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MINIREVIEW

Genomic Targets of Nuclear Estrogen Receptors

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Estrogen influences the physiology of many target tissues in both women and men. The long-term effects of estrogen are mediated predominantly by nuclear estrogen receptors (ERs) functioning as DNA-binding transcription factors. Tissue-specific responses to estrogen therefore result from regulation of different sets of genes. However, it remains perplexing as to what regulatory sequence contexts specify distinct genomic responses. First, this review classifies estrogen response sequences in mammalian target genes. Of note, around one third of known human target genes associate only indirectly with ER, through intermediary transcription factor(s). Then, computational approaches are presented both for refining direct ER-binding sites and for formulating hypotheses regarding the overall genomic expression pattern. Surprisingly, limited evolutionary conservation of specific estrogen-responsive sites is observed between human and mouse. Finally, consideration of the cellular functions of regulated human genes suggests links between particular biological roles and specific types of estrogen response elements, although with the important caveat that only a restricted set of target genes is available. These analyses support the view that specific, hormone-driven gene expression programs can result from the interplay of environmental and cellular cues with the distinct types of estrogen-response sequences. (*Molecular Endocrinology* 18: 1859–1875, 2004)

ESTROGENS ARE IMPLICATED in a wide variety of physiological processes that affect multiple tissues in the human body. In particular, this group of steroid hormones plays important roles in cellular growth, differentiation, and specific organ functions (1). Estrogens mediate responses via diffusion through the plasma membrane and signaling through intracellular hormone-specific estrogen receptors (ERs). Two distinct types of signaling can be mediated, often referred to as the genomic and the nongenomic or nongenotropic pathways. In the genomic pathway, estrogens bind the receptor in the nucleus, inducing a conformational change in the receptors that causes dissociation from chaperones (2), dimerization, and activation of the receptor's transcriptional domain. The canonical model for ER-mediated regulation of gene expression involves the direct binding of dimeric ER to DNA sequences known as estrogen response elements (EREs), which are specific, inverted palindromic sequences. In addition, ER can indirectly associate with promoters through protein-protein interactions with other DNA-binding transcription factors (3, 4). In either case, interaction of ER liganded with estrogen leads to transcriptional activation of the associated genes via recruitment of coactivators and components of the basal transcriptional machinery, as extensively reviewed elsewhere (5–9). In addition to the nuclear ERs, plasma membrane-associated ER mediates the nongenomic signaling pathway (10–13), which can lead both to cytoplasmic alterations and to regulation of gene expression (14). The genes regulated directly by estrogen-induced nongenomic pathways have not yet been extensively analyzed.

Many recent studies have shown that regulation of transcription by nuclear ER is more complicated than the classical paradigm would predict (for reviews see Refs. 4, 6, 8, 15, and 16). The two nuclear ERs, ERα and ERβ, exhibit distinct transcriptional properties and can form both homodimers and heterodimers (17–19). In addition, nuclear ER can regulate gene expression through sequestration of other DNA-binding proteins in the nucleoplasm. Typically, estrogen-induced interactions between ER and such transcription factors prevents binding of the secondary transcriptional activators to their responsive promoters, thereby inhibiting transcription (20–25). Finally, ERs are regulated by posttranslational modifications (26–29), which can alter ER activities even in the absence of estrogen. Recent, exciting studies point to the specific constellation of coactivators and corepressors and to signal-

Abbreviations: ATF, Activating transcription factor; AP-1, activator protein 1; CREB, cAMP response element-binding protein; ER, estrogen receptor; ERE, estrogen-response element; NMDA, *N*-methyl-D-aspartate; SFRE, steroidogenic factor-response element.

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ing pathways as critical modifiers of the role of ERs in distinct environments (30, 31). Activities not only of the ERs, but also of such coregulatory molecules, are modulated by signaling pathways (31).

To understand the connection between physiological and molecular functions of ER, the field requires an in-depth understanding of the spectrum of genes regulated in each tissue and cell type. This review will focus on the current state of knowledge regarding estrogen response sequences in regulatory regions of genes directly targeted by nuclear ERs.

ASSOCIATION OF ERs WITH DNA REGULATORY SEQUENCES: A FUNDAMENTAL MEANS OF GENE REGULATION IN RESPONSE TO HORMONE

The first identified transcription regulatory sequences bound by ER upon ligand activation were 13-bp palindromic response elements. More and more variability has subsequently been uncovered among sequences with which ERs associate in response to hormone (4, 32). To categorize and analyze EREs further, known, primary, ER-regulated mammalian genes were compiled (see Tables 1–4). Only mammalian genes were included, as the ultimate goal was to understand human physiology, which mammalian genes are more likely to reflect. Two experimental criteria were used to identify primary response genes and to prevent inclusion of secondary response genes (genes indirectly regulated by estrogen, *e.g.* they may be stimulated by a transcription factor the expression of which is directly activated by ER). Secondary response genes would skew analysis of sequence contexts. The first criterion was demonstration that a promoter/enhancer region directly responded to estrogen, usually coupled with mutational analyses to delineate specific sequences that directed this responsiveness. Generally, these results were obtained by transient transfection assays in which the promoter/enhancer was linked to a reporter gene. The second criterion was demonstration of *in vitro* association of ER with this regulatory region, or *in vivo* association in cells through chromatin immunoprecipitation assays.

Given the strict criteria, many genes that are estrogen responsive in their expression (33–35), but have not been demonstrated to be primary target genes or to directly associate with ER, are not included. In particular, in cell types that are not readily transfectable, the primary and secondary response genes have not generally been distinguished. With the advance of chromatin immunoprecipitation methodology to identify ER-bound regulatory regions, such barriers should soon be overcome. Nonetheless, the current set of primary response genes were mainly identified as estrogen responsive in breast cancer cells, with only a few studied in bone (*e.g.* progesterone receptor, c-*fos*, IGF-I; see references in Ref.33), vascular [*e.g.* vascular endothelial growth factor, progesterone receptor, c-*fos*; (34)], neuronal [oxytocin, *N*-methyl-D-aspartate (NMDA) receptor 2D subunit; see references in tables], or liver cells [low density lipoprotein receptor (36)]. A number of the genes expressing general housekeeping functions are widely expressed, however, and may be estrogen responsive in many ER-positive cell types.

In general, the primary, ER-regulated promoter/enhancers fall into two major categories (4, 32): those in which ER directly binds DNA (Tables 1–3) and those in which ER associates with the regulatory sequences due to interactions with other DNA-binding proteins (Table 4).

