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13. ABSTRACT ***(Maximum 200 Words)***

Estrogens are one of the major agents responsible for the development of breast cancer disease. The effects of estrogens are mediated by estrogen receptor (ER) alpha and beta. The ER interacts with specific DNA sequences (EREs) of estrogen responsive genes and modulates gene transcription. The biochemical and physiological features of the newly discovered ER beta remain largely unknown. We found that ER beta, in contrast to ER alpha, lacks the ability to induce transcriptional responses synergistically when binding to multiple copies of ERE. The events leading to ER-mediated synergistic induction of gene transcription are not well understood. We found that the N-terminal region of ER alpha, which contains transactivation function-1, is critical for the synergistic response induced by ER alpha. To understand the underlying mechanism for the low transactivity of ER beta, we examined the biochemical properties of ER. ER alpha and ER beta use the same nucleotides for DNA contacts and bind to EREs with similar affinity and preferences independent from ligands. Both receptors can interact with p160 family co-activators in a ligand-dependent manner. Howerver, ER alpha, not ER beta, can also interact with a ligand-dependent co-factor through a distinct surface. The ability of ER alpha to differentially recruit a co-factor through distinct interacting surfaces could contribute to ER subtype-specific gene responses.

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INTRODUCTION

Estrogen hormones trigger a broad array of physiological responses. They are also one of the major factors involved in the initiation and progression of breast cancer. Estrogen receptor (ER) alpha and beta are high affinity protein transcription factors that regulate expression of estrogen regulated genes by binding to a *cis* acting element called estrogen responsive element (ERE) when activated by estrogen. Most highly estrogen responsive genes contain multiple copies of estrogen responsive elements. Synergism is observed for ER alpha in the presence of estrogen when gene is induced by two or more EREs, which is greater than the sum of induction by individual ERE present alone (1,2). It contributes to the high level of transcription activation of ER alpha in mammalian cells. Since estrogen receptors play an important role in the etiology of breast cancer, understanding of the molecular mechanism of synergy would be helpful for developing new treatment for breast cancer.

In addition to natural estrogenic hormones, a variety of compounds bind ER as agonists and/or antagonists. Antagonists such as tamoxifen counteract the estrogenic effect of 17 beta-estradiol (E_2) and are used as drug to fight breast cancer. However, tamoxifen can function as either antagonist or partial agonist depending on specific promoter and cell type, which limit its efficiency as a therapeutic agent. The molecular mechanisms of tamoxifen action are not completely understood. Understanding both estrogenic and antiestrogenic properties of tamoxifen will help in the design of better therapeutic agents.

The newly identified ER beta is highly homolous to ER alpha in DNA binding domain. It is 30-60% homologous at other regions (3,4). The physiological function of ER beta remains unclear. It was recently shown that ER beta acts as a transdominat inhibitor of ER alpha transcriptional activity (5), although studies with ER beta knock-out mouse (6) showed that the absence of ER beta had minimal effects on reproduction. Analyzing the DNA binding property and transcriptional responses would shed light on the mechanism of ER beta action.

BODY

Generation of Stably Tansfected Cells

The genomic DNA wrap around histone proteins and form chromatin structures. The chromatin structure plays an important role in the regulation of gene expression. Recent studies show that transiently transfected DNA is both structurally and functionally different from cellular chromatin (7). In order to determine whether ER alpha or ER beta can act synergistically when binding to multiple EREs in the chromatin context, we constructed stably transfected cells with ERE containing reporter genes integrated into genomic DNA.

To simplify the system, we first constructed the reporter gene driven by simple promoter and zero, one or two EREs (shown in Fig.1). The luciferase reporter cDNA and the promoter were inserted into pCI-neo mammalian expression vector (Promega, Madison, WI) containing selective marker. CHO cells transfected with the luciferase vectors were selected with antibiotic G-418. The integration of the luciferase reporter was screened through PCR on genomic DNA extracted from survival colonies.

