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Transcriptional regulation of bidirectional gene pairs by 17β-estradiol in MCF-7 breast cancer cells

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Abstract

Using cDNA microarray analysis, we previously identified a set of differentially expressed genes in primary breast tumors based on the status of estrogen and progesterone receptors. In the present study, we performed an integrated computer-assisted and manual search of potential estrogen response element (ERE) binding sites in the promoter region of these genes to characterize their potential to be regulated by estrogen receptors (ER). Publicly available databases were used to annotate the position of these genes in the genome and to extract a 5'flanking region 2 kb upstream to 2 kb downstream of the transcription start site for transcription binding site analysis. The search for EREs and other binding sites was performed using several publicly available programs. Overall, approximately 40% of the genes analyzed were potentially able to be regulated by estrogen via ER. In addition, 17% of these genes are located very close to other genes organized in a head-to-head orientation with less than 1.0 kb between their transcript units, sharing a bidirectional promoter, and could be classified as bidirectional gene pairs. Using quantitative real-time PCR, we further investigated the effects of 17β-estradiol and antiestrogens on the expression of the bidirectional gene pairs in MCF-7 breast cancer cells. Our results showed that some of these gene pairs, such as *TXNDC9/EIF5B*, *GALNS/TRAPPC2L*, and *SERINC1/PKIB*, are modulated by 17β-estradiol via ER in MCF-7 breast cancer cells. Here, we also characterize the promoter region of potential ER-regulated genes and provide new information on the transcriptional regulation of bidirectional gene pairs.

Key words: Breast cancer; Estrogen receptor; Gene expression; Bidirectional promoters

Introduction

Estrogens are crucial for the development and maintenance of reproductive organs and play important roles in several target tissues, including bone and the cardiovascular and nervous systems. In the mammary gland, the estrogens mediate key physiological processes that are essential for normal growth, differentiation, and survival. A large body of evidence shows that estrogens, especially 17β-estradiol (E_2), also play a pivotal role in promoting mammary carcinogenesis (1). Most of the complex effects of estrogens on cell proliferation, differentiation, and survival are mediated by the estrogen receptors ERα and ERβ via the transcriptional regulation of ER target genes. In the classical mechanism of action, the estrogen receptor complex binds with high affinity to estrogen response elements (EREs) in the promoter region of target genes, interacts with the basal transcription machinery, and regulates gene expression. The consensus ERE, which was first identified in the promoter region of the *Xenopus vitellogenin* gene, is a perfect palindromic DNA motif composed of two inverted sequences of 5 bp separated by three nucleotides: 5'-GGTCAnnnTGACC-3' (2). Computational and experimental studies have revealed that most promoter regions in estrogen target genes in the human genome do not contain the consensus ERE, but contain degenerate palindromic sequences showing one or more substitutions, ERE half-sites, direct repeats of half-palindromes, and other transcription factor binding sites, such as AP1, SP1, NF-κB, C/EBP, FoxA1, and octamers (2-5). ER also interacts with EREs containing variations in base substitution, although some nucleotide substitutions can be more harmful than others (6). Some symmetrical substitutions on each side of the palindrome may more dramatically affect the interaction with the ER than a unilateral

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replacement (6). ERE half-site sequences associated with SP-1 sites, direct repeats of ERE half-sites, and EREs with different spacing between the palindromic sequences also display estrogen responses (7-9).

Genome-wide analysis has indicated that 10% of human genes are organized in a head-to-head orientation and located on opposite DNA strands in close proximity to the transcription start sites (TSS), separated from them by less than 1 kb (10,11). These genes are classified as bidirectional gene pairs and share an intervening sequence containing regulatory elements called bidirectional promoters (12,13). Bidirectional gene pairs are evolutionarily conserved, suggesting that the close proximity might have been maintained for some functional reason (14). One possibility is that genes sharing promoters might be coordinately regulated in different tissues; however, the mechanisms underlying the transcriptional regulation of bidirectional promoters are still poorly understood (15,16). Much evidence indicates that bidirectional gene pairs might play a role in cancer; DNA repair genes that are involved in tumorigenesis are frequently found to be organized as bidirectional gene pairs (10,11). The expression profiles of ovarian and breast cancer show an enrichment of bidirectional gene pairs that include DNA repair genes, such as BRCA1, BRCA2, CKEK1, and FANC family members (17). Furthermore, the silencing of bidirectional gene pairs by DNA methylation has been reported in various human cancer cell lines and primary breast tumors (18-20).

In the present study, we performed an integrated computational and manual search to identify potential ER binding sites in the promoter region of 83 genes previously identified as being differentially expressed in primary breast tumors based on the presence of estrogen and progesterone receptors (21). We identified a subgroup of genes arranged in a head-to-head orientation and separated by a small intergenic region that could be classified as bidirectional promoters. Selected candidate ER target genes were further examined for their potential to be modulated by 17β-estradiol in MCF-7 breast cancer cells.

Material and Methods

ERE binding site search

The NCBI (http://www.ncbi.nlm.nih.gov), USCS Genome Bioinformatics (http://genome.uscs.edu) and ENSEMBL (http://www.ensembl.org) databases were used to annotate the position of 83 genes in the genome and to extract sequences 2 kb upstream to 2 kb downstream of the TSS for binding site analysis. The search for EREs and other binding sites was performed using several publicly available programs (Transfac 6.0, http://www.gene-regulation.com, and Dragon Genome Explorer, http://research.i2.a-star.edusg/promoter/). A list of ERE sequences was prepared from the reports extracted from PubMed and used for a manual search (6,22-24). The present study was approved by the Institutional Ethics Committee.