Estrogen-Response Sequences Containing a Direct ER-Binding Site

Even among the promoters directly binding ER, the types of response elements vary widely. The predominant sequence for direct binding is, indeed, palindromic EREs (Table 1). Other enhancers have been reported to include only half-ERE sites in multiple copies (Table 2), in which distant half-EREs may cooperatively bind ER (37). Alternatively, some of these sequences may represent low-affinity, full EREs (see below). Yet other regulatory regions contain only extended half-site response elements, tcaaggtca, of the orphan nuclear hormone receptor steroidogenic factor 1 (SFREs) (Table 2). ERα, but not ERβ, is capable of binding to SFREs (38), presumably due to an ERα-specific C-terminal extension adjacent to the zinc finger DNA-binding domain (4). Finally, estrogen responsiveness can be driven by a half-ERE site in strict combination with a nearby Sp1 site, both of which must be occupied for maximal activation (Table 3). Gene-regulatory regions can incorporate a combination of direct ER-interaction sites, such as the TGFα promoter that contains two full nonconsensus EREs plus an Sp1-binding site with an adjacent ERE half-site, all of which are functional in estrogen responsiveness (see Tables 1 and 3 for references). It is tempting to speculate that ER may regulate expression of the same gene under differing cellular environments through distinct types of responsive sequences.

Overall, the palindromic ERE binding sequence for ER is well defined and represents a typical DNA-binding element for a transcription factor, with variability permitted to some extent at all positions in the recognition sequence (Fig. 1; shown as a matrix and pictogram derived from sequences in Table 1). However, the rules for binding to half-EREs (with or without accompanying Sp1 sites) and SFREs are less defined; less sequence variability in the single half-ERE site may be tolerated given the already lower affinities of ER for these half-site(s), and possibly additional protein interactions may be required in the genomic context for efficient binding. For most classes of mammalian transcription factors, half-binding sites have not been identified as functional elements; thus, such response sites are unusual.

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Table 1. Genes Regulated by ER that Contain Palindromic ERE Sequences

Gene	Organism	Estrogen-Responsive Region[a]	ERE Sequence(s)[b]	Strand[c]	Ref.
Consensus ERE: two consensus half-sites					
EBAG9 (RCAS1)	Human	−60 to −48	cg **GGTCA** ggg **TGACC** tc	+	67, 68
Efp (ZNF147)	Human	3′-UTR	ag **GGTCA** tgg **TGACC** ct	+	69
COX7RP	Human	+311 to +327[d] (intron 1)	gg **GGTCA** agg **TGACC** cc	+	67
EREs with one consensus half-site					
TERT	Human	−2677 to −2655	tt **GGTCA** ggc **TGATC** tc	+	70
pS2 (TFF1)	Human	−405 to −393	aa **GGTCA** cgg **TGGCC** ac	+	71
Lactoferrin (also Table 2)	Human	−414 to +69	ca **GGTCA** agg **CGATC** tt	+	72
Keratin 19	Human	+2414 to +2430[d] (intron 1)	ta **GGTCA** gta **AGACC** tc	+	73
Oxytocin[e] (Neurophysin I)	Human	−164 to −146	gg **GGTCA** agg **TCACC** gc	−	74
Hageman factor XII	Human	−45 to −29	tt **GGTCA** agc **TGCCC** tc	−	75
Complement 3	Human	−235 to −22	ag **GGTCA** ggg **CCACC** tg (2 other estrogen-responsive regions whose sequences have not been precisely pinpointed)	−	76
Angiotensin	Human	−26 to −10	cg **GGTCA** cga **TGCCC** ta	−	77
Lipocalin 2 (NGAL)	Human	−916 to −800	ga **GGTCA** ctg **AGACC** at	−	78
Cathepsin d[f] (also Table 3)	Human	−145 to −101[d] (multiple TSS)	cc **GGTCA** cgt **GGGCG** cg	−	79–81
Hepatocyte Growth Factor	Mouse	−872 to −860	aa **GGTCA** gaa **AGACC** at	+	82
Uteroglobin (also Table 4)	Rabbit	−265 to −252	ca **GGTCA** cca **TGCCC** tc[g]	+	83
Calbindin D9K	Rat	+50 to +66	ca **GGTCA** ggg **TGATC** tt	+	84, 85
Oxytocin receptor	Rat	Roughly −4000	tg **GGTCA** tct **GGACC** aa	−	86
EREs with no consensus half-site or with unusual spacing					
TGFα (also Table 3)	Human	−252 to −200	gg **GGTCA** gctg **TGCCC** cg	+	88, 89
			gc **GGCTA** ccg **TCACC** tc	−	
VEGF (also Table 4)	Human	−1527 to −1511	cc **AGTCA** gtc **TGATT** at	−	90
HMG CoA reductase	Rat	−93 to −81	gc **CGTCA** ggc **TGAGC** ag	+	91
Prolactin[h]	Rat	−1581 to −1569	tt **TGTCA** cta **TGTCC** ta	+	61
Progesterone receptor	Rat	+617 to +629 (relative to distal promoter)	tc **AGTCA** tga **CGACC** cg	−	92, 93
VEGF	Rat	+395 to +411	ag **GGGCA** aag **TGACT** ga	+	94
		3′UTR	ga **GGGCA** ggg **TGCTC** tt	−	
LH B	Rat	−1173 to −1159	at **GGACA** gatgg **TGTCC** cg	+	95

Note that ERE sequences are denoted in *red* in the figures.

[a] The locations of the estrogen-responsive regions were obtained from the references indicated.

[b] The ERE-containing sequences are oriented such that the consensus half-site is on the *left*.

[c] For ERE-containing regulatory regions "+" indicates on coding strand; "−" indicates on noncoding strand.

[d] The locations of these estrogen-responsive elements are listed relative to the translation initiation codon, often due to presence of multiple transcription start sites (TSS). See supplemental Table 2 for relative position of TSS.

[e] A recent report suggests that the estrogen-responsive element in the oxytocin promoter may not be a true binding site for ER, but instead for other factors (118).

[f] The human cathespin D gene requires the factor USF for maximal activation by estrogen (62).

[g] The sequence from the GenBank accession number (see supplemental Table 2) does not precisely agree with the sequence from the literature referenced.

[h] The rat prolactin gene requires the factor Pit-1 for maximal activation of the full ERE (61).

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Table 2. Genes Regulated by ER that Contain Half-ERE or SFRE Sequences

Gene	Organism	Estrogen-Responsive Region[a]	ER Binding Sequence(s)[b]	Strand[c]	Ref.
c-H-ras1	Human	+49 to +78[d] (multiple TSS) multiple half-sites	ggttctg**GATCA**gctggat**GGTCA**gcgcactctt	−	96
ERα (P1 promoter)	Human	−892 to −420 three half-sites	tggttcg**TGACC**Atgaggttatgtttggtatgaaaa**GGTCA**catttta N_{422} ccccag**GGTCA**tcctatg	+	55
Prothymosin α	Human	−1051 to −750 two half-sites	acggcag**TGACC**gctcgggacagac N_{276} ggcctcg**TGACC**tcgttgctcgtcg	+	54
NMDA receptor, 2D subunit	Human	3′-UTR multiple half-sites	human: g**GGTCA** N_{159} a**GGTCA** N_{59} g**GGTCA** N_{29} g**GGTCA** N_{7} g**GGTCA**gg**GGTCA** N_{15} **TGACC**a N_{25} g**GGTCA** N_{30} g**GGTCA**	−	67, 97
	Rat		rat: g**GGTCA** N_{99} a**GGTCA** N_{47} g**GGTCA**tcgaggtct N_{23} g**GGTCA** N_{23} g**GGTCA** N_{34} g**GGTCA**		
Lactoferrin (also Table 1A)	Human	SFRE (within −414 to +69)	tcaa**GGTCA**tc	+	72
Osteopontin	Mouse	−736 to −728	taaa**GGTCA**	+	38
		−708 to −700	tcag**GGTCA**		
		−571 to −563 (all SFREs)	tcca**GGTCA**		

ER binding sequences appear in *blue* in the figures.
[a] The locations of the estrogen-responsive regions were obtained from the references indicated.
[b] The half-ERE-containing sequences are oriented such that the consensus half-site is on the *left*.
[c] For half-ERE-containing regulatory regions "+" indicates on coding strand; "−" indicates on noncoding strand.
[d] The locations of these estrogen-responsive elements are listed relative to the translation initiation codon, often due to presence of multiple transcription start sites (TSS). See supplemental Table 2 for relative position of TSS.

