATGTTGACCT	
H11	Hs.111676	-7364	AGGTCtTTGTGACCT	87
H2AFZ	Hs.119192	2777	ctGTCAAAATGACCT	95
H3F3A	Hs.181307	-4907	AGGcCAGCCTGACCa	95
HM13	Hs.444601	2754	GGGTCAAGGTGtCCa	94
HMGB1	Hs.434102	-5191	AGcTCACTGcGACCT	108
HMOX2	Hs.284279	-6954	AGGTCAGAcctACCT	109
		-2454	GGGTCcCAGTGACCT	
HPCAL1	Hs.3618	-6710	AGGTCtTTGTGACCT	133
		4293	AGGTtgCTGTGACCC	
HPS1	Hs.404568	2429	GGGgCAGCCTGACCa	133
HSPA8	Hs.180414	-9233	AGGTCgCAGTGAgCC	95
		-2702	GGcTCACTGcGACCT	
HSPB1	Hs.76067	-7227	tGGTCACGCTGgCCT	95
		-6805	GGGcCATTCTGACCC	
		1428	AGGgCACACTGACCg	
		4951	GGGTCAGGCTGgCCC	
HSPCA	Hs.446579	-3795	AGGgCAATCTGACCa	95
HSPD1	Hs.79037	-4771	taGTCAGTGTGACCT	101
IDH2	Hs.5337	-4929	AGcTCACTATGACCT	91
IER3	Hs.76095	-7195	AGcTCAGCCTGACCC	54
		-5668	tGGgCAAATTGACCT	
IFRD2	Hs.315177	1836	GGcTCACTGcGACCT	94
IGFBP4	Hs.1516	-4124	AGGTCATTGTGACaC	54,91
		273	cGGTCATGCTGcCCC	
IL1R2	Hs.25333	-6432	GGGaCAAGGTGACCC	94
IL8RB	Hs.846	-2298	GGcTCACTGTGACCT	133
		3946	tGGTCAGCCgGACCC	
INHBB	Hs.1735	-8211	AaGTCACCAcGACCC	94
		-3738	AGGcCAGCTTGACCC	
INPP5A	Hs.408063	1410	AGGcCgGTCTGACCT	133
IRS1	Hs.390242	-6253	GGGTCACCATGgCCC	110
		-6096	AGGTCACCATGtCCC	19
		-462	AGGTaAGACTGACCC	112
		1921	GGGTCAGCCccACCT	113
ITPK1	Hs.408429	-6389	GGcTCACTGcGACCT	54
		-2279	AGGcCAGCCTGACCa	
ISG20	Hs.105434	-7373	AGGTCACACaGACCT	114
JUNB	Hs.400124	-2609	GGGTCACAGgGACCC	94
KCNIP2	Hs.97044	3108	tGGTgAGAATGACCT	91
KIAA0062	Hs301743	-4025	AGGTCgCAGTGAgCC	91
KIAA0073	Hs.1191	-764	AGGTCATTTTGACaa	133
KIAA0153	Hs.82563	-9574	GGcTCACTGTGACCa	54
		2042	AGaTCAGCCTGACCa	
		4055	tGGTCACGATGACCT	
KIAA0864	Hs.430725	-4360	AGGTtgCAGTGACCT	113
KRT19	Hs.309517	-9590	AGGTCAGGCTGACga	115
		-7850	AGGTCAGGATGgCCC	
		2484	AGGTCtTACTGACCT	
KRT6B	Hs.432677	-2049	tGGcCAATTTGACCT	91
KRT7	Hs.23881	2161	GGGcCAATGTGACCC	116
		2659	tGGTCATTGTGAgCC	
KRTHB3	Hs.182506	-8336	GGGTCACAGaGACCa	116
L3MBTL	Hs.300863	3633	GGGTCcTGTTGACCa	94
LDHA	Hs.2795	-1074	AGaTCAGCCTGACCa	95
		-265	AGGTCgTCCTGACtC	
		3181	AGGTggCAGTGACCC	
		4478	tGGTCAATATcACCT	
LOC91012	Hs.458450	-5009	AGGTtgCAGTGACCC	54
		-882	AGGTCgCAGTGAgCC	
LTF	Hs.437457	-1166	AGGTgAGTCTGACCa	85
		-81	AGaTCgCCTTGACCT	
LY6E	Hs.77667	-9749	GGGTCAGACTGgCCC	54
		-499	GGGaCAAGATGACCT	
		-298	cGGTCACCCcGACCT	
		3400	AGGTCAGGCcCACCC	
MAP1B	Hs.103042	-2800	AGGTgAAATTGACCa	91
MATN1	Hs.150366	-8045	GGGTCAGGAccACCC	133
MGC8407	Hs.145156	-7041	AGGTCAGGGTGACaT	54
MJD	Hs.419756	-518	AGaTCAGCCTGACCa	133
MMP12	Hs.1695	-5633	AGcTCAAAGTGACCC	133
		-3556	tGGTCATCTTGACtC	
MMP2	Hs.367877	-2578	AGGTCtTTATGACCT	117