Cell lines and culture conditions

Human breast cancer cells, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (ATCC). Cells were cultured at 37°C in phenol red-free RPMI supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco, USA) in an atmosphere of 5% CO_2/95% air. Before the treatments, cells were washed twice with PBS to remove residual serum and were grown in phenol red-free media containing 5% charcoal-stripped FCS for 48 h. After hormone deprivation, the cells were incubated with 10 nM 17β-estradiol for 2, 6, and 24 h or treated with 1 µM 4-hydroxytamoxifen or 1 µM ICI 182.780 (Faslodex) alone or in combination with 17β-estradiol for 24 h. Cells maintained in phenol red-free media and supplemented with 5% FBS were used as controls.

RNA extraction

After the treatments, the cells were washed twice with PBS and harvested. Total RNA was extracted from cultured cells using the guanidine isothyocyanate method (25). RNA samples were analyzed using 1% agarose gel electrophoresis and ethidium bromide staining. The quality of the RNA samples was estimated by the ratio of 28S:18S (2.0 and higher). All RNA samples were treated with DNaseI for 1 h at 37°C to eliminate genomic DNA contamination.

Quantitative real-time PCR (qPCR)

qPCR was performed using the GeneAmp 7500 Sequence Detection System (Applied Biosystems, USA). cDNA was generated using the High Capacity cDNA Archive kit (Applied Biosystems), and each cDNA sample was analyzed in duplicate. PCR was carried out in a total volume of 50 µL according to manufacturer instructions for SYBR Green PCR Core reagent (PE Applied Biosystems). Primer pairs were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/). The primers used for qPCR analysis were as follows: *DIDO1*, 5'-AGAAAGCTGCAAATCCAAGTG-3' and 5'-GGCGTGTTTGAGGATACAGTC-3'; *C20orf11*, 5'-TTTGACAGTCCCGAGGAGTC-3' and 5'-GTGTTGACTCGCGATTTTCAT-3'; *TXNDC9*, 5'-GTGAAAATGTGGTTTGCCATT-3' and 5'-TGCTTTTTCCACATTCAGCTT-3'; *EIF5B*, 5'-AGATTCGAGGCCTCCTGTTAC-3' and 5'-GGGTAAACCAGCCAATGTTTT-3'; *GALNS*, 5'-CAGGATCACCTCGGTCGT-3' and 5'-GAATGGATTCTGGAGGTGTCA-3'; *TRAPPC2L*, 5'-GTCACCAACTCCAAGGTGAAG-3' and 5'-TTGTAGAAGGGGTTGCACATC-3'; *SERINC1*, 5'-GTGATGGATCACTGGAGGATG-3' and 5'-TCACGAGAGGGTTCATACCTG-3'; *PKIB*, 5'-CGGAATGCCTTACCAGACATC-3' and 5'-TTCTTCATTTTGAGGCTTTTCC-3'; *GTPBP2*, 5'-CCTACACACCACCATCTTTGG-3' and 5'-ATGAAGAAGGGCACTTTCAGG-3'; *MAD2L1BP*, 5'-GCAGAGGAGATGCTGAAGAAG-3' and 5'-GGCTGAGGACACTCTCCAGTT-3'; *GAPDH*, 5'-CCTCC

AAAATCAAGTGGGGCG-3' and 5'-GGGGCAGAGATG ATGACCCTT-3'.

The relative gene expression was normalized using GAPDH expression as an internal control. MCF-7 cells maintained in phenol red-free medium and supplemented with 5% FCS served as calibrator samples for hormone-treated cells. The results are reported as *n*-fold differences in the expression of the target gene relative to the expression of *GAPDH* and a calibrator sample. The relative expression was calculated by $2^{-\Delta\Delta CT}$, where CT is the fluorescence threshold value, ΔCT = CT of the target gene - CT of the reference gene, and $\Delta\Delta CT = \Delta CT$ of the tumor sample - ΔCT of the calibrator sample.

Statistical analysis

Statistical analyses were performed by one-way ANOVA using the SPSS 10.0 software (Statistical Package for the Social Sciences; SPSS Inc., USA), and P values were calculated using the Bonferroni test and considered to be significant at $P < 0.05$.

Results

In a previous study using cDNA microarray, we identified differentially expressed transcripts between ER/progesterone receptor (PR)-positive and ER/PR-negative primary breast tumors, providing a new set of potential target genes for the ER signaling pathway. In the present study, in order to identify DNA binding sites that mediate the estrogen response, we performed a computer-assisted and manual search for putative ERE binding sites in the promoter region extracted from 83 of these potential ER target genes. The manual search was performed using a list of putative ERE binding sites based on data available in the literature. A summary of the results obtained from this analysis is shown in Figure 1. Overall, 43% (36/83) of the candidate genes showed a good potential for being regulated by estrogen via ER. The promoter regions of these genes contain perfect or imperfect palindromic sequences, SP1, AP1, and/or FOXA1 binding sites.