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Table 3. Genes Regulated by ER that Contain Half-ERE Sequences in Proximity to Sp1-Binding Sites

Gene	Organism	Estrogen-Responsive Region[a]	Sp1 Sites and Half-ERE Sequence(s)	Ref.
TGFα (also Table 1)	Human	−625 to −581	c**CCCGCC**cc 30 bp a**GGTAA**	88, 89
Cathepsin D[b] also Table 1	Human	−199 to −165[c] (multiple TSS)	**GGGCA** 23 bp **GGCGGG**	79–81
RARα	Human	−82 to −62	**GGTGA** ttggtcggtg **GGCGGG**	98
Progesterone receptor (A)	Human	+565 to +601 (relative to PR B promoter)	**TGACC** agc **GCCGCC** ctcc **CCCGCC** c	99
Creatine kinase B (also Table 4)	Rat	−568 to −523	ttaggg**CCCGCC**c aa**GGTCA**gaaCACCCtg ggtgcttccg**GGCGGG**acc	100, 101

Sp1 sites and half-ERE sequences appear in *purple* in the figures.

[a] The locations of the estrogen-responsive regions were obtained from the references indicated.

[b] The human cathespin D gene requires the factor USF for maximal activation by estrogen (62).

[c] The location of this estrogen-responsive element is listed relative to the translation initiation codon, due to presence of multiple transcription start sites (TSS).

Biological Relevance of Specific ERE Sequences. The consensus ERE was initially described based on the estrogen-responsive sequence in the *Xenopus laevis* vitellogenin A2 promoter: 5′-GGTCANNNT-GACC-3′ (39). As is true for most DNA-binding transcription factors, only a fraction of the known mammalian estrogen-responsive palindromic EREs reflect this consensus, instead consisting of variations on this sequence (Table 1). In a synthetic promoter context, in which the promoter activity is due mainly to the inserted ERE, the affinity of the ER-ERE interaction can reflect the degree of transcriptional activation by ER (40, 41). However, within native promoter contexts, the affinity of ER to the respective ERE is not the major determinant of the degree of stimulation of the gene by estrogen. Specifically, upon comparison of reported induction by different promoters in reporter gene assays, no correlation was discernible between the fold induction and either the degree of similarity of an ERE to the consensus sequence or the type of direct ER-binding site [data not shown; (32)]. Furthermore, the degree to which liganded ER activates any particular promoter is often cell type dependent. Thus, the overall context, including the other transcription factor-binding sites in the promoter/enhancer, the chromatin structure of the regulatory region, and the spectrum of cellular coregulators, is critical for translating the binding of ER into activation of gene expression.

Despite the potential continuum of simply decreasing affinity of dimeric ER with sites ranging from consensus EREs to half-EREs, the ERE is not only a quantitative determinant of the affinity of the interaction, but also a qualitative determinant of the conformation of bound ER. Protease digestion patterns and reactivity to peptide libraries have demonstated highly specific alterations in the conformation of the receptor when bound to different response element sequences (40–44). In fact, different coactivators can interact with ER, depending on the DNA site to which it is bound (44). Because each specific direct binding site can act as a differential allosteric modulator of ER conformation, the ERE is one determinant of the overall promoter context, and the specific sequence to which ER binds could therefore contribute to differential responses to hormone.

Response Elements in which ER Association with DNA Is Indirect

Another category of estrogen-responsive DNA sequences contain no sequence similarity to the ERE and do not directly interact with ER. The promoters that lack any ERE-like sequences require a second DNA-binding transcription factor to mediate ER association with the DNA, and make up roughly 35% of the categorized human primary responsive genes (Table 4). In nonhuman mammalian species, known estrogen response sequences are predominantly direct ER-binding sites, with 10 of 15 promoters containing a palindromic ERE element (Table 1) and another three promoters containing nonpalindromic ER-binding sites (Tables 2 and 3). Therefore, considering known response elements, human regulatory regions appear to be more diverse in the sequences responsible for estrogen response.

Of the human genes in which indirect binding of ER can result in estrogen regulation, Sp1 is the predominant mediator, implicated in 12 of 13 known regulatory regions (Table 4). In response to estrogenic stimulation, Sp1 binds its site in the estrogen-responsive DNA-regulatory region, with ER enhancing the binding of Sp1 to the DNA and contributing to coactivator recruitment; the DNA-binding domain of ER is dispensable for such activation (for references, see Table 4). Other intermediary factors through which ER can associate with promoter/enhancers include: activating transcription factor (ATF)-2/c-*jun* or ATF-2/cAMP-response element binding protein (CREB) for the cyclin D1 gene, ATF-1/CREB for the Bcl2 gene, activator protein 1 (AP-1) (jun/fos) for the IGF-I gene, and nu-

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Table 4. Genes Regulated by ER that Contain Sp1 or Non-Sp1-Regulatory Sequences through which ER Indirectly Associates

Gene	Organism	Estrogen-Responsive Region[a]	Intermediary Transcription Factor(s)	Binding Site(s) for Intermediary Transcription Factor(s)	Ref.
Adenosine deaminase	Human	−79 to −73	Sp1	gagggcgggc	102
IGFBP-4	Human	−569 to −540	Sp1	cgGGGGCGGGag	103
		−83 to −54		cgCCCGCCCCgg	
Hsp27	Human	−105 to −84	Sp1	agGGGCGGcc	104
Thymidylate synthase	Human	−150 to −142[b] multiple TSS)	Sp1	gaagaggcggagcg	105
EGF receptor	Human	−110 to −84[b] (multiple TSS)	Sp1	cttcgcgtccgcccgagtcccc gcctcgccgccaacgcca[c]	106
LDL receptor	Human	−142 to +35	Sp1	aaactcctcctcttgc 35 bp aaactcctccccctgc	36
RAGE	Human	−189 to −166	Sp1	GAGGCTGGTagtacccaGGGGTGG	107
c-fos	Human	−1168 to −1161	Sp1	ggggcgtggc	108
Progesterone receptor (B)	Human	−80 to −34	Sp1	taGGGAGGGGCtttGGGCGGGGCct	109
VEGF (also Table 1)	Human	−66 to −47	Sp3, Sp1 (inhibition)	tcccggcggggcggagccatg	110
Uteroglobin (also Table 1)	Rabbit	−229 to −222	Sp1	ctt gccacacccct gc[c]	83
Bcl2	Human	−1603 to −1534	Sp1, ATF-1/CREB	cgggct 8 bp ggagggc 28 bp gtgtgacgtt[c]	111
Cyclin D1	Human	−143 to −110	Sp1	cgcccgcgccccctccccc tgcgcccgccccccgc	112, 113
		−96 to −29	ATF-2/c-jun, CREB/ATF-2	agtaacgtcaca	
E2F1	Mouse	−146 to −54	Sp1, NF-Y	cggccaatgga [CCAAT box] 3 Sp1 sites upstream of 2 CCAAT boxes	114
IGF-1	Human	−324 to −124[b]	AP-1	ttgtcaccatgcccaaaaaagtcct tactcaataactttgccagaagagg gagagagagagaaggcaaatgttcc cccagctgtttcctgtctacagtgt ctgtgttttgtagataaatgtgagg attttctctaaatccctcttctgtt tgctaaatctcactgtcactgctaa attcagagcagatagagcctgcgca a	115, 116
Brain creatine kinase (also Table 3)	Rat	−95 to −46	Multiple factors required	ccaat 13 bp tataaata 4 bp ccaat	117