A recent study (8) showed that the effectiveness with which ER alpha or ER beta activates transcription is strongly dependent on promoter and cell context. Presumably the function domains of each receptor respond differently to the protein activators unique to individual promoter. To investigate how ER alpha or ER beta induces transcription upon binding to multiple EREs within specific promoter context, we generated cell lines stably

expressing luciferase reporter driven by the moderately strong thymidine kinase (TK) promoter. The constructed reporter plasmids are shown in Fig.1. The promoter regions of estrogen responsive genes contain other elements in addition to ERE, such as AP-1 and SP-1. It was shown that ER alpha and ER beta can induce different transcriptional responses on AP-1 site upon binding to ligands (9). To investigate the responses of natural genes to ER alpha and ER beta in cells, we generated cell lines stably expressing luciferase reporter driven by pS2 or Cathepsin D gene promoters, which contain ERE and are estrogen responsive. The pS2 and Cathepsin D gene promoters are cloned through PCR from genomic DNA library and inserted into luciferase promoter region in the expression vector.

We transiently transfected ER alpha or ER beta expression vectors into various stably transfected cells described above in the presence of estradiol or 4-hydroxyltamoxifen and measured the expression levels of luciferase in response to ER. However, we could not detect any increase of luciferase expression in ER alpha or ER beta transfected cells comparing to those transfected with control vectors. The reason could be the low efficiency of transient transfection we used. The increase of luciferase expression in a small portion of cells is difficult to detect among the major untransfected cells. We try to solve this problem by delivering ER alpha or ER beta expression vectors into the stable cell lines by retrovirus system. However, we did not succeed in developing this retrovirus system.

The Effects of ER ligands on ER transcriptional activation function upon binding to multiple Estrogen Responsive elements

ER belongs to the nuclear receptor superfamily. It exists at inactive state without estrogen. Estrogen triggers its activation through a multi-step process. The process involves conformational change, dimerization, binding to ERE and interacting with co-activators. The binding of tamoxifen induces a different conformational change of ER from that of estrogen (10,11,12). To examine how ER ligands affect estrogen responses induced by individual ER subtype upon binding to multiple EREs, we transiently transfected ER negative mammlian cells with ER and reporter luciferase gene driven by promoters containing 0, 1 or 2 EREs in tandem. We incubated the cells in the absence or presence of different ER ligands and then examine the promoter activity by measuring the expressed luciferase activity.

We initially use the simple promoter with only TATA box and ERE to simplify the system. It is easy for examing ER effect by using this simplified system. In transfected COS-1 cells, 17beta-estradiol (E_2) only induces very low transcriptional responses for both ER alpha and ER beta when the receptors bind to single ERE in the promoter region (Fig. 2A). ER alpha induces a much higher transcriptional response when binding to two ERE in tandem (Fig. 2A, left panel). This confirms our observation that ER alpha can induce estrogen responses synergistically (2). In contrast, ER beta appears to induce transcriptional responses additively (Fig. 2A, right panel). As a result, the level of transcriptional response induced by ER beta from two EREs is very low. We also performed the same experiments in different ER negative mammalian cell lines. Similar results have been obtained in Hela (Fig. 2B) and CHO cells (data not shown).

In the presence of 4-hydroxyltamoxifen (4-OHT), the active metabolite of tamoxifen, and another type of antiestrogen, ICI 182,780, ER has no effect on the transcriptional level of luciferase gene. 4-OHT and ICI also inhibit the effect of E_2 on ER alpha activity when co-exist in COS-1 cells (Fig. 2C). To determine whether the synergistic response of ER alpha can be

induced in the presence of tamoxifen when it functions as partial agonist, we measured ER transactivity in HepG2 cell line, which is a model cell line for tamoxifen to behave as a partial agonist (13). As shown in Fig. 2D, ER alpha still induces transcriptional responses synergistically as observed in other cell lines. Although in the presence of 4-OHT and ICI ER alpha can increase the transcriptional responses, the mode of transcriptional activation appears to be additive.

It has been shown that ER transactivation function is dependent on specific promoter and cell type (8). To determine the effect of promoter context on ER activity, we constructed reporter plasmids with 0, 1 or 2 EREs in tandem embedded in thymidine kinase (TK) promoter region, which is a moderately strong promoter. This promoter contains not only ERE but also other enhancer elements, which can be regulated by other transcription factors. As shown in Fig. 2E, ER alpha still induces synergistic response in the context of TK promoter although the extent of increase is smaller than simple promoter context. This is because the basal level is much higher is in TK promoter than in simple promoter.