		-801	GGGTCtTTGTGACCT	118
		2295	GGGTCtTTGTGACCC	119
		3822	AGGTgATCTTGACCa	
MSX2	Hs.89404	-9630	AGGTCAGAATGAtCa	54
MTHFD2	Hs.154672	-4241	GGcTCACTGTGACCT	91
MYBL2	Hs.179718	3441	AGGTggAGCTGACCt	132
NFATC3	Hs.172674	-2740	GGGTCcAGATGACCT	94
NME1	Hs.118638	-5636	tGGTCAGACTGAtCT	95
NOS3	Hs.446303	-4618	AGGTCAAGGcGtCCC	120
		-1593	ctGTCACCTTGACCC	
NPM1	Hs.411098	-1876	AGGTCACAGTGAgCC	121
NPR2	Hs.78518	-6835	AGGTCgCTTTGACaC	133
		-2519	AGGaCACTGcGACCT	
		705	GGGTcgCTGTGtCCC	
NR4A1	Hs.1119	3478	tGtTCACTCTGACCT	94
NR4A3	Hs.279522	-1929	GGGTCAGGGcGcCCC	94
		3743	GGGcCgTAGTGACCC	
NRIP1	Hs.155017	-706	**GGGTCACTTTGACCC**	54,91
NUCB2	Hs.423095	-8257	GGGTtgATTTGACCC	91
OVGP1	Hs.1154	-156	GaGTCACAGTGACCC	74
		200	GGGTCcCTCTGACCT	133
OXT	Hs.113216	-2596	AGGTCATCGTGcCCa	79
		-2465	AGGTCAGCCTGACCg	
		-166	cGGTgACCTTGACCC	
P2RY6	Hs.16362	4581	AGGTCAGCCTGgCCa	94
PA2G4	Hs.374491	-5274	GGcTCACCGTGACCT	95
PCNA	Hs.78996	-9730	tGGTCAGGCTGgCCT	100
		-5397	AGGTCAAAATtACCa	132
PDE4B	Hs.188	4409	GGGTCATTCTtACCC	94
PDE4C	Hs.437211	-2035	GGcTCACTGcGACCT	133
		460	cGGTCACACaGACCC	
PDK4	Hs.8364	557	AGGTggCCTTGACCT	133
PKMYT1	Hs.77783	-3298	AGGTCACAAaGACCT	132
		-722	tGGTCACCTaGACCC	
POLA2	Hs.201897	-2268	AGGTCgAGGaGACCT	132
PPP2R4	Hs.400740	-267	GGGTCAGTAccACCC	122
PRC1	Hs.344037	4225	AGGTtgCAGTGACCT	133
PRKAR1A	Hs.280342	-8067	AGGTtgCAGTGACCT	87
		3744	AtGTCATTGTGACCT	
PRKCB1	Hs.349845	1396	tGGTCgGAGTGACCT	133
		2434	GGGgCAAAGTGACCa	
PSMB4	Hs.89545	-1786	GGGTCgCAGTGAgCC	95
PTGER3	Hs.527970	-5430	GGGaCAGAGTGACCT	132
PTMA	Hs.459927	4361	AGGcCgCCGTGACCT	123
PTPN2	Hs.446126	-9369	AGGTCgCAGTGAgCC	94
PTPRO	Hs.160871	-6656	AGGTCgCTGTGAcCT	94
PTPRU	Hs.19718	-5945	cGGTCtTTCTGACCT	94
		3505	tGGcCAGCCTGACCT	
PTTG2	Hs.511755	-3351	GGaTCAAGCTGACCa	94
PTX3	Hs.2050	844	AGGcCAGGCTGACCa	133
QPCT	Hs.79033	190	GGGTCAGCCctACCT	133
RAN	Hs.10842	2869	GGcTCACTGcGACCT	95
		3007	tGGTCAGGCTGAtCT	
RARA	Hs.361071	-8627	GtGTCACCGTGACCC	54,93
RBBP8	Hs.437224	-9688	AGGTCACTGcaACCT	54
RFC4	Hs.35120	-3314	AGGTCACTGcaACCT	132
RFP	Hs.440382	-7698	tGGTCAGGCTGAtCT	95
		-5598	AGGcCAGCCTGACCa	
		-522	AGGTtgCAGTGACCC	
		581	GGGTCACGTTGgCCa	
RPS6KA1	Hs.149957	-5213	tGGgCAAGCTGACCC	91
SCD	Hs.119597	-7982	GGGgCAGTATGACCC	91
SCNN1A	Hs.446415	-4605	GaGTCATTGTGACCa	54
		-559	AGGTCAGCCTcACCC	133
SDS	Hs.511764	-8602	GGGgCATTCTGACCa	133
		-1020	AGGTggCCCTGACCT	
SERPINA5	Hs.76353	3152	AGcTCAGTGTGACCC	54
SERPINA6	Hs.1305	1967	GGGTCtTTGTGACCC	54
SF3A3	Hs.77897	-8338	cGGaCATGATGACCC	95
SHMT2	Hs.75069	-2501	GGGTCAGGGTGcCCa	91
		-1575	AGaTCAGCCTGACCa	