The binding sites for intermediary transcription factors appear in *green* in the figures.

[a] The locations of the estrogen-responsive regions were obtained from the references indicated.

[b] The locations of these estrogen-responsive elements are listed relative to the translation initiation codon, often due to presence of multiple transcription start sites (TSS). See supplemental Table 2 for relative position of TSS.

[c] The sequence from the GenBank accession number (see supplemental Table 2) does not precisely agree with the sequence from the literature referenced.

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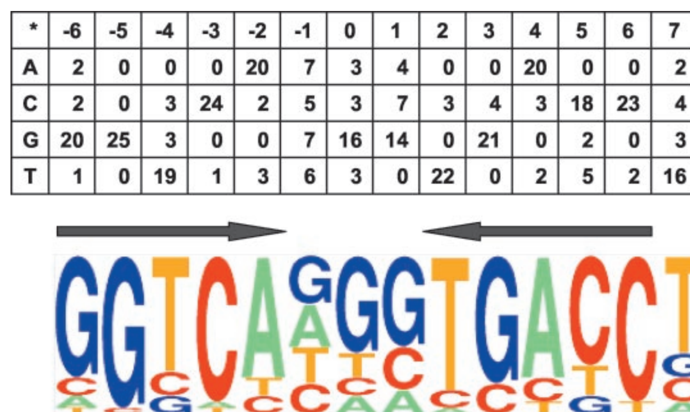

*	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7
A	2	0	0	0	20	7	3	4	0	0	20	0	0	2
C	2	0	3	24	2	5	3	7	3	4	3	18	23	4
G	20	25	3	0	0	7	16	14	0	21	0	2	0	3
T	1	0	19	1	3	6	3	0	22	0	2	5	2	16

Fig. 1. Count Matrix and Pictogram Obtained from Alignment of 25 EREs

The optimal alignments of each sequence were determined computationally, as described in the text. In this alignment, some EREs were reversed relative to the direction shown in Table 1: pS2, lactoferrin, keratin 19, angiotensin, lipocalin 2, cathepsin D, hepatocyte growth factor, uteroglobin, and oxytocin receptor. The positions in the ERE are labeled from -6 to $+7$; positions beyond this to either side did not demonstrate a sequence preference. The numbers in the body of the matrix give the counts of each nucleotide observed at each position in the alignment. The graphical representation of the ERE count matrix is presented according to the guidelines at http://genes.mit.edu/pictogram.html.

clear transcription factor-Y for the mouse E2F1 gene (Table 4). The DNA recognition sequences for the AP-1 family of transcription factors are related, but not identical, to those for the ATF/CREB transcription factors. It has been suggested that AP-1-mediated estrogen-responsive genes contribute to the tissue-specific responses to estrogen analogs, via differential activation of ERα and ERβ (45–47). Proof of this hypothesis awaits demonstration of additional AP-1-dependent estrogen-responsive genes.

Overall, the ability of ER to associate indirectly with promoter/enhancers through binding to other transcription factors dramatically expands the set of target genes capable of responding to estrogen induction and may also determine how estrogen responsiveness can be modified in different cellular environments.

Given the wide diversity in EREs, we considered whether distributions of distances from the transcription initiation sites would vary with the type of sequence elements. Distances were deduced either from experimental determinations of the transcription start sites or, when these values were not available, were computed using databases of expressed sequence tag and cDNA sequences (see supplemental Table 2, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org, for details and specific values). Upon plotting such distributions of distances, all are in fact similar, with binding sites concentrated in the few hundred bp upstream of the transcription initiation sites, and more scarce at greater distances (see supplemental Fig. 5, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). We note that these distributions do have potential for bias, in that investigators may have focused on the immediate upstream region when searching for functional sites. However, the presence of a number of distant sites suggests that the bias may not be overwhelming.

COMPUTATIONAL ANALYSES OF ER-BINDING ELEMENTS

This compilation of mammalian ER-responsive elements allowed analysis of the sequence requirements for transcriptional regulation mediated by direct contact of ER with DNA, to extend known patterns of sequence requirements. As expected, when ER directly interacts with the promoter/enhancer, binding to a full ERE is apparently the dominant mode of interaction (Table 1). Of the 27 full EREs listed, all but two have a 3-bp spacer between the two half-sites, the exceptions being response elements in the human TGFα promoter, with a 4-bp spacer, and in the rat LH B promoter, with a 5-bp spacer. As ER does not significantly bind ERE half-sites separated by 2, 4, or 5 bp *in vitro* (32), ER may recognize these latter two elements as single half-sites, or as EREs with a 3-bp spacer and one extremely degraded half-site. We favor the latter case, as even half-EREs may actually represent degenerate full EREs (see below). An alternative model, however, is that DNA bending *in vivo*, caused by other factors, may allow ER to recognize such longer spacers (48).

Analysis of the full EREs with a 3-bp spacer indicate possible evolutionary pressure for an ERE to contain at least one consensus, or perfect, half-site. Considering the consensus half-site of 5′-ggtca-3′ (or its complement), three of the full EREs with a 3-bp spacer have two perfect half-sites, 15 have one perfect half-site (oriented in Table 1 with the con-

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sensus half-site on the *left*), and seven have only imperfect half-sites (oriented in Table 1 with the half-site most similar to the consensus on the *left*). This indicated slightly more pairings of one perfect with one imperfect half-site than expected by randomly pairing 21 perfect with 29 imperfect half-sites. We also observed that the central base pair in the 3-bp spacer exhibits a striking asymmetry. When the perfect half-site is aligned on the *left*, a G:C generally occupies the central position in the spacer, generally with the G on the same strand as the GGTCA.