These results suggest that ER alpha can induce transcriptional responses synergistically independent from the cell types. Under the same conditions, ER beta induces much lower transcriptional responses and the mode of induction is additive.

Mechanistic studies to understand multiple ERE effects

Described in appended manuscript: STRUCTURAL REGIONS OF ERα CRITICAL FOR SYNERGISTIC TRANSCRIPTIONAL RESPONSES CONTAIN CO-FACTOR INTERACTING SURFACES

The Biochemical Properties of ER beta vs. ER alpha

Described in appended manuscript: ERE AND ER LIGAND INDUCED STRUCTURAL CHANGES ARE INDEPENDENT DETERMINANTS FOR THE RECRUITMENT OF CO-FACTORS AND TRANSCRIPTIONAL RESPONSES BY ERα AND ERβ

KEY RESEARCH ACCOMPLISHMENTS

- Construction of estrogen responsive reporter plasmids containing various promoters including multiple EREs embedded in simple or moderately strong promoters and natural gene promtoers
- Generation of cell lines with reporter gene and its promoter integrated into genome
- Assess the effect of ER ligands on the transactivation function of ER alpha and beta upon binding to multiple EREs in the context of different promoters and from different mammlian cell lines.
- Identify structural regions of ER alpha critical for the synergistic response when it binds to multiple EREs.

- Test the DNA binding affinities of ER alpha and ER beta to various consensus and non-consensus ERE in the absence and presence of estrogen and antiestrogens

- Identification of the contact sites of consensus and non-consensus ERE by ER alpha and ER beta

- Determine the effect of ERE sequences and ER ligands on the conformation of ER and the recruitment of co-factors to ER.

REPORTABLE OUTCOMES

Manuscript:
Muyan, M., Yi, P., Sathya, G., Willmert, L., Driscoll, M. D., , Hilf, R., Bambara, R. A. (2001) Fusion Estrogen Receptor Proteins: toward the Development of Receptor-Based Agonists and Antagonists *Mol. Cell. Endo.(in press)*

Abstracts:
Muyan, M., Yi, P., Sathya, G., Willmert, L., Driscoll, M. D.,, Hilf, R. (1999) Engineering of a Biologically Active Homofusion Estrogen Receptor. *The Program and Abstracts of the 81st Annual Meeting of the Endocrine Society,* p.266:P1-621.

Sathya, G., Muyan, M., Hilf, R., Bambara, R. A. (1999) Estrogen Receptor Alpha Amino-Terminal Activation Function (AF-1) is Required for Estrogen-Induced Synergistic Response at Multiple EREs. *The Program and Abstracts of the 81st Annual Meeting of the Endocrine Society,* p.184:P1-233.

Yi, P., Muyan, M., Driscoll, M.D., Sathya, G., Hilf, R., Bambara, R.A. (1999) Characterization of the Biochemical and Functional Properties of Estrogen Receptor beta
4th Annual Scientific Symposium, University of Rochester Cancer Center p.73.

Yi, P., Bhagt, S., Hilf, R., Bambara, R.A. Muyan, M. (2000) Does the Binding Affinity of Estrogen Receptor beta to Various Estrogen Responsive Elements Correlate with Transcriptional Strength of the Receptor? *Era of Hope, Department of Defense Breast Cancer Research Program Meeting,* Porceedings Vol. I, p.355.

Muyan, M., Yi, P., Hilf, R., Bambara, R.A. (2000) A Fusion Estrogen Receptor Variant Generated by Genetic Conjugation Displays an Effective Dominant Negative Phenotype. *The Program and Abstracts of the 82nd Annual Meeting of the Endocrine Society,* p.190: 771.

Yi, P., Bhagt, S., Hilf, R., Bambara, R.A. Muyan, M. (2000) Does the Binding Affinity of Estrogen Receptor beta to Various Estrogen Responsive Elements Correlate with Transcriptional Strength of the Receptor? *The Program and Abstracts of the 82nd Annual Meeting of the Endocrine Society,*p. 359: 1493.

Yi, P., Bhagt, S., Hilf, R., Bambara, R.A. Muyan, M. (2001) The Absence of Functional Interaction between the N and C-termini of ER beta Contributes to the Inability of the Receptor

to Induce Gene Transcription Synergistically. *The Program and Abstracts of the 83rd Annual Meeting of the Endocrine Society,* P1-41.