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Table 3. Continued

Gene	UniGene #	Pos.	ERE Sequence	REF	Gene	UniGene #	Pos.	ERE Sequence	REF	Gene	UniGene #	Pos.	ERE Sequence	REF
SLC29A1	Hs.25450	4242	cGGTCACGTTGACCT	54	TERT	Hs.439911	-5663	GGGTCACTCTGaCCC	124	VDR	Hs.2062	-3231	GGGTCACTTTGtCCC	127
SLC7A5	Hs.184601	4963	AaGTCAGAATGACCT	54,91			-2687	AGaTCAGCCTGACCa		VEGF	Hs.73793	-9667	AGGcCACTGTGACCC	132
SLK	Hs.105751	-3049	AGGTCACTGcaACCT	132	TFF1	Hs.350470	-392	tGGcCACCGTGACCT	91,133	VTN	Hs.2257	-4209	AGGTCACAATGAgCT	87
SMC2L1	Hs.119023	-9528	AGGTCtTTGTGACCT	94	TFF3	Hs.82961	-8655	AGGTCtTTATGACCT	54,124	VWF	Hs.440848	-9547	AGGTCACAGcGAgCC	133
		-8678	tGcTCAGCTTGACCT				-4315	GaGTCATCAcGACCT	125	WISP2	Hs.194679	-2526	AaGTCgGAGTGACCT	97
SNRK	Hs.79025	-1342	AGGTCAGAGTGAtCC	94	TFPI2	Hs.438231	-4126	tGGTCATGTTtACCT	54			-452	GGGTCACACccACCT	
SNRPA	Hs.173255	2000	AGGTCgCAGTGAcCT	54	THBD	Hs.2030	-2152	GGGTggACATGACCC	94	WNT1	Hs.248164	-9908	GGGTCAAGATGAtCT	128
SOS1	Hs.326392	-3457	tGGgCATGTTGACCT	94	TNFRSF8	Hs.1314	-6502	AGGTCAAAAgGACCT	94	WNT10B	Hs.91985	3215	GGGTCAAGATGAtCT	129
SP4	Hs.2982	-1480	GGGTCACTTcCACCT	94			-2999	GGGTgACTCTGACCC		WNT9B	Hs.326420	1839	GGGTCACTGcaACCT	130
SPRY1	Hs.436944	138	GGGTCAGCCaGACCg	94	TOB1	Hs.178137	-3159	AGaTCAGCTTGACCa	54			3736	GGGTtgCACTGACCC	
SPRY2	Hs.18676	1041	GGGTCAGCCcGAgCT	94	TOPBP1	Hs.91417	-1262	tGaTCACCTTGACCT	133	XPNPEP1	Hs.390623	-799	AGGTtgCAGTGACCT	94
SSNA1	Hs.18528	-8128	GGGTCgTGATGAgCC	107	TP53	Hs.408312	-1529	AGGTCgATCTGtCCT	126	XRCC4	Hs.150930	-7225	AGGTtATCTTGACCa	133
		-1829	cGGTCtCCCTGACCC		TPBG	Hs.82128	-4705	ttGTCAAATTGACCC	54	ZDHHC4	Hs.5268	-9951	GGGTCACCTTGcCCg	97
		3468	AGGTCATTCTGACtg				820	tGGTgAGCCTGACCT		ZNF17	Hs.185796	3231	GGaTCATTGcGACCT	94
STAG1	Hs.138263	-4456	caGTCACTATGACCT	133	TPO	Hs.71304	-609	caGTCATGGTGACCT	94	ZNF230	Hs.193583	-9478	GGcTCACAGcGACCT	94
STK6	Hs.250822	3170	GGGTCAATTccACCT	132	TPX1	Hs.2042	-7640	GGGTCATCTTGAaCa	133			82	GGGTCgCAAcGACCC	
SUOX	Hs.16340	-7499	**AGGTCACAGTGACCT**	95			-3900	AGGTCtTTATGACCT		ZNF75	Hs.355015	-6059	AGGTCATGATGAgCa	94
		306	AaGTCAGTCTGACCC		TRIM31	Hs.493275	-288	AGaTCAGAATGACCg	133			-62	GGGTCACATTGACaa	
		1718	GGGTCAGGGTGAgCT		TSHB	Hs.406687	-4099	AGGTCAGCTTGACaT	59	ZNF9	Hs.2110	2272	AGGTCgCAGTGAgCC	95
TAC1	Hs.2563	167	GGGTCACCCcGcCCC	94	USF2	Hs.454534	305	GGGcCgCGCTGACCC	97	ZNF91	Hs.8597	-5023	AtGTCACAGTGACCC	131
TCF2	Hs.408093	996	AGGTCAATGTGtCCg	94			2061	GGGTCcCCCTGACCa						