Interestingly, during the annotation of the 83 potential ER target genes, we identified 17 genes located in close proximity to other genes and organized in a head-to-head orientation, possibly sharing the same promoter region. Using criteria of an intergenic distance of <1000 bp spanning the TSS of the divergent gene pair to define bidirectional promoters (11), we identified 14 (16.8%) bidirectional gene pairs (Table 1). The size of the intergenic sequences ranged from 18 to 973 bp, and all of these bidirectional promoters contain CpG-rich sequences. Figure 2 shows a schematic representation of three of the bidirectional gene pairs identified. We also investigated whether the identified bidirectional gene pairs maintained their divergent head-to-head organization in other species. Although limited, our analysis showed that the majority of the bidirectional gene pairs found in this study is evolutionarily conserved, as shown in Figure 3 for the *GALNS/TRAPPC2L* divergent gene pair.

Our computer-assisted search for EREs revealed that the promoter regions of the majority of the bidirectional gene pairs showed good potential to be regulated by estrogen. As shown in Table 1, all 14 bidirectional promoters analyzed contain putative ERE binding sites (at least half EREs) and other binding sites for other transcription factors, such as SP1 and AP1 or FOXA1 binding sites, indicating that they have the potential to be regulated by ER. The putative EREs were also found to be conserved in different species (Figure 3). In addition, approximately 80% of these bidirectional promoters contain GABP binding sites (CCGGAA(GTG)). We further evaluated the expression of five of these bidirectional gene pairs in response to 17β-estradiol in MCF-7 breast cancer cells. The gene pairs *GALNS/TRAPPC2L*, *TXNDC9/EIF5B*, and *SERINC1/PKIB* were modulated by estradiol (Figure 4). However, the expression of the gene pairs *GTPBP2/MAD2L1BP* and *DIDO1/C20orf11* was not affected in the MCF-7 cells exposed to 17β-estradiol or to the antiestrogens tamoxifen and ICI 182,780 (data not shown). The transcripts of *GALNS* and *TRAPPC2L* showed increased expression in MCF-7 cells maintained in stripped serum (deprived of steroids and growth

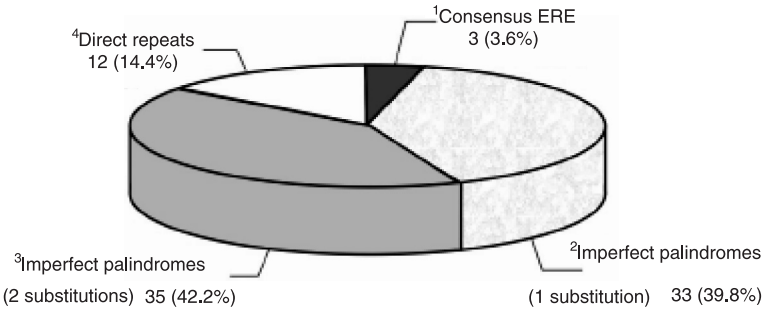


Figure 1. Summary of the results of the estrogen response element (ERE) search for 83 potential estrogen receptor target genes. The NCBI (http://www.ncbi.nlm.nih.gov) and USCU Genome Bioinformatics (http://www.genome.uscs.edu) databases were used to annotate the position of the 83 differentially expressed genes in the genome and to extract sequences 2 kb upstream and 2 kb downstream of the start sites for binding site analyses. The search for EREs and other binding sites was performed using several publicly available programs (Transfac 6.0, http://www.gene-regulation.com, and Dragon Genome Explorer, http://research.i2.a-star.edusg/promoter/). [1]Palindromic ERE consensus derived from vitellogenin A2 (GGTCAnnnTGACC) separated by 1 to 7 bp. [2]Imperfect palindromic ERE carrying a single substitution. [3]Imperfect palindromic ERE carrying two substitutions. [4]Direct repeats separated by 3 bp.

Table 1. List of the bidirectional gene pairs identified.