Poster Presentations:
University of Rochester Cancer Center, 3rd Annual Scientific Symposium, Rochester, New York, September, 1998

The Endocrine Society's 81st Annual Meeting, San Diego, California, June, 1999

University of Rochester Cancer Center, 4th Annual Scientific Symposium, Rochester, New York, September, 1999

Department of Defense Breast Cancer Research Program Meeting, Atlanta, Georgia, June, 2000

The Endocrine Society's 82nd Annual Meeting, Toronto, Canada, June, 2000

The Endocrine Society's 83rd Annual Meeting, Denver, Colorado, June, 2001

Degree obtained:

Ganesan Sathya	Ph.D., 2000
Ping Yi	M.S., 2000

Employment or Research Opportunities Received:

Ganesan Sathya	Post-doctoral position in Duke University Medical Center, Pharmacology and Cancer Biology

CONCLUSIONS

We determined the effect of ER ligands on the transcritional responses induced by ER upon binding to multiple EREs. We found that ER alpha induces synergistic response in the presence of estradiol when binding to two EREs in tandem. The synergy observed for ER alpha is independent of cell type. Although tamoxifen functions as partial agonist in HepG2 cells, ER alpha only induces addititve responses when binding to it. Under the same conditions ER beta lacks the ability to induce transcriptional responses synergistically. As a result, the extent of transcriptional responses induced by ER beta is much lower than that of ER alpha.

Using full length and truncated variants of ER alpha, we show in transfected mammalian cells that although the carboxyl (AF-2) and the amino (AF-1) terminal activation domains are functionally integrated to induce transcription, AF-1 is critical for mediating synergy. Partial characterization of AF-1 sub-domains revealed that both Box-1 and Box-2 regions (amino acids 41-64 and 87-108, respectively) are essential for a synergistic response to estrogen. We show that members of the p160 family of co-factors and TIF-1 interact with the AF-2 domain of ER alpha. We also found that TIF-2, a member of the p160 family, can interact with the Box-1 region of AF-1. Apparently, structural regions required for the ability of ER alpha to induce transcription synergistically from tandem ERE sequences are also critical for the interaction of the receptor with the co-regulatory proteins.

We observed that ER alpha and ER beta use the same nucleotides for DNA contacts and bind to EREs with similar affinity and preferences independent from ligands. Although EREs and ligands induce conformational changes in ERs, these changes are not integrated to provide the receptors with novel functional features. We found that ERE determines the receptor affinity and thereby the relative amount of receptor binding, while the nature of ligand dictates the interaction of ligand-dependent co-factors by altering the affinity of ERs to co-factors. We also found that preferential interaction of a ligand-dependent co-factor through a distinct surface with ER alpha occurs independently from ligands, but the extent of interaction is dependent upon the ERE sequence. In transfected cells, ER alpha is more transcriptionally active than ER beta. The identity of ERE sequence, however, determines the potency of transcription when ERs bind to an agonist. Antagonists prevent ERs from inducing transcription independently from ERE sequences. Thus, ERE and ligand induced structural changes are independent determinants for the recruitment of co-factors and transcriptional responses by ERs. The ability of ER alpha to differentially recruit a co-factor through distinct interacting surfaces could contribute to ER subtype-specific gene responses.