The listed EREs were compiled by comparison with upregulated E2 target genes identified in gene expression studies as cited. The positions given are distances to the most upstream mRNA 5′-end for each gene (negative distances correspond to elements located upstream of the 5′, and conversely). ERE sequences in *bold* correspond to consensus elements. *Underlined* genes contain EREs that are conserved in mouse (see supplemental data) according to criteria defined in *Materials and Methods.*

ERα bound to the TSHB ERE whereas no binding of TBP and P-PolII was detected either on the reported start site (58) or on another site described in NCBI (National Center for Biotechnology Information) AceView (Fig. 4). Note that regulation of TSHB by E2 has been observed in mouse pituitary (59). It is possible that another transcriptional start site may be used in MCF7 cells, or that this gene is inactive in these cells despite ERα binding to upstream sequences.

Results from ChIP experiments demonstrate that it is possible to identify *bona fide* ER binding sites both in proximal and distal promoter sequences of E2 target genes, suggesting that the search for ER binding sites should not be limited to promoter sequences immediately upstream of the transcriptional start site. In addition, our results indicate that access to some high-affinity binding sites is restricted *in vivo*, possibly in a tissue-specific manner.

DISCUSSION

In this study, our goal has been to define high-affinity *in vitro* ER binding sequences and to characterize the occurrence of these motifs throughout the human and mouse genomes. This approach differs from those of previous studies, which have aimed at characterizing a larger array of potential binding sites for ERs or nuclear receptors in defined stretches of genomic sequences (60, 61). These approaches are based on nucleotide frequency matrices constructed via compilation of the relatively small number of known natural response elements and can identify many variant sequences. Although the sensitivity of the detection can be lowered for screening of genomic sequences, these types of detection programs cannot be easily used for genome-wide studies (the maximal length of input sequences is ~100 kb for Dragon ERE Finder version 2.0 and 30 kb for the NUBIScan program). On the other hand, we chose to identify only high-affinity binding sites in a genome-wide approach to determine their distribution with respect to transcriptional start sites, their conservation between the mouse and human genomes, and their functionality as receptor binding sites *in vivo*. Our choice of elements was based both on functional validation of variant EREs by *in vitro* binding assays, and on the variations observed in known near-consensus sequences. Although not within the scope of this study, our functional characterization of EREs both *in vitro* and *in vivo* is also expected to contribute to the development of refined nucleotide frequency matrices for the detection of a wider range of elements in genomic sequences of interest.

Our screen identified a large number of EREs (71,119 in the human genome, or about one in every 43 kb of genomic DNA). Not surprisingly, the frequency of ERE occurrence was found to be highly dependent on their sequence. For instance, elements containing CG dinucleotides are drastically less represented than other EREs. CG dinucleotides are represented at a frequency of about 0.8% in the genome, which is five times less than the expected frequency based on the typical fraction of Cs and Gs (42). Elements containing two CG dinucleotides are found at only about 1–2% of the number of consensus EREs, which do not contain CG dinucleotides except possibly in the spacer. On the other hand, consensus EREs are slightly more represented than the expected fre-

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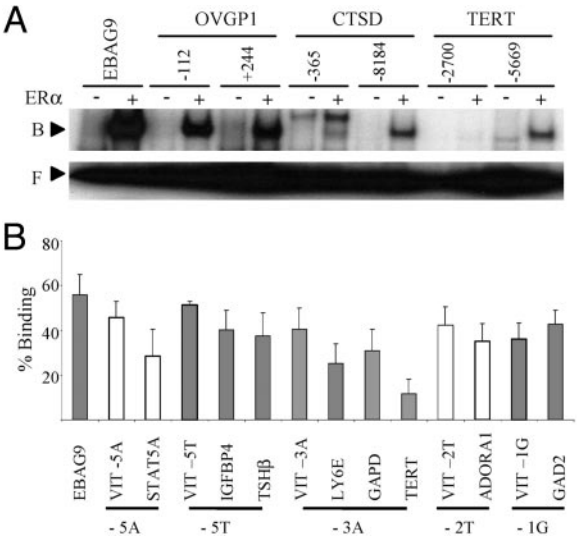

Fig. 3. *In Vitro* Binding of ERα to EREs Identified in This Study

A, Relative binding of hERα to known and newly characterized EREs in estrogen target genes. B, Binding of hERα to the novel EREs characterized through genomic screening. Binding is expressed as the percentage radioactivity of specifically bound probe *vs.* bound plus free probe.

quency in random sequences (122% in human and 150% in mouse).