Annotated gene	Bidirectional partner	Chromosome localization	ERE position*	ERE sequences	Intergenic distance (bp)
C16orf61	*CENPN*	16q23.2	-1158 to -1141	CG**GGTCA**CTG**CAACC**TC	236
			-664 to -648	CT**GGTCA**CA**TGACC**AC	
			+484 to +501	CC**GGTAA**AAT**GGACC**TT	
			+1663 to +1681	TT**GGCCA**GGCT**TGTCC**TG	
			+775 to +783	FOXA1	
			-1345 to -1338	GABP	
DIDO1	*C20orf11*	20q13.33	-1131 to -1110	CT**CGACC**TCCTGG**GCTCA**AG	166
			-532 to -515	AC**TGCCC**CCT**GGGCA**CC	
			+193 to +211	AG**TGTCC**CTGA**GGTCC**AG	
			-55 to -46	GABP	
GALNS	*TRAPPC2L*	16q24.3	-1952 to 1934	TA**GGTCC**TCCC**TGACA**CA	119
			-1452 to -1432	CT**GGGCA**CGCCTC**TCACC**TT	
			-1116 to -1099	AT**GATCA**TCT**TGGCC**AA	
			-865 to -846	GA**GGGCA**GAGCA**TGTCC**AG	
			-221 to -242	CG**TGACC**CGCCGCT**GGTCA**CG	
			-168 to -148	CT**GGTCA**CGAGGC**AGTCC**AG	
			-107 to -91	CG**AGTCA**CG**TGGCC**GT	
			-44 to -24	TA**GGTCG**GCTCGC**TGGCC**GG	
			+361 to +379	GC**GGTCA**CCGA**TCACC**AC	
			+668 to +684	AA**GGTCA**GG**TGTCC**CT	
			+732 to +748	GG**GGTCA**GG**GGTCA**CA	
			+816 to +834	TG**GGGCA**TGGG**TCACC**GT	
			+923 to +941	GG**GGTTA**CACC**TGTCC**CG	
			-68 to -61	GABP	
GTPBP2	*MAD2L1BP*	6p21-p12	-1100 to -1084	CA**GGTCC**CG**GGACC**CC	342
			-1057 to -1041	CA**GGTGA**CA**GGACC**TC	
			-422 to -404	CC**TGACC**TCA**GGTGA**TC	
			-264 to -248	GC**TGGTCC**CA**GGACC**TC	
			+1057 to +1076	TT**GGTCA**GGC**TGGTC**TC	
			+1080 to +1099	GC**TGACC**TCT**GGTGA**TC	
			-1096 to -1089	GABP	
HNRPK	*RMI1*	9q21.32	-1800 to -1782	CC**TGACC**TTGT**GGTCC**GC	67
			+328 to +345	CG**TGGCC**TCG**GGCCA**AT	
			+881 to +899	AC**GGTCA**GGGC**TGCCT**CG	
			-1729 to -1720	GABP	
KARS	*TERF2IP*	16q23-q24	-930 to 910	GC**GCTCA**GCCTCC**TCACC**CC	243
			-188 to -172	GA**GCTCA**AC**TGACT**GA	
			+1566 to 1586	CT**TGACT**TTATTA**AGTCA**AA	
			-1551 to -1543	FOXA1	
			-82 to -72	GABP	
LRRC49	*THAP10*	15q23	-1824 to -1807	AA**GGTCA**GCA**TGATG**GT	233
			-1793 to -1778	AG**GGACA**G**TGGCC**TT	
			-553 to -523	GT**GGTCA**GAGCGA**TGACC**GAG C**GGTCA**TT	
			-252 to -235	CC**CTACC**TCT**GGTCA**CC	
			-262 to -252	FOXA1	

Continued on next page

Table 1 continued

Annotated gene	Bidirectional partner	Chromosome localization	ERE position*	ERE sequences	Intergenic distance (bp)
			-237 to -228	GABP	
			-84 to -75		
MRPL27	*EME1*	17q21.3-q22	-1312 to -1296	AG**GATCA**CT**TGAGC**CC	18
			-321 to -300	GG**GGGCC**TCATCTC**TGACC**CT	
			+109 to +126	GA**GGTGA**CAC**TGATC**CC	
			+189 to +205	CT**GGTCA**CG**TGACA**CA	
			+816 to +832	AG**GATCA**CC**TGAGC**TC	
			+957 to +977	AG**GGACA**GGAGAG**TGACT**TG	
			+1748 to +1767	TG**GGTCA**TGGTT**GGACC**TC	
			+1273 to +1265	FOXA1	
			-22 to -13	GABP	
SERINC1	*PKIB*	6q22.31	-1166 to -1149	CC**TGGCC**TTG**TGACC**CG	109
			-1323 to -1306	CG**GCTCA**CTG**CAACC**TC	
			+674 to +690	CA**TGCCT**TG**TGCCT**AA	
			+1534 to +1556	GG**GGTCT**AAGTGTAT**TGGCC**GG	
			-78 to 68	FOXA1	
			-96 to -87	GABP	
SLC5A6	*C2orf28*	2p23		Half EREs	320
				SP1 sites	
TCF19	*CCHR1*	6q21.3	-2052 to -2032	CC**TGGCC**AGA**GGTGA**GA	736
			-1939 to -1920	CA**TGACT**CTTG**GGTCC**TT	
			+198 to +216	GA**TGACT**GGAG**GGTCA**GC	
			+250 to +268	CA**GGGCA**GCTT**TGCCC**CT	
			+439 to +457	TA**GCTCA**TACA**GGACC**TG	
TMEM186	*PMM2*	16p13.13	-1519 to -1501	GT**GGTTA**CTAT**AGACC**AC	268
			-1289 to -1267	AA**GGTCA**CACGCATT**TGACC**TG	
			-1166 to -1144	AT**GGCCA**GTGTTCAG**TGGCC**AA	
			-1134 to -1112	GG**GGCCA**GGAGAGCC**TCACC**TC	
			-1022 to -1000	GC**GGGCA**GCCTGTAA**TAACC**TA	
			-791 to -771	AA**GGTCA**CACAGA**TGTCA**AG	
			-751 to -734	TA**GGTCG**GTT**TGACC**TG	
			+1129 to +1148	GT**GGGCA**GCAGT**TCACC**CA	
			+1474 to +1493	CA**GGACA**CATAC**TGTCC**CA	
			+1884 to +1903	TC**AGTCA**GATAA**TGGCC**AG	
			-29 to -21	FOXA1	
			-215	GABP	
TXNDC9	*EIF5B**	2q11.2	-1958 to -1943	TA**GGACT**G**TGACC**CT	973
			-717 to -698	TA**GGTCC**CCTCT**TGACT**CT	
			-660 to -640	AG**GGTGA**AGCTGA**AGACC**TA	
			-560 to -540	GA**GGTCC**CTATTT**TGTCC**TG	
			+338 to +354	CG**TGGCC**CA**GGCCA**GT	
			+1660 to +1679	TT**GGTCA**GGC**TGGTC**TC	
			-1095 to -1089	GABP sites	
			+609 to +616		