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APPENDICES

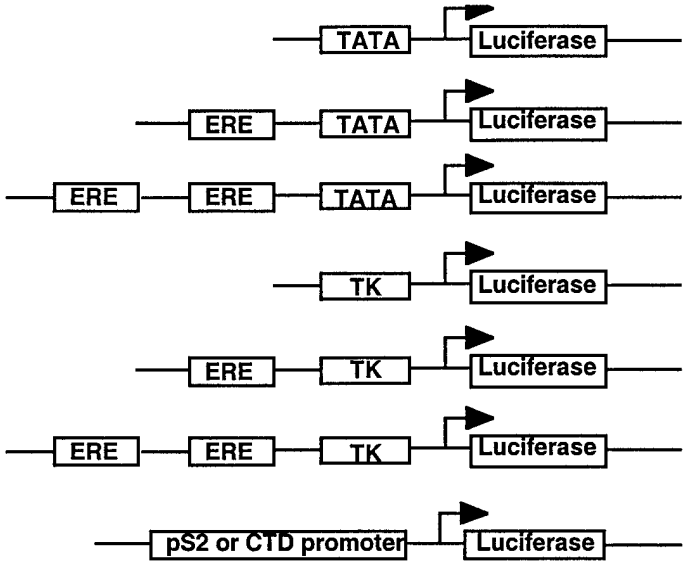
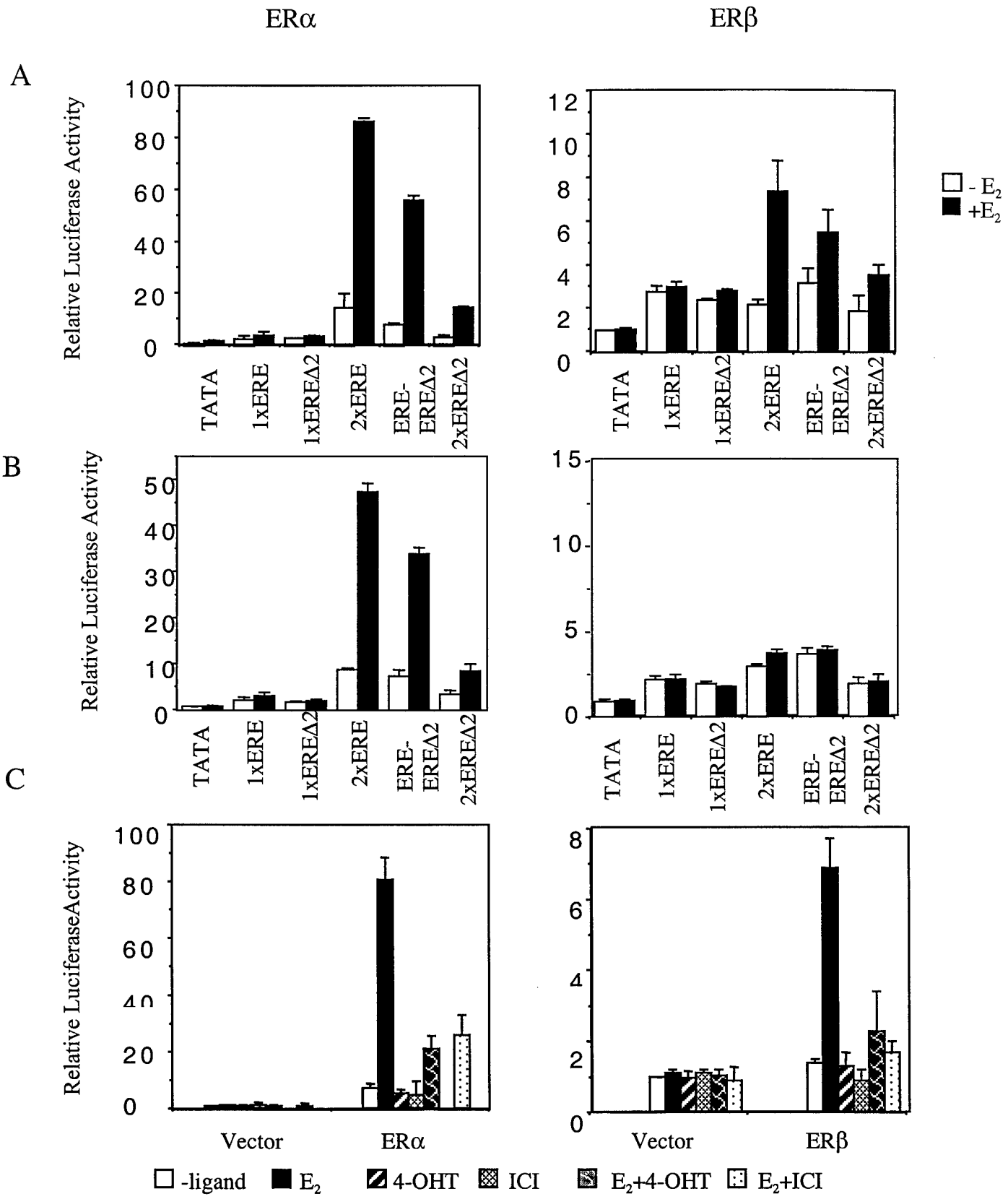


Fig.1 Constructed plasmids to test the synergy of ER upon binding to multiple EREs within chromatin context and under control of different promoters. TK: thymidine kinase promoter, moderately strong promoter. pS2 or CTD (Cathepsin D): natural estrogen responsive gene containing ERE in the promoter region.