The sequence bias for some of the elements and the observation that some high-affinity EREs are found in tandem repeats precluded evaluation of which proportion of near-consensus EREs are under positive selective pressure, as could be expected if they mediate important regulatory roles. However, it was possible to evaluate the relative distribution of EREs in the vicinity of human or mouse genes. We have calculated that the number of EREs found in the human genome in the −1 to + 2-kb region around the 5′-end of mRNAs exceeds the number of elements expected from their frequency in the genome by 64%. It is unclear whether this represents conservation of elements found in the vicinity of transcriptional start sites, or bias for undetermined causes. A contributing factor may be the increased GC content in CpG islands, which are associated with the 5′-end of genes. Consensus EREs have an average GC content varying between 40 and 73% (depending on the identity of the variable bases). For most sequences, this is higher than the genome-wide average of 41%, but compatible with the 60–70% average in CpG islands (42). It is surprising, however, that the main peak of ERE representation is in the 0 to +1-kb region around the 5′-end of mRNAs both in human and mouse. Although the basis for this observation is unclear, it is worth noting that only the most 5′-mRNA start site for each gene was taken into consideration in this representation. Thus, some of these EREs may regulate promoters of downstream initiation sites.

We performed next a direct comparison of elements found in human and mouse orthologs to assess the fraction of total EREs that is conserved in the two species. Of the 9944 known orthologs (Mouse Genome Informatics database), 660 contained one or several conserved EREs at similar relative positions in the 5′-flanking sequences, *i.e.* at 2 kb from each other (708 conserved elements in total). This corresponds to about 1% of total elements in the human or mouse genome. Note that our criteria for conservation are relatively stringent, including both limited sequence variations (no more than two differences from consensus) and positional constraints (relative positions of EREs not further apart than 2 kb). Other possible sources of under-representation of conserved EREs are the incomplete identification of human/mouse orthologs, and the lack of systematic annotation of the mRNAs 5′-ends in the human and mouse genomes. Nevertheless, we have estimated that there is an approximately 74% overrepresentation of EREs compared with chance occurrence of these elements in both the human and mouse orthologs within 2 kb of each other (708 *vs.* 407 elements; see *Materials and Methods*). This overrepresentation suggests functional conservation of EREs throughout evolution, although we cannot rule out that functions unrelated to recruitment of ERs may contribute to this conservation (see below). The distribution of the conserved EREs was similar to that of total EREs in the vicinity of human or mouse genes but displayed a more marked concentration in the vicinity of the transcriptional start sites (see Fig. 2C). Interestingly, the percentage of conserved elements dropped markedly upstream of −5 kb, suggesting that upstream EREs are less likely to be conserved than elements closer to the initiation site, albeit these EREs still represent 24% of the total number of conserved elements between −10 and +5.

Apart from conservation due to functional importance, the most likely reason for preservation of an ERE is its location within the coding sequence of a gene. This parameter might contribute to the overrepresentation of EREs located downstream of the initiation start sites. In addition, some upstream elements may also be found in the coding sequences of other genes. The possible contribution of coding sequences in preserving ERE motifs in the human and mouse genomes was examined in the extreme case of EREs found to be totally conserved in sequence between the two species, including the Pu/Py and spacer base pair. Of 41 distinct EREs flanking 47 genes, 14 were found in coding sequences in both species, and four additional elements overlapped a CDS. It is unclear whether these elements could function as binding sites *in vivo*. Binding of yeast transcription factors such as Gcn4, Sbf, Mbf, and Rap1 to elements within open reading frames occurs reportedly less frequently than in promoter sequences (62–64) and, in the case of Gcn4, results in recruitment of histone acetyltransferase and SWI-SNF coactivator complexes, but not of the Mediator complex (64). Finally, 23 EREs were not