Continued on next page

Table 1 continued

Annotated gene	Bidirectional partner	Chromosome localization	ERE position*	ERE sequences	Intergenic distance (bp)
NPC2	*ISCA2*	14q24.3	-1745 to -1729	AG**AGTCA**CC**AGTCA**TC	500
			-564 to -545	TC**GGCCA**GGGAG**TGACC**GC	
			-106 to -89	CA**GGTCG**CC**TGACT**GG	
			+33 to +54	GC**GCTCA**GCACCGC**TGCCC**AG	
			+182 to +199	GC**GGCCA**GGC**TGAGC**CT	
			+1531 to +1549	TA**GGTGA**GTCC**TGATC**TC	
			-159 to -151	GABP	

Half-estrogen response elements (half-EREs) are listed in bold and underlined**.** *Numbers refer to the location of the EREs listed relative to the transcription start site of the annotated gene.

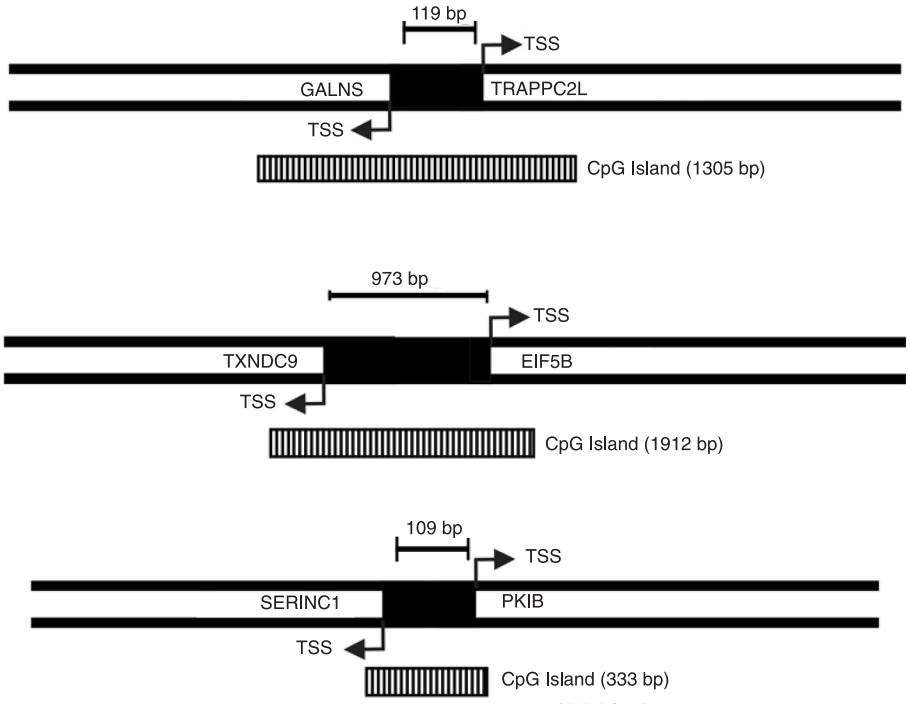
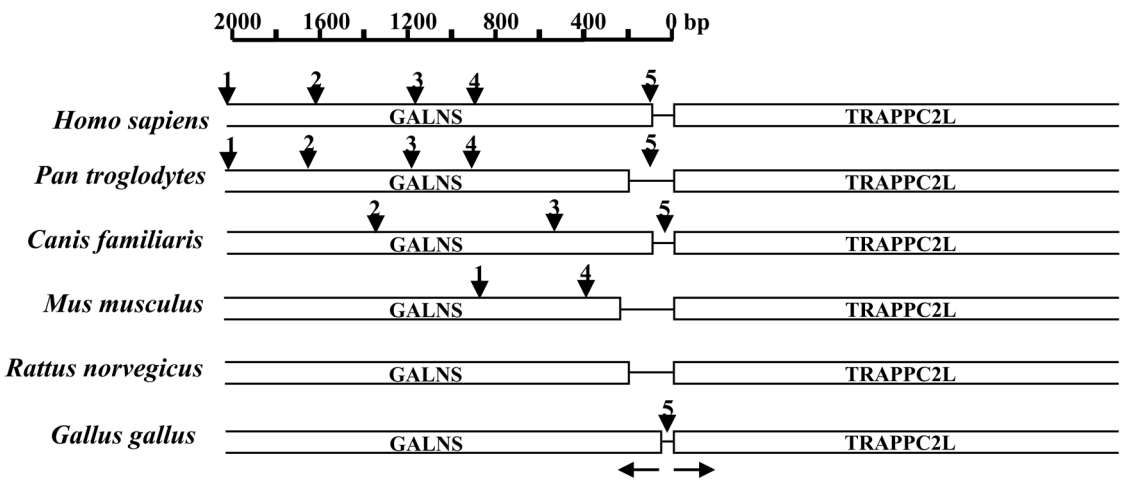


Figure 2. Schematic presentation of the bidirectional gene pairs *GALNS/TRAPPC2L*, *TXNDC9/EIF5B*, and *SERINC1/PKIB*. The transcriptional units of the bidirectional gene pairs are organized in a head-to-head orientation on chromosomes 16q24.3 (*GALNS/TRAPPC2L*), 2q11.2 (*TXNDC9/EIF5B*), and 16q24.3 (*SERINC1/PKIB*) (http://genome.uscs.edu). The transcript start site (TSS) for each gene is indicated by black arrows, which show the transcriptional direction. Open boxes indicate the first exons of each gene. Black boxes indicate intergenic sequences for each gene pair. The boxes with vertical bars represent CpG-rich sequences.