Sequences of a *cis*-element are often represented by a matrix of numbers. The simplest construction is the count matrix (49), which records counts of the four nucleotides observed in each position in an alignment of *cis*-elements. For palindromic *cis*-elements (*e.g.*, the ERE), the sequences must be aligned without bias, so as to maintain asymmetric properties. Using a Gibbs sampling algorithm (50, 51) that includes an automatic width determination (52), either the direct or the complementary strand for each ERE with a 3-bp spacer was optimally aligned. The resulting count matrix and pictogram for the ER recognition sequence are shown in Fig. 1. As was suggested by manual alignment (above), the central spacer position significantly prefers a G in the orientation shown, as does one additional spacer position. In agreement with previous reports (32, 53), an A is also preferred preceding the ggtca, but only for one of the half-sites (shown here as a T at the end of the sequence); no preferences are apparent at more distant positions.

Although some regulatory regions responding to ER are reported to contain only ERE half-sites, ERα does not avidly bind individual half-sites *in vitro* (32). Only at high concentrations can binding be observed (54, 55). Thus, binding *in vivo* could be due either to the presence of cryptic, full EREs in the regulatory regions, to highly cooperative binding between two half-EREs (37), or to cooperative binding with unrelated transcription factors. Using the ERE matrix represented by Fig. 1 and the matrix comparison algorithm Possum (http://zlab.bu.edu/~mfrith/possum/), we investigated whether cryptic full EREs could be identified in regulatory regions containing SFREs or multiple ERE half-sites. Indeed, potential, weak full EREs were discovered overlapping or within the ER-binding regions in almost all cases. Examples include agctga tcc agaacc for human c-H-ras1 and agacct cga tgaccc for rat NMDA receptor 2D subunit (*underlined*, Table 2). Only for the regulatory regions of the human NMDA receptor 2D subunit and human ER genes were the predicted full EREs somewhat questionable (for details of all predicted sites, see supplemental Table 3, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). The relevance of such sequences, however, remains to be experimentally determined.

Rules for Relative Orientations of ERE Half-Sites to Sp1-Binding Sites

The primary ER-regulated promoters include five examples in which interaction of ER with an ERE half-site is assisted by binding of Sp1 to a nearby GC-rich motif (Fig. 2). Somewhat unexpectedly, there appears to be no consistent pattern regarding the relative orientations or positions of these sites. In most cases, the sequence ggtca of the half-ERE is on the same strand as ggcggg of the GC-rich motif, but not in all. The distance between the sites, as well as the position and strandedness of these elements relative to the transcription start site, is variable. These observations suggest that ER and Sp1 do not form a rigid complex on the DNA sequence, but rather a flexible structure, perhaps facilitated by hinge regions in the proteins.

Summary: Directly Interacting ER-Response Sites

In conclusion, transcriptional regulation by direct interaction of ER with DNA is predominantly mediated by full EREs containing two inverted half-sites, separated by a 3-bp spacer. Our newly constructed ERE matrix (Fig. 1) should assist in identifying novel, biologically relevant EREs. In a few cases, ER can bind to half-sites when assisted by the binding of Sp1 to nearby GC-rich motifs. However, in other circumstances, reported half-EREs may either bind ER cooperatively, or actually reflect more divergent full EREs.

LIMITED EVOLUTIONARY CONSERVATION OF ESTROGEN RESPONSE SEQUENCES BETWEEN HUMAN AND MOUSE

Additional information regarding gene-regulatory sequences can sometimes be inferred from conservation

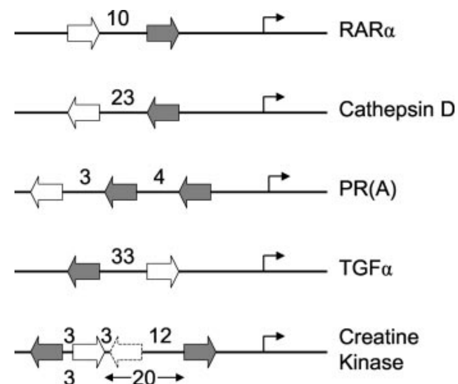

Fig. 2. Schematic of the Organization of *cis*-Elements in Five Sequences in which ER and Sp1 Jointly Interact with DNA

The *white arrows* indicate the ERE half-site sequence 5′-ggtca-3′; the *gray arrows* indicate the GC-rich motif sequence 5′-ggcggg-3′; the *numbers* indicate the distances between sites (bp). The *thin hooked arrows* indicate the transcription start site.

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of sequences between evolutionarily related species. Given the availability of mouse genome sequence, we compared estrogen-responsive regulatory regions from human with those of mouse. Conservation of both direct and indirect ER-binding sites was analyzed using the whole-genome human-mouse alignments generated by the Mouse Genome Sequencing Consortium (http://genome.ucsc.edu/) (56, 57). Such alignments cover about 40% of the human genome (the remaining 60% corresponding to lineage-specific insertions or deletions); it is therefore noteworthy that 35 of 42 estrogen-responsive human binding sites are contained completely within aligning regions between the human and mouse genomes (supplemental Table 3).

For evaluation of whether or not a specific estrogen response sequence is evolutionarily conserved, the preferable method is to determine the extent to which the aligned mouse sequence matches a count matrix for the transcription factor, such as that for ER or Sp1. Simple sequence conservation does not prove functional conservation, as a single base pair change in a position critical for DNA-protein recognition may abolish the biological function and, conversely, divergent sequences can bind a transcription factor with similar affinities. Using the matrix comparison tool Possum, which determines probabilistic scores [log (base e) likelihood ratios for the site *vs.* background] that a sequence represents a specific transcription factor binding site, we compared predicted binding probabilities for both human and mouse aligned estrogen-response sequences. For the set of palindromic ERE sites (using Fig. 1 as the matrix), only four of the 16 human sites are similar in mouse, 11 are absent or severely degraded, and one is borderline in the degree of conservation of a functional ERE (supplemental Table 3A). Thus, fewer than one third (31%) of these EREs should function in mouse. This level of conservation is strikingly low compared with protein-coding genes, 80% of which are conserved as 1:1 orthologs between these species (57). Of the other types of estrogen-responsive binding sites, only one in four (25%) ERE + Sp1 sites and seven of 13 (54%) Sp1 sites were functionally conserved (supplemental Table 3B). The statistical significance of the apparent differences in degrees of conservation among different types of response sequences cannot be established, due to the low sample sizes. Consistently, however, the degree of conservation of estrogen-response sequences is remarkably low. In contrast, in another human-mouse comparison study, 60–68% of a variety of functional human transcription factor-binding sites remained functional in mouse (58).

Some of the most notable differences between the human and mouse genomes, in fact, involve gene families associated with reproductive physiology (57). This physiological divergence could explain the degree of conservation of estrogen response sequences being lower than that for other regulatory programs. An alternative viewpoint is that many of these genes may be similarly regulated in human and mouse, despite the low degree of conservation of specific sites, due to binding site turnover. In other words, mutations that weaken one binding site may have been compensated by mutations that strengthen an alternative binding site (58, 59). In other evolutionary studies, enhancer elements encompassing many binding sites may exhibit significant functional and sequence conservation, whereas individual binding sites within them are not notably conserved (Ref. 60 and E. Davidson, personal communication).