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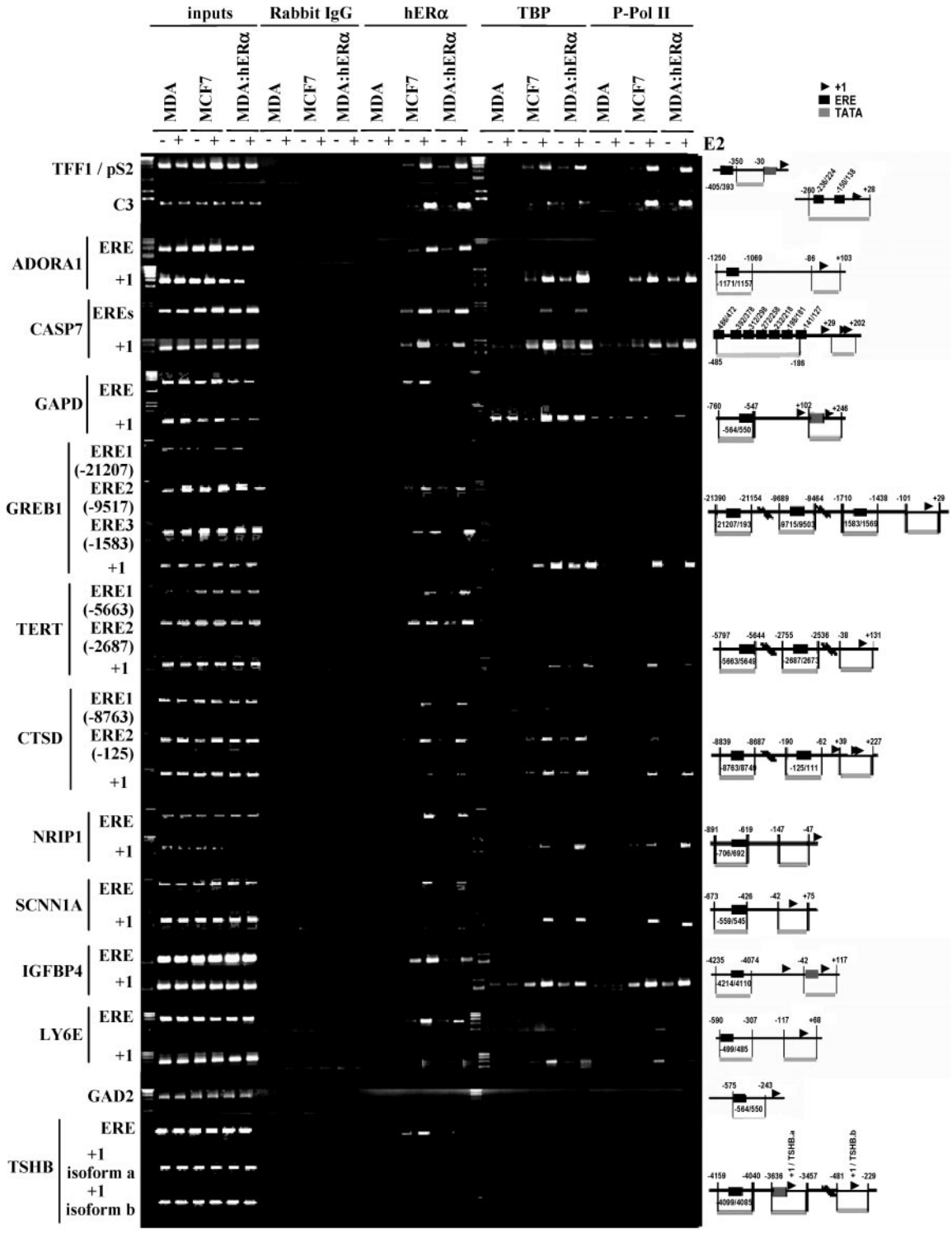

Fig. 4. *In Vivo* Binding of Human ERα to EREs in ER-Positive Breast Cancer Cells

Interaction of hERα with selected EREs in the absence or presence of estrogen was investigated by chromatin-immunoprecipitation assay in ERα-negative MDA-MB231 (MDA) cells, in ERα-positive MCF7 cells, or in MDA-MB231 cells stably transfected with an expression vector for hERα (MDA::hERα) and treated or not with E2 for 2 h. Fragments containing putative EREs were immunoprecipitated from formaldehyde-fixed chromatin preparation with antibodies against hERα, TBP, or phosphorylated polymerase II (P-PolII), or with preimmune rabbit IgG. A separate fragment spanning the transcription start site was used for PCR amplification when the start site was situated at more than 400 bp of the ERE. Whenever available, experimentally mapped transcription start sites were included in the +1 fragments. Experiments were performed two to three times and a representative result is shown.

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found in coding sequences in either species. Interestingly, more than half of these EREs (12 elements) were located between −1 and +1 kb of the transcriptional start site, including eight elements between 0 and +1 kb. Therefore, although the presence of EREs in coding sequences may account, in part, for element conservation, it does not explain the large proportion of elements found immediately after the annotated transcriptional start site. Of interest in this regard, a recent publication indicated that an important fraction (40%) of NFκB *in vivo* binding sites in chromosome 22 is located in intronic sequences (65).