A



B

EREs (*Homo sapiens*)

1: **CCACC**CCT**CCACC**

2: **GGACACGGACC**

3: **GGACA**GGTG**TAACC**

4: **GGACACCTGACC**

5: **GGCCACGTGACT**

C

		*** * ** *** ****	
Homo sapiens	-1685	ACTGCTGTCA**GGACACGGACC**CCTGGGCGCT	-1655
Pan troglodytes	-1704	ACTGCTGTCA**GGACACGGACC**CCTGGGCGCT	-1674
Canis familiaris	-1482	TAAAGCATGG**GGACA-GGACC**CGTGGGCAGG	-1453

D

		* * * * * * * * * * *	
Homo sapiens	-190	GGGCCTGGAC**GGCCACGTGACT**CGCGGGGCGG	-159
Pan troglodytes	-190	GGGCCTGGAC**GGCCACGTGACT**CGCGGGGCGG	-159
Canis familiaris	-62	GGGGCGGGGC**GGCCACGTGACT**GGCGGCGGGG	-31
Gallus gallus	-47	ACCGCCTCCC**CGCCACGTGACG**CG-GGCGGCG	-16

Figure 3. Evolutionarily conserved estrogen receptor binding elements (EREs) in the potential promoter region of the bidirectional gene pair *GALNS/TRAPPC2L* across vertebrates. *A*, Schematic presentation of the bidirectional gene pair *GALNS/TRAPPC2L* in humans, chimpanzees, dogs, rats, and chickens. The black arrows and numbers 1 to 5 indicate the location of conserved EREs relative to the position of the transcript start site in the *TRAPPC2L* gene. *B*, 1 to 5 ERE sequences; EREs 2 (C) and 5 (D) and the surrounding sequences are shown in the alignment. The half-EREs are in bold. Asterisks denote highly conserved bases.

factors) compared to the cells grown in normal serum (control cells) and were negatively modulated in the presence of 10 nM 17β-estradiol for 24 h (Figure 4A). A similar reduction in these transcripts was observed in MCF-7 cells treated with 1 µM tamoxifen or 1 µM tamoxifen plus 100 nM 17β-estradiol compared to the expression in MCF-7 cells maintained in stripped serum. However, in MCF-7 cells treated with 1 µM ICI 182,780 alone the *GALNS*/*TRAPPC2L* transcripts showed increased expression compared to control cells and remained elevated in cells treated with 1 µM ICI 182,780 plus 100 nM 17β-estradiol. The transcripts of the bidirectional gene pair *TXNDC9* and *EIF5B* were positively regulated by 10 nM 17β-estradiol after 6 h of treatment (Figure 4B). In contrast to the gene pair *GALNS/TRAPPC2L*, the *TXNDC9* and *EIF5B* transcripts were not significantly altered in MCF-7 cells grown in stripped serum compared to control cells, but both transcripts were significantly induced (>2-fold) in MCF-7 cells exposed to 10 nM 17β-estradiol for 6 h. After 24 h of 17β-estradiol treatment, the expression of these transcripts was similar to that observed in control cells. However, the transcripts of the *TXNDC9* and *EIF5B* gene pair were not affected by treatment with the antiestrogens tamoxifen and ICI 182,780 alone or in combination with 17β-estradiol for 24 h compared to cells growing in normal or stripped serum. Interestingly, the transcripts of the bidirectional gene pair *SERINC1/PKIB* were divergently modulated by 17β-estradiol (Figure 4C). Expression of the *PKIB* transcripts was reduced in MCF-7 cells maintained in stripped serum and was substantially stimulated by 10 nM 17β-estradiol after 6 h of treatment, whereas the *SERINC1* transcripts increased in MCF-7 cells grown in stripped serum and expression was significantly inhibited in the presence of 17β-estradiol. In addition, cells treated with 1 µM tamoxifen showed a reduction of protein kinase inhibitor beta (PKIB) levels, which was reversed in the presence of 100 nM 17β-estradiol. Treatment with ICI 182,780 dramatically reduced the expression of PKIB but did not affect the expression of *SERINC1* transcripts compared to cells grown in stripped serum.