To assess this possibility, the degree of conservation between human and mouse promoter sequences was plotted for 2-kb regions surrounding each human ERE. These conservation graphs (http://genome.ucsc.edu/) (56) take into account the rate of neutral evolution (representative plots are in Fig. 3A; method details and all graphs in supplemental Fig. 6 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). The y-axis represents a log scale of the degree of conservation, with an increasing score indicating greater similarity of sequence between human and mouse. For some EREs, particularly where binding sites are functionally conserved between human and mouse (as determined by matrix scores), visual examination suggests that the estrogen-responsive sites are located as anticipated within peaks of similarity between mouse and human sequences (*e.g*. EBAG9, adenosine deaminase, c-H-ras1). However, exceptions are noted where functionally conserved binding sites are located in regions of limited sequence similarity (*e.g*. ERE in lactoferrin). Sites that are not functionally conserved (by matrix comparisons) are predominantly located in regions of low conservation or in unalignable regions (*e.g.* complement 3, c-*fos*, SFRE in lactoferrin, one Sp1 site in IGF-binding protein 4). However, again exceptions appear, where nonconserved binding sites reside in regions of overall similarity (*e.g*. the other Sp1 site in IGF-binding protein 4, angiotensin).

For a more precise determination of the relationship between functional conservation of binding sites and conservation of the surrounding regulatory sequences, we compared the degree of human-mouse conservation of the site itself with the average sequence conservation of 100 bp of flanking sequences (50 bp to either side), for all regulatory regions that could be aligned between human and mouse. By plotting the matrix score (representing the strength of the binding site) of the human palindromic ERE-regulatory sites against the ratio of sequence conservation of the ERE to its flanking sequences, there appeared a general trend that the stronger the binding site, the more its sequence was conserved during evolution relative to flanking sequences (Fig. 3B). More strikingly, for sites predicted to be functionally conserved from human to mouse (*solid diamonds*), the sequence conservation in the site itself was always greater than the sequence conservation of the surrounding sequences (mean ratio of 6.9, indicating that

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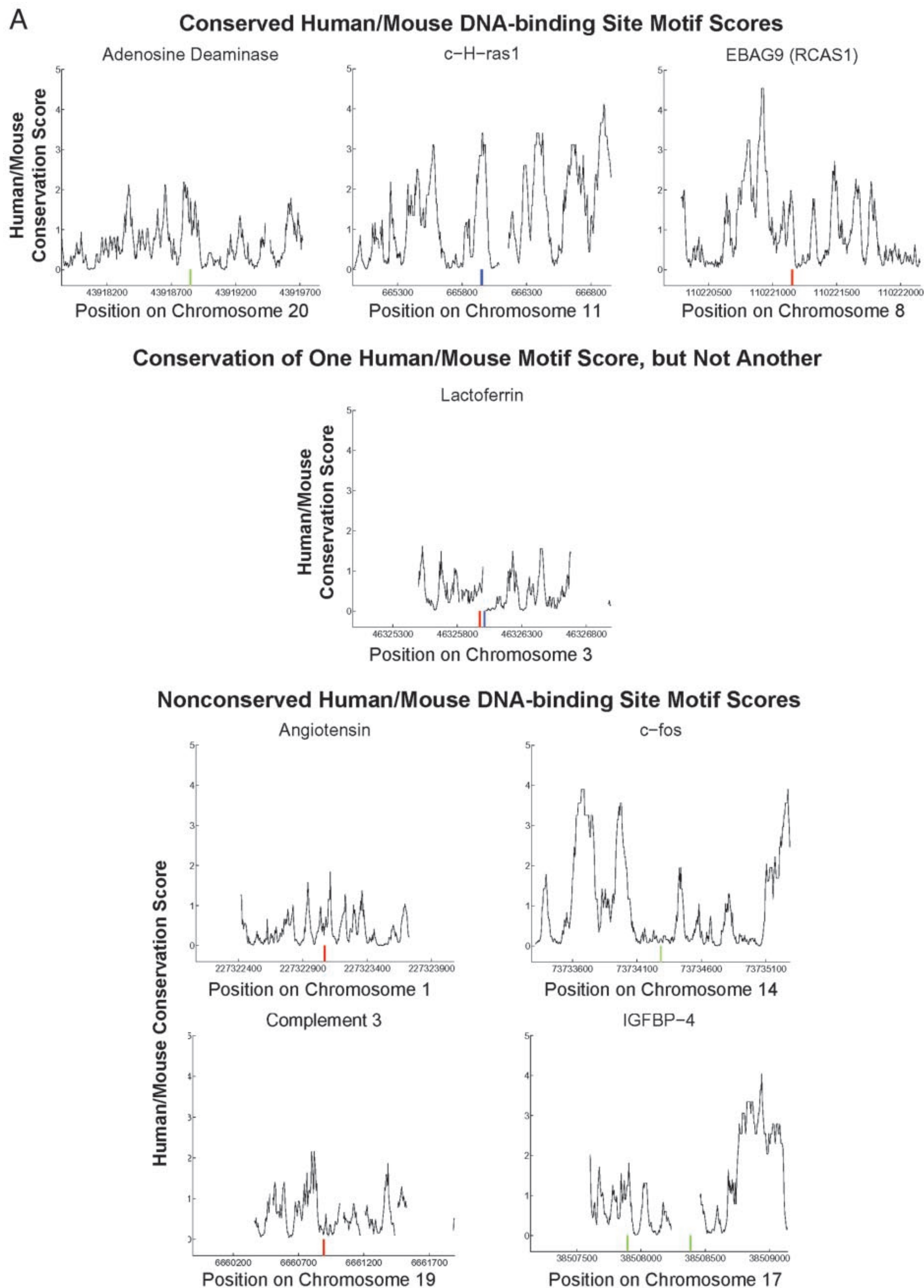

Fig. 3. Sequence Conservation between the Human and Mouse Genomes in Regions Surrounding the Human Estrogen-Responsive Sites

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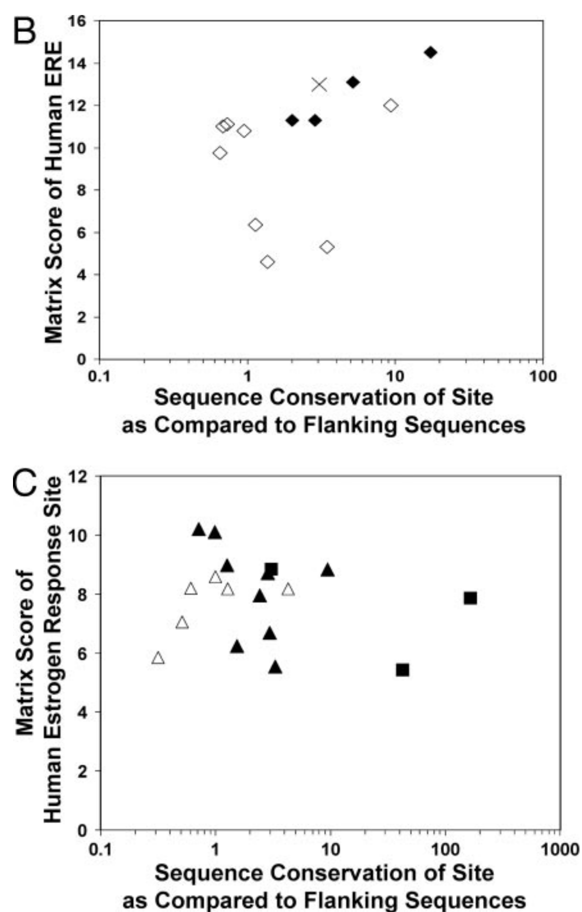

Fig. 3. *Continued*

A, Representative examples of sequence conservation in 2-kb regions surrounding sites that associate, directly or indirectly, with ER. The *curves* reflect the probability that the degree of conservation observed in 50-bp windows would occur by chance under neutral evolution (see supplemental data and http://genome.ucsc.edu/) (56). The degree of conservation is presented as $-\log(\text{probability})$; the maximum value of the y-coordinate of 5, indicating the greatest conservation between human and mouse, corresponds to a probability of 10^{-5}. *Breaks in the curve* indicate regions of the human genome that could not be aligned to mouse by Blastz. Positions of binding sites are indicated with *colored blocks*: *red*, full ERE; *blue*, SFRE or half-ERE; *purple*, ERE/Sp1; *green*, indirect ER-binding sites. B, EREs predicted to be functionally conserved from human to mouse are more highly conserved at the sequence level than flanking sequences. For each ERE, the relative mouse-human sequence conservation of the ERE compared with the average sequence conservation of its 100 bp of flanking sequence (50 bp to either side of the site) was determined (see supplemental data for details). Estrogen-responsive sites predicted to be functionally conserved from human to mouse were identified by comparing the functional conservation scores [log (base e) likelihood ratios] for EREs in the human sequence and the aligned mouse sequence; likelihoods were calculated using the Possum algorithm and the count matrix for ERE shown in Fig. 1 (see supplemental Table 3). Mouse sequences with site matrix scores similar to the human scores (likelihood within 10-fold) were taken to be functionally conserved; those in which the likelihood of representing a binding site was 100-fold less than the human site were defined to be functionally nonconserved. *Solid diamonds*, Functionally conserved EREs; *open diamonds*, functionally nonconserved EREs; *cross*, functionally borderline ERE. By the Wilcoxon one-tailed test, the nonconserved sites displayed relative sequence conservation (conservation of the site compared with flanking sequences) significantly less than that of the conserved sites ($P < 0.036$). C, Other estrogen-response sites (exclusive of palindromic EREs) predicted to be functionally conserved from human to mouse are more highly conserved at the sequence level than flanking sequences. The analysis shown in panel B was repeated for non-ERE, estrogen-response sites. The functional conservation of the sites was determined by comparison against count matrices for the relevant transcription factor-binding sites, as available through TRANSFAC (see supplemental data). *Solid triangles*, Functionally conserved Sp1 sites and half-ERE/Sp1 sites; *open triangles*, functionally nonconserved Sp1 sites and half-ERE/Sp1 sites; *solid squares*, functionally conserved AP-1, ATF, CREB-binding sites. By the Wilcoxon one-tailed test, the nonconserved sites displayed relative sequence conservation values (conservation of the site compared with flanking sequences) significantly less than that of the conserved sites ($P < 0.021$). Using all the data in panels B and C, an even more significant difference was observed in the relative sequence conservation for functionally conserved *vs.* nonconserved estrogen-response sites, $P < 0.0026$ by the Wilcoxon one-tailed test.

sequence conservation in the ERE site is, on average, 6.9-fold higher than that in ERE-flanking sequences). In contrast, sequences for functionally nonconserved sites were maintained, on average, approximately to the same extent as their surrounding sequences (*open diamonds*; mean ratio of 2.3). These differences in the relative conservation of the site to flanking sequence between the two groups is statistically significant ($P < 0.036$). A similar analysis of other types of estrogen response sites (Sp1 sites and half-ERE/Sp1 combinations, *triangles*; and AP-1, ATF, CREB binding sites, *squares*; Fig. 3C) provided a comparable conclusion: for the group of sites predicted to be functionally conserved, 10 of 12 showed higher conservation of their specific sequences than their surrounding sequences (*solid triangles* and *squares*; mean ratio of all sites of 20), whereas for functionally nonconserved sites, only two of six were more highly conserved at the sequence level than surrounding sequences (*open triangles*; mean ratio of 1.3). This is consistent with the loss of functionality of these sites across evolution. Once again, the difference in these groups is statistically significant ($P < 0.021$). These results suggest that there is significant evolutionary pressure to specifically preserve biologically relevant transcription factor binding sites, as opposed to the entire surrounding regulatory region. Conversely, where there is no biological imperative, the degree of sequence conservation in the binding sites may simply reflect that of flanking regions.

Overall, there is limited association of estrogen-responsive binding sites with regions of striking human-mouse sequence conservation. We conclude that either hormonal regulation of these genes is mod-

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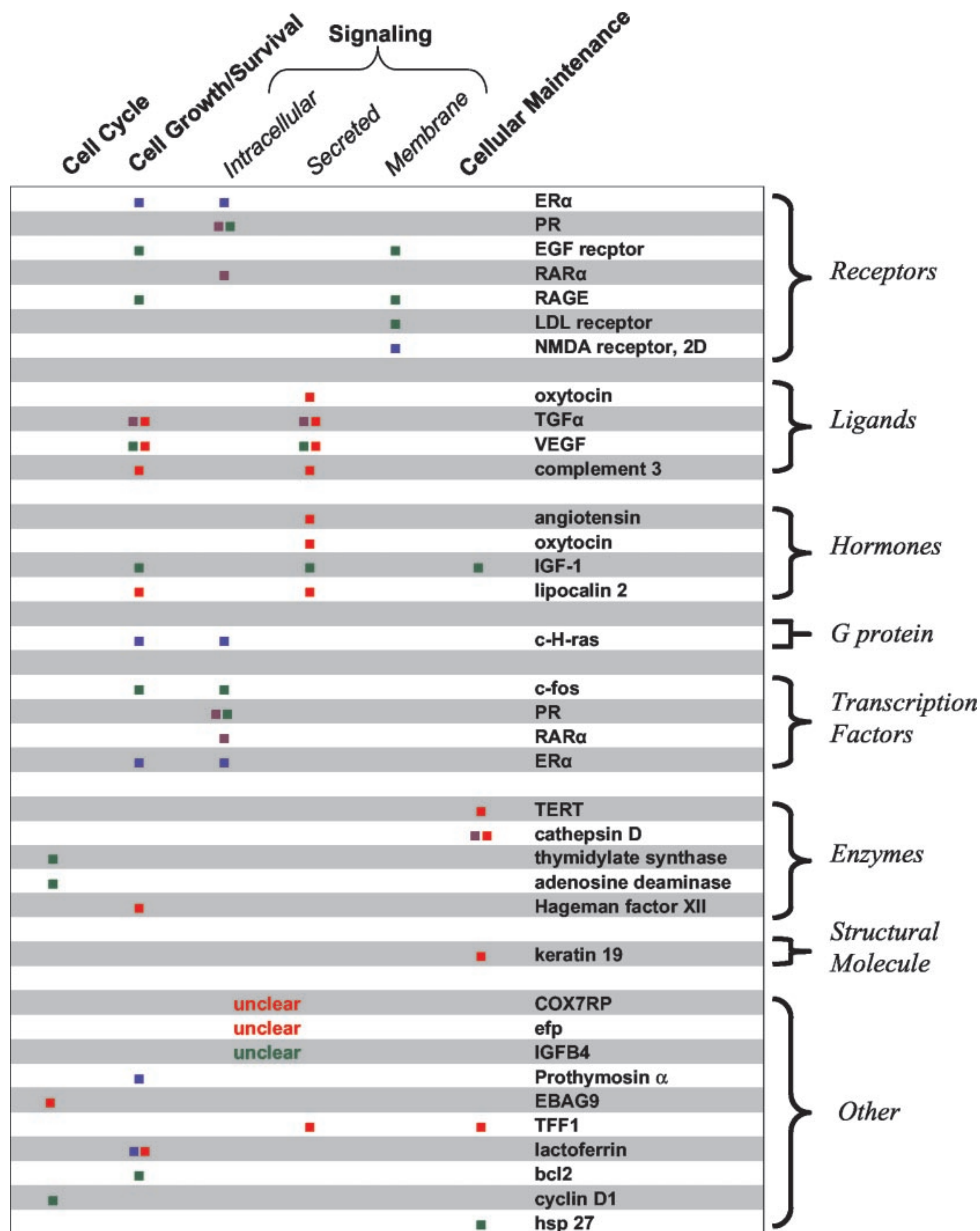

Fig. 4. Protein Functions and Cellular Roles of Human Gene Products Regulated by ER, as Related to the Types of EREs in Their Regulatory Regions

Binding sites are indicated with *colored boxes* in the grid, as follows: *red*, full ERE; *blue*, SFRE or half-ERE; *purple*, ERE/Sp1; *green*, indirect ER-binding sites. Protein functions are listed to the *right* of the grid, and cellular functions are listed across the *top*. Descriptions of the products were obtained mainly from LocusLink (http://www.ncbi.nlm.nih/LocusLink). Genes were grouped into four categories of cellular function based on the following considerations. 1) Cell Cycle: gene products directly involved in regulation of cell cycle, as well as those required for or involved in cell cycle progression, such as in biosynthesis of nucleotides. 2) Cell Growth, Cell Survival: gene products involved in signaling pathways that promote cell growth and/or survival, including those exerting protective effects against cell death caused by any insult. 3) Signaling molecules (intracellular, secreted, or membrane associated): gene products directly involved in a cellular signaling pathway, either as a ligand, as a membrane-bound or intracellular receptor involved in transmission of the signal, or as an effector in the signaling pathway. 4) Cellular Maintenance: gene products involved in cellular processes contributing to normal cell function.

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ified significantly between human and mouse, or that selection for conserved regulatory function by estrogen imposes such weak sequence constraints that it is not readily detectable by sequence conservation.

POTENTIAL CORRELATIONS OF TYPES OF ESTROGEN-RESPONSE SEQUENCES TO CELLULAR FUNCTIONS OF REGULATED GENES

The identified primary human ER-regulated genes (Tables 1–4) encode diverse types of protein products, including receptors, ligands, G proteins, transcription factors, enzymes, chaperones, and structural molecules. These proteins function in a multitude of cellular pathways, serving a variety of cellular roles: cell cycle progression, cellular maintenance, cell growth and survival, and signaling cascades. In general, the set of primary target genes induced in any particular circumstance would initiate a cascade of downstream events, leading to the overall physiological changes induced by estrogen.

We probed the hypothesis that particular types of elements may generate a coordinated cellular response, such as cell cycle progression, cell growth and/or survival, or cellular maintenance. In particular, we searched for correlations between the types of estrogen-response sequences regulating the known human primary target genes (color coded according to the type of response sequence) and either the biochemical functions of these gene products (Fig. 4, categories to the *right*) or the cellular functions of the gene products (Fig. 4, categories at the *top*). This analysis revealed several encouraging trends. 1) Of the four genes involved in cell cycle progression, three contain response elements in which Sp1 mediates ER binding; these three gene products also encode enzymes, or required subunits of enzymes. 2) Of the genes encoding secreted, signaling molecules, eight of nine are regulated by full EREs. 3) Of the genes encoding cell surface receptors, three of four genes are regulated through Sp1 sites. 4) Finally, induction of three of four genes encoding transcription factors requires Sp1, with or without associated ERE half-sites. The remaining functional categories of genes are more evenly distributed with respect to the types of response elements they contain. We note that both the types of genes the regulation of which has been studied and the types of estrogen-response sequences that have been uncovered may have been influenced by the goals of past researchers, and that the numbers are too small to garner statistical significance. Although these observations provide only clues and not proof, they nonetheless support the hypothesis that the expression of sets of functionally related, estrogen-responsive genes are coordinately regulated in many cellular settings, due to common motifs in their promoters. This would provide one mechanism for generation of the appropriate physiological responses to estrogen.

TOWARD AN UNDERSTANDING OF THE ESTROGEN GENOMIC RESPONSE

Coordinated expression of specific sets of target genes initiate the characteristic cell type-specific, long-term effects of estrogen in each tissue. The overall response is generated by cross-talk among cellular signaling pathways, the genomic regulation by liganded nuclear ERs, the nongenotropic estrogen pathway, and nuclear sequestration of secondary transcription factors by ER. Nonetheless, enumeration of primary ER response genes in each cell type is critical for uncovering the molecular basis of the tissue-specific physiological responses. The computational analyses described here suggest refinements to the understanding of DNA sequences directly binding ER, both in the details of canonical, palindromic EREs, and an alternative view for half-EREs. Furthermore, the cross-species comparisons suggest that estrogen responsiveness at a genomic level may be less conserved than for other transcriptional regulatory programs. Given the variability in types of estrogen-response sequences, an attractive hypothesis, partially supported by investigation of existing human target genes, is that estrogen regulation of a pathway of genes may relate to particular sequence contexts and response elements in those genes. In other words, the cell type and activity of cellular signaling pathways may differentially impact estrogen regulation, depending on the gene-regulatory elements.

For predictions of coregulated sets of genes involving nuclear ERs, the constellation of secondary transcription factor binding sites within the sequence context of the estrogen-responsive regulatory regions is also likely to be crucial. For some genes, transcription factors have already been experimentally identified as essential for regulation by ER: Pit-1 for the rat prolactin promoter (61), and upstream stimulatory factor for the human cathepsin D promoter (62). We and others have developed algorithms to identify binding sites that are overrepresented in regulatory regions from sets of coexpressed genes (52, 63–65). Armed with a group of binding sites important for estrogen regulation either in a particular cell type or in combination with a particular signaling cascade, regulatory regions containing clusters of transcription factor-binding sites can then be predicted using clustering algorithms (66). Combining such computational predictions with experimental testing of hypotheses should accelerate progress toward understanding the underlying molecular bases of the physiological responses to estrogen.

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