Even though EREs appear less represented and/or conserved when located farther upstream of the transcriptional start site (−1 to −10 kb), our results suggest that they may contribute to estrogen target gene regulation. Indeed, some of these distal EREs are conserved between the human and mouse genomes and bind ERα *in vivo*, as demonstrated by our ChIP experiments. These observations validate the choice of the −10 to +5-kb window around transcriptional start sites used in this study to identify potential ER binding sites. However, the frequency of near-consensus EREs found within this range strongly suggests that not all of these elements mediate transcriptional regulation of neighboring genes in a given cell context. It is unclear at this point whether access to some of these elements is restricted, or whether bound receptors may be unable to transactivate neighboring genes. The number of EREs in bulk genomic DNA also indicates that if all elements were accessible, binding sites would likely outnumber the molecules of receptor dimers in estrogen target cells. Indeed, the number of estrogen binding proteins in rat uterine cells was estimated via either biochemical or autoradiographical methods to vary between 5,000 and 30,000 molecules per cell (66–69). Large-scale studies of transcription factor binding sites using ChIP assays have also indicated that the number of *in vivo* binding sites for different transcription factors in the genome is high. For instance, in ChIP experiments performed in HeLa S3 cells, 15% of the genes in chromosome 22 were found to contain functional NFκB binding sites within 10 kb (65). In addition, 11% of the promoters were found to contain one or several high-affinity c-*myc* binding sites, suggesting competition between target sites in chromatin for limiting amounts of Myc protein levels (70). Alternatively, another possibility that arises from these observations is that stochastic and/or dynamic binding of transcription factors may allow usage of a larger number of binding sites.

We are aware that the total number of potential ER binding sites may be much higher than reported here. Elements not included in our search may represent good binding sites *in vivo*, including those that display a relatively low affinity *in vitro*. Whereas the compiled list of EREs is clearly not exhaustive, our ChIP experiments support the notion that the near-consensus EREs identified in estrogen-target genes represent likely binding sites *in vivo* (13 of 14 elements bound). Nevertheless, access to some of these elements may be restricted irrespective of their strength as ER binding sites *in vitro*. Neither a perfect ERE located within about 20 kb of the transcriptionally active GREB1 estrogen target gene, nor a near-consensus ERE located in the nontranscribed GAD2 gene were bound in ChIP experiments. Although results obtained in two breast cancer cell lines were very similar, it will be of interest in future studies to examine further how binding site recognition and coactivator recruitment are affected by cellular context. The future availability of large-scale gene expression studies performed in different tissues will allow us to expand the list of known E2 target genes and to determine whether the near-consensus EREs in their flanking sequences are bound in a tissue-specific manner. In addition, our data will facilitate future studies comparing the patterns of coactivators recruited by distal/proximal elements. For instance, it is possible that upstream EREs would recruit histone acetyltransferase and/or SWI/SNF complexes, resulting in long-range opening of chromatin and facilitating access of enhancer proteins to far upstream flanking sequences, whereas only proximal promoter sequences may be able to recruit mediator complexes. Alternatively, chromatin loops may allow upstream elements to participate in the recruitment of the basal machinery on the transcriptional start site. It is our hope that the database of high-affinity EREs derived from this study (URL: http://mapageweb.umontreal.ca/maders/eredatabase/) will prove a useful tool for the characterization of primary E2-regulated genes in various human and mouse target tissues and will ultimately enhance our understanding of the molecular mechanisms underlying the physiological actions of estrogens.

MATERIALS AND METHODS

Bioinformatics

The algorithms developed (Nagai, Y., V. Bourdeau, and S. Mader, in preparation) enable a search of the NCBI fasta and gbs files of the Human Genome (Built 33; June 6, 2003) or of the Mouse Genome (Built 30; March 11, 2003) for a specified group of sequences and extract the positions of matching motifs in the genome contigs as well as the coordinates of the surrounding genes, mRNAs, and CDS within a preset cutoff distance of each motif. The programs, written in C, were run on an SGI Origin 2000 with 32 CPUs IP 27, R10000, 300-MHz processor (16 Go) using UNIX IRIX 6.5. Results presented in this article were generated using a cutoff of −10 to +5 kb of the mRNA 5′-ends (database available at http://mapageweb.umontreal.ca/maders/eredatabase/). Statistics for the total number of EREs occurring in the genomes were derived from lists of elements generated before the cutoff was applied. The total number of consensus EREs in the human genome was 891, and the number in the mouse genome was 923. Expected frequency in random DNA sequences was calculated as the total number of base pairs in the genome divided by the frequency of occurrence of a sequence with specified base pairs at 10 positions and two base pair choices at two positions ($3{,}069334246/4^{11} = 732$

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EREs in the human genome; $2{,}578250392/4^{11} = 615$ EREs in the mouse genome).