To determine whether 17β-estradiol or antiestrogens are able to modulate the transcripts of *GALNS/TRAPPC2L*, *TXNDC9/EIF5B*, and *SERINC1/PKIB* in ER-

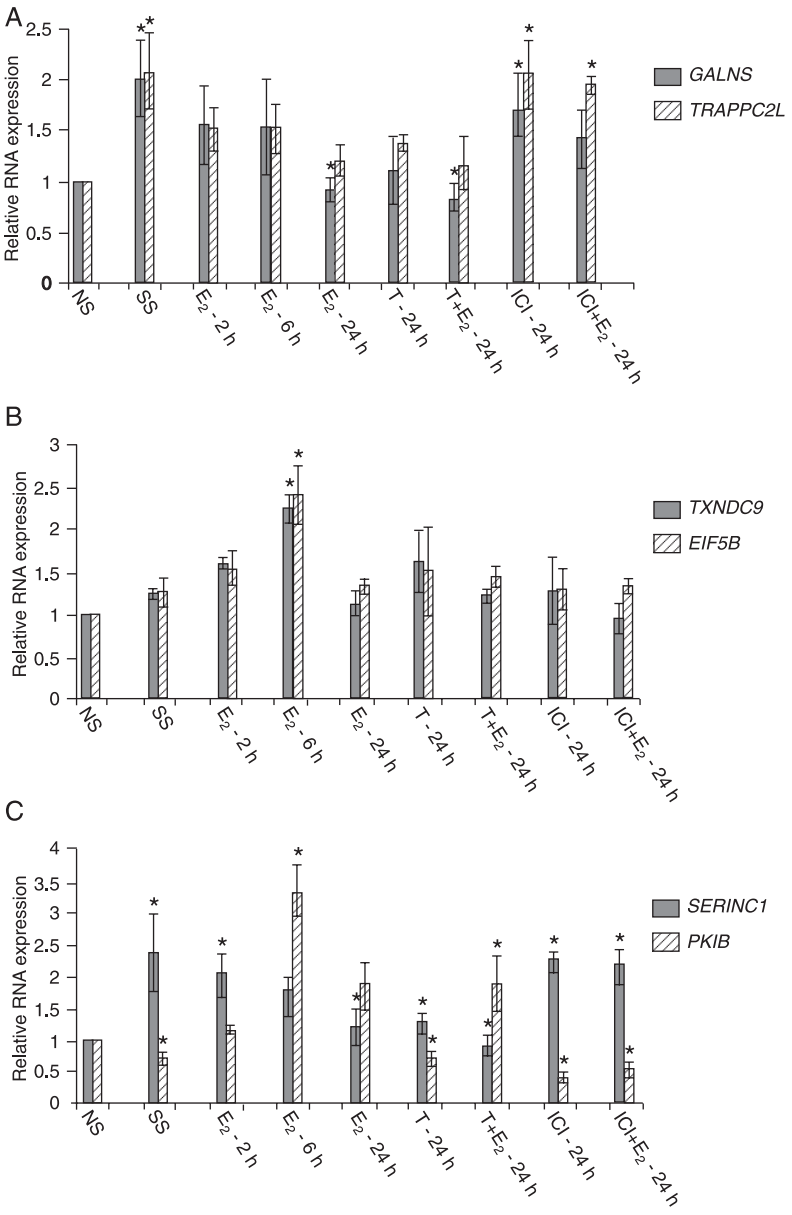


Figure 4. Effects of 17β-estradiol (E_2) and the anti-estrogens tamoxifen (T) and ICI 182,780 (ICI), on the transcriptional regulation of the bidirectional gene pairs *GALNS/TRAPPC2L* (A), *TXNDC9/EIF5B* (B), and *SERINC1/PKIB* (C) in MCF-7 breast cancer cells. Synchronized MCF-7 cells were cultured in: NS, medium supplemented with 5% fetal calf serum (FCS); SS, medium supplemented with 5% charcoal-stripped FCS; E_2 - 2 h, exposed to E_2 (10 nM) for 2 h; E_2 - 6 h, exposed to 10 nM E_2 for 6 h; E_2 - 24 h, exposed to 10 nM E_2 for 24 h; T - 24 h, exposed to 1 µM tamoxifen for 24 h; T+E_2 - 24 h, exposed to 1 µM tamoxifen plus 100 nM E_2 for 24 h; ICI - 24 h, exposed to ICI 182,780 for 24 h, or ICI+E_2 - 24 h, exposed to ICI 182,780 plus E_2 (100 nM) for 24 h. Data are reported as means ± SD of three experiments. *P < 0.05 compared to NS and/or SS (one-way ANOVA followed by the Bonferroni post-test).

negative breast cancer cells, MDA-MD-231 cells was also treated with 17β-estradiol and/or antiestrogens for 6 and 24 h. Estrogen or antiestrogens were unable to regulate the transcripts of these bidirectional gene pairs (data not shown) in MDA-MB-231 cells.

Discussion

In the present study, we employed an integrated computer-assisted and manual search to identify EREs in the promoter region of a set of candidate ER target genes previously identified as being differentially expressed in primary breast tumors based on their estrogen and progesterone status (21). Using this approach, we characterized the promoter region of 83 genes for their potential to be regulated by ER. Although only a small percentage of the candidate ER target genes showed the presence of an ERE consensus sequence in their promoter region, more than 40% of these genes were found to have putative ERE, SP1, AP1, and/or FOXA1 binding sites in their promoter regions, indicating the potential to be regulated by estrogen. The maximum ER transcriptional transactivation is thought to be displayed by the interaction of a homo- or heterodimer of ER with the ERE consensus sequence (CA**GGTCA**nnn**TGACC**TG). However, the ERE consensus sequence is rarely found in the promoter regions of human genes (2). The promoter regions of estrogen-responsive genes usually contain more than one imperfect palindromic sequence separated by one, two, three, or more interstitial bases, direct repeats of ERE half-sites, and widespread ERE half-sites (2). Half-EREs have been shown to act synergistically, but the degree of synergy between EREs is modulated by the physical distance between EREs and by the distance between the ERE and the TATA-box (26). In addition, perfect or imperfect ERE half-sites adjacent to AP1 or SP1 binding sites in the promoter region of known estrogen-regulated genes, such as HSP27 and PR, were also found to be effective in conferring estrogen responsiveness (27,28).

During the process of gene annotation, we identified a subgroup of divergent gene pairs organized in a head-to-head orientation that could be classified as bidirectional gene pairs. Surprisingly, the prevalence of bidirectional gene pairs found in the set of candidate ER target genes analyzed was higher than the predicted frequency of 9.4-11% found in the human genome by computational genome-wide studies (11,29). An incremental prevalence of gene pairs organized in a head-to-head orientation has also been observed among the genes that encode proteins that function in DNA repair (10,17).

Bidirectional gene pairs show evolutionary conservation, which suggests a selective pressure to facilitate the expression of functionally related genes or those that need to be co-regulated by the same stimulus or in a cell-specific manner (10,11,29). In agreement with the literature, the majority of the bidirectional gene pairs identified in the present study were shown to be conserved during evolution, keeping the same organization in vertebrates and indicating that these genes organized in a head-to-head orientation play important roles (29,30). Our data also showed that some of the putative ERE binding sites were conserved in the bidirectional gene pairs in other species. Different degrees of conservation of ERE or ERE-SP1 sites have been reported in the literature (4,9,31). In addition, the majority of the bidirectional promoters analyzed also contain GABP binding sites, which were previously shown to be over-represented in bidirectional promoters (32,33). However, whether or not they are all likely to be functionally conserved is still being questioned.

The bidirectional head-to-head orientation suggests that the potential promoter regions of these gene pairs share cis-elements, such as ERE and SP1 sites, that could be transcriptionally regulated by estrogen. To validate our results, we evaluated the effects of estrogen and antiestrogens on the transcriptional regulation of five bidirectional gene pairs in the breast cancer cell line MCF-7. To our surprise, the expression of two of the bidirectional gene pairs, *GTPBP2/MAD2L1BP* and *DIDO1/C20orf11*, was not modulated in MCF-7 cells exposed to estrogen and antiestrogens. Since the activity of transcription factors in transcriptional transactivation depends on the state of the chromatin (34,35), we can speculate that, in MCF-7 cells, the E_2-ER complex has no accessibility to the binding sites in the promoter regions of these genes, possibly due to the state of chromatin organization. Further studies using different cell lines and agents that affect chromatin organization are required. On the other hand, distinct patterns of gene regulation by estrogen and antiestrogens were observed for the bidirectional gene pairs *GALNS/TRAPPC2L*, *TXNDC9/EIF5B*, and *SERINC1/PKIB* in MCF-7 cells. In contrast, in ER-negative breast cancer cells, MDA-MB-231, 17β-estradiol or antiestrogens (tamoxifen or ICI) were unable to modulate the mRNA expression of these bidirectional gene pairs (data not shown), indicating that the presence of ER is essential for the transcriptional transactivation of these bidirectional promoters by estrogen. In MCF-7 cells, two of the gene pairs analyzed displayed a positive correlation, with both gene transcripts being up-regulated (*TXNDC9/EIFB5*) or down-regulated (*GALNS/TRAPPC2L*) by estrogen. The differences observed in the effects of the antiestrogens tamoxifen and ICI 182,780 on the transcriptional regulation of these bidirectional gene pairs can be attributed to their mechanisms of action. Tamoxifen is a selective estrogen modulator that acts by inhibiting estrogen binding to its main target estrogen receptor; however, it also acts as an estrogen agonist in certain tissues or in specific regulation of ER-responsive genes (36). Compared to tamoxifen, ICI 182,780 is considered to be a pure antagonist that blocks ER-mediated transactivation down-regulating ER protein levels. Additionally, the gene pair *SERINC1/PKIB* showed a negative correlation with the up-regulation of PKIB tran-

scripts and down-regulation of SERINC1 transcripts when MCF-7 cells were exposed to estrogen. The *SERINC1/PKIB* gene pairs may have distinct elements in the bidirectional promoter and further studies using reporter assays are needed to better define the critical sequences involved in this divergent regulation. On the other hand, in this study we evaluated the presence of EREs in the proximal promoter region only; however, the presence of EREs located in distal regions or of other cis-regulatory elements regulated by other factors may be necessary to modulate the divergent expression of this bidirectional gene pair.

Although genome-wide analysis showed that 45-68% of the gene pairs organized in a head-to-head orientation are co-expressed (16,29), the mechanisms involved in the transcriptional regulation of these bidirectional gene pairs appear to be complex and still poorly understood (13,29,37,38). Diverse patterns of gene regulation have been reported for different bidirectional gene pairs, including co-regulation, and divergent or asymmetric regulation (20,30,39,40).

In the present study, we characterized the promoter region of potential ER-regulated genes and provided new information on the transcriptional regulation of gene pairs organized in a head-to-head orientation. Further clinical and experimental studies are required to determine whether these genes play a role in breast cancer. The identification of ER target genes may improve our understanding of the role played by estrogens in breast cancer, permitting a better tailored hormone dependence and improved patient response to hormonal therapy.

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