ERα
ERβ
A
B
C
Relative Luciferase Activity
Relative LuciferaseActivity
100
80
60
40
20
0
12
10
8
6
4
2
50
30
15
5
TATA
1xERE
1xEREΔ2
2xERE
ERE-EREΔ2
2xEREΔ2
- E2
+E2
Vector
ERα
ERβ
-ligand
E2
4-OHT
ICI
E2+4-OHT
E2+ICI

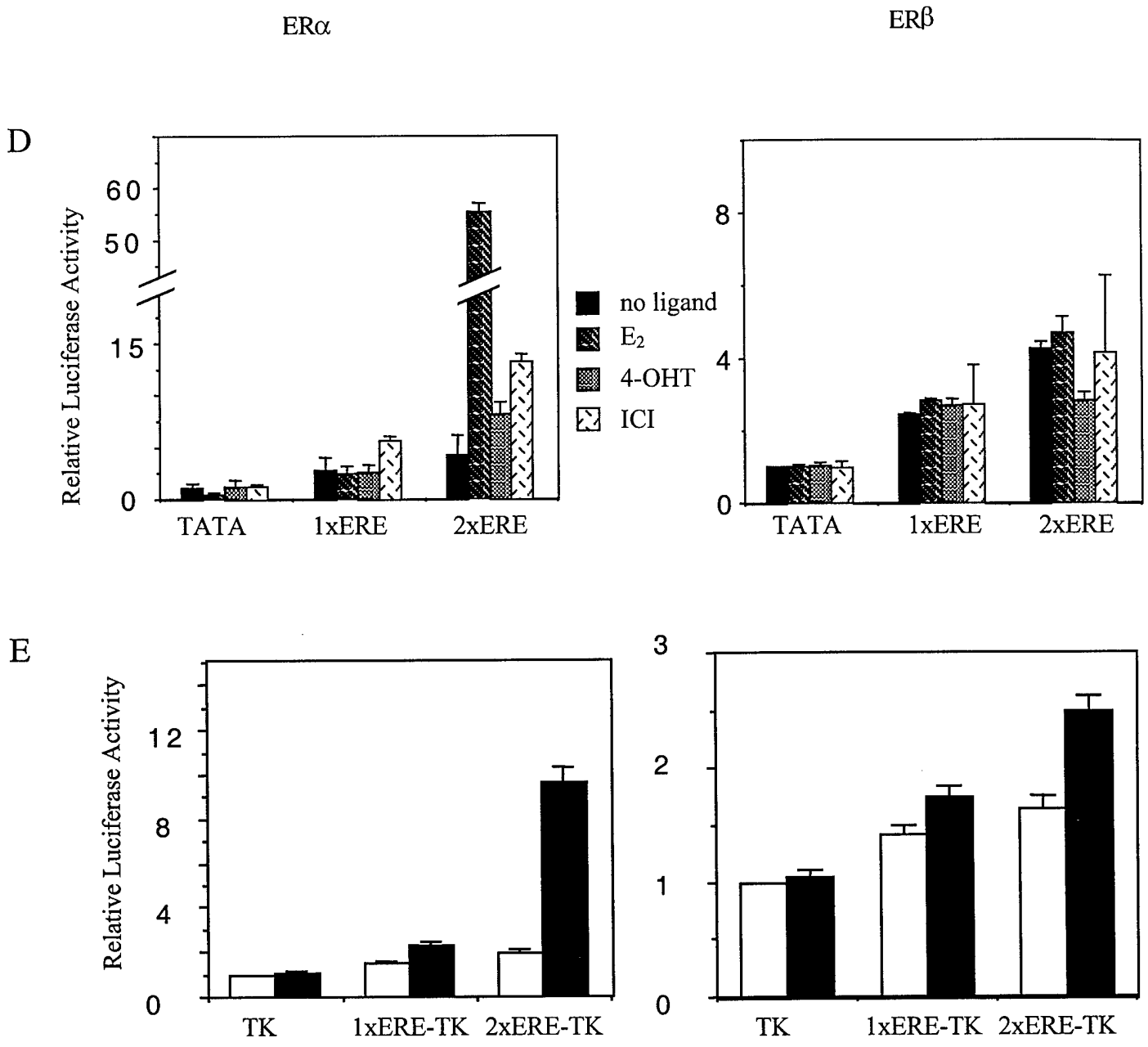


Fig. 2. The transcriptional responses of reporter luciferases gene induced by ERs in transient transfected COS-1 cells. The expression vector carrying ERα (right panel) or ERβ (left panel) cDNA was co-transfected with the luciferase cDNA driven by the TATA box promoter (A-D) or TK promoter (E) without (TATA, TK, respectively) or with one (1X) or two (2X) consensus or non-consensus EREs in tandem. The experiments were done in the absence and presence of 10^{-9}M E_2. **A, E.** COS-1 cells, **B.** Hela cells, **C.** The effect of ER ligands on ER induced transcriptional responses from two ERE in tandem in transfected COS-1 cells. **D.** The effect of ER ligands on ER induced transcriptional responses transfected HepG2 cells.

STRUCTURAL REGIONS OF ERα CRITICAL FOR SYNERGISTIC TRANSCRIPTIONAL RESPONSES CONTAIN CO-FACTOR INTERACTING SURFACES

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Abstract
Most highly estrogen responsive genes are synergistically activated by multiple copies of estrogen responsive elements (EREs) capable of binding to the estrogen receptor, ER. The extent of synergistic activation depends on the number of EREs, their spatial separation and stereo-alignment with respect to the promoter. The events leading to ER-mediated synergistic induction of gene transcription are not well understood. Here we examined the structural features of the receptor necessary to interact with co-regulatory proteins and to produce a synergistic pattern of activation from multiple EREs. Using full length and truncated variants of ERα, we show in transfected mammalian cells that although the carboxyl (AF-2) and the amino (AF-1) terminal activation domains are functionally integrated to induce transcription, AF-1 is critical for mediating synergy. Partial characterization of AF-1 sub-domains revealed that both Box-1 and Box-2 regions (amino acids 41-64 and 87-108, respectively) are essential for a synergistic response to estrogen. We show that members of the p160 family of co-factors and TIF-1 interact with the AF-2 domain of ERα. We also found that TIF-2, a member of the p160 family, can interact with the Box-1 region of AF-1. Apparently, structural regions required for the ability of ERα to induce transcription synergistically from tandem ERE sequences are also critical for the interaction of the receptor with the co-regulatory proteins.

INTRODUCTION

Physiological effects of estrogens are mediated by nuclear estrogen receptor (ER) α and β present in target organs. ERs are members of the steroid/nuclear receptor superfamily, and display conserved structural and functional organization ((Evans, 1988; Beato et al., 1989; Tsai and O'Malley, 1994; Glass and Rosenfeld, 2000; McKenna et al., 1999). The 17β-estradiol (E_2)-activated ER binds to estrogen responsive element, ERE (Klein-Hitpass et al., 1988; Peale et al., 1988), often found clustered as multiple copies of the consensus 5'-GGTCAnnnTGACC-3' or related non-consensus sequences along with binding sites for other transcription factors in the regulatory regions of responsive genes (Truss and Beato, 1993). Presence of multiple EREs results in a synergistic pattern of response to estrogen, in which the total response is greater than the sum of responses to individual EREs (Chang et al., 1992; Klein-Hitpass et al., 1988; Martinez and Wahli, 1989; Ponglikitmongkol et al., 1990; Sathya et al., 1997). Previous work from several laboratories, including ours, has shown that the degree of synergy depends on the number of EREs, and also their spacing and distance from the promoter (Klein-Hitpass et al., 1988; Martinez et al., 1987; Martinez and Wahli, 1989; Massaad et al., 1998; Ponglikitmongkol et al., 1990; Sathya et al., 1997).

Steroid hormone receptor-mediated synergy is thought to result from cooperative binding to adjacent response elements mediated by direct protein-protein interactions, or from interactions between DNA bound receptor dimers and the transcriptional apparatus (Tsai and O'Malley, 1994). The transcription complex is comprised of multiple activators and modulators bound to their cognate sites interacting through surfaces that are complementary to target surfaces on each other (Carey, 1998). Although, the contribution of cooperative binding of ER to synergistic response from multiple EREs remains controversial (Driscoll et al., 1996; Martinez and Wahli, 1989; Ponglikitmongkol et al., 1990), it is likely that ERs bound to multiple EREs *in vivo* present surfaces that interact with co-activators and transcription machinery. This could provide a means for ERα to regulate gene transcription synergistically in response to E_2.

ERα has six distinct functional domains, including an activation function AF-1 at the amino terminal A/B domain, a DNA binding domain C (Kumar et al., 1986), and a second activation function AF-2 within the carboxyl terminal ligand binding domain (LBD) E/F (Lees et al., 1989; Tora et al., 1989). It is well documented that the amino terminal activation function AF-1 and the ligand dependent carboxyl-terminal AF-2, independently and cooperatively act to induce transcription in a cell and promoter specific manner (Beekman et al., 1993; Kraus et al., 1995; Tora et al., 1989; Tzukerman et al., 1994). Although the underlying mechanisms remain largely unknown, the ER mediated transactivation is thought to occur through recruitment of co-regulators which have specific functions in transcription (Glass and Rosenfeld, 2000; McKenna et al., 1999). Recent studies have indicated that ER-induced transcriptional responses are mediated by the AF-1 and AF-2 through interactions with a variety of co-regulators. AF-2 mediated responses in ERαrequire agonist-modulated conformational changes in the LBD domain that realign the helix 12 together with helices 3, 4 and 5 to form co-factor interacting surfaces in the LBD. The interaction of co-factors with ERα occurs through the nuclear receptor interacting domains (NID) that contain a signature LXXLL motif wherein L denotes leucine and X refers to any amino acid. Co-regulator proteins mediate both the interactions between receptors and the basal transcription apparatus, as well as the remodeling of chromatin structure. These events are critical for the formation of the transcription complex (Glass and Rosenfeld, 2000; McKenna et al., 1999). Among the co-regulators, the p160 family of co-regulators that include steroid receptor

cofactor-1 (SRC-1), transcription intermediary factor 2 (TIF-2), and a protein amplified in breast cancer (AIB-1), as well as other nuclear receptor co-factors including receptor interacting protein 140 (RIP-140) and TIF-1 have been shown to interact with the AF-2 domain of ER in an agonist-dependent manner (Anzick et al., 1997; Onate et al., 1995; Voegel et al., 1996).

Although the nature of AF-1 function is poorly understood, studies indicate that AF-1 activity can be modulated through phosphorylation by growth factor signaling pathways. Reports indicate that p68 RNA helicase protein (p68; Endoh et al., 1999) can potentiate ERα function by directly interacting with the AF-1 domain in a ligand independent manner. In addition, recent studies from this (Yi et al., 2001; Muyan et al., 2001) and other laboratories (Ding et al., 1998; Webb et al., 1998; Benecke et al., 2000) suggest that some members of the p160 family of co-factors can interact with both the AF-1 and AF-2 domains in a ligand-independent and -dependent manner, respectively.

Given the importance of both AF-1 and AF-2 domains for the ability of ERα to induce transcription, these results collectively suggest that the co-factor interaction surfaces of ERα are also structural regions required for the ability of the receptor to induce synergistic transcription of the responsive gene containing multiple ERE sequences in tandem. Previous studies addressed structural determinants of ERα in the synergistic regulation of transcription by using amino- and carboxyl-terminal truncation mutants of the receptor containing only AF-1 or AF-2 (Klein-Hitpass et al., 1988; Tora et al., 1989; Xing et al., 1995). Results indicated that these mutants not only exhibited cell specific differences in their activation levels, but also differed in their ability to synergistically activate estrogen response depending on the presence of other *cis*-acting elements in complex promoter regions. Assigning a role for a specific region of the receptor in synergistic activation is complicated by the potential interaction between ER and upstream regulatory factors bound to complex promoter regions. To avoid this problem, we used a minimal promoter consisting of only the