Conserved EREs were identified as elements present in both human and mouse gene orthologs (listed at the Mouse Genome Informatics database) at distances comprised between −10 to +5 kb from their respective mRNAs 5′-ends, and differing by less than +/− 2 kb. Because in numerous instances transcriptional start sites are mapped at the ATG codon in at least one species, or at the 5′-end of the gene for alternative upstream start sites, we have included EREs present in orthologs that were distant from each other by more than 2 kb when positions were calculated with respect to mRNAs 5′-ends, but less than 2 kb with respect to gene 5′-ends or initiator ATGs to minimize the underrepresentation of conserved EREs due to differential annotation in the human and mouse genomes. For example, the EBAG9 gene element, which is perfectly conserved in sequence, would not be selected on the basis of the distances to the 5′-end of mRNA because the mouse transcriptional start site was annotated at the ATG, but was included due to similar location with respect to the annotated 5′-end of the genes. Note that the 5′-end of the mRNA now coincides with the 5′-end of the gene in the most recent version of the mouse genome (Built 32).

The probability of an ERE to be found by chance in both the human and mouse orthologs was calculated by multiplying the probability of finding an element within −10 to +5 kb of the start site of one gene (14,074 elements in that window of the human genome/34,699 total genes) by that of finding an ERE in a 4-kb window (±2 kb) of corresponding sequence in the other species (12,828 elements × 4 kb/33,914 total mouse genes × 15 kb) and by the total number of orthologs (9,944 gene pairs) giving 407 expected elements.

Cell Culture

Hela cells were maintained in DMEM (Wisent, St-Bruno, Québec, Canada) supplemented with 5% fetal bovine serum (FBS, Sigma, Oakville, Ontario, Canada). Cells were switched 3 d before experiments to medium without phenol red containing charcoal-stripped serum. For gel shift assays, Hela cells were electroporated (10^7 cells, 0.24 kV, 950 μF in a Bio-Rad Gene Pulser II apparatus; Bio-Rad Laboratories, Mississauga, Ontario, Canada) with 80 μg expression vector (pSG5-ERα, pSG5-ERβ or parental vector alone). Note that pSG5-ERβ was generated by subcloning the open reading frame of hERβ (71) from pCMVSport-ERβ (a kind gift from Dr. T. Willson, Glaxo Wellcome, Inc., Research Triangle Park, NC) into the *Bam*H1 site of pSG5 (72) by PCR amplification. Cells were treated with 25 nM E2 (Sigma Chemical Co., St. Louis, MO) 1–2 h before harvesting (48 h post transfection). Whole-cell extracts were prepared by three freeze-thaw cycles in gel retardation buffer as previously described (73).

For ChIP experiments, MCF-7 and MDA-MB231 cells were grown in DMEM (Sigma) supplemented with 10% fetal calf serum (Sigma). The medium was changed to phenol-red free DMEM supplemented with 2.5% dextran-charcoal treated fetal calf serum 48 h before hormone addition and replaced each 24 h. The MDA::hERα cell line stably expressing hERα was generated from hERα-negative MDA-MB231 cells by transfection of pCDNA3.1/Hygro-hERα (25).

Gel Shift and ChIP Assays

For gel shift assays, whole-cell extracts expressing ERα or ERβ or control extracts from cells transfected with the parental pSG5 vector were diluted to 120 mM KCl and assayed for binding to ^{32}P-labeled, double-stranded oligonucleotide probes (50,000 cpm/sample) as described previously (73). Radioactivity associated with bound or free probe was quantified using a Molecular Imager FX with the Quantity One software (Bio-Rad).

For ChIP assays, chromatin was cross-linked using 1.5% formaldehyde for 5 min at 37 C and fragmented by sonication as previously reported (25, 33), yielding fragments of average size approximately 350 bp. Antibodies against a C-terminal epitope of hERα (HC20) and against TBP were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany), and the antibody against phosphorylated polymerase II was purchased from Upstate Biotechnology, Inc. (Buckingham, UK). The sequences of the primers used in ChIP assays (synthesized by MWG GmbH, Ebersberg, Germany) are available upon request.

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Note Added in Proof

A database of estrogen-responsive genes was published while this article was in revision (ERGDB, Ref. 134). This database, compiled from a literature review of experimentally identified estrogen target genes, includes putative ERE sequences found in some of these genes through screening of their promoter-proximal sequences with Dragon ERE Finder version 2.0. Our database includes all ERGDB EREs that correspond to sequences used in our screen (although positions may vary, as our study used a later release of the human and mouse genomes), but also ERE sequences found beyond the cutoff distance used for the ERGDB (−4500 to +500 bp from transcriptional start sites), or located in genes not currently known to respond to estrogen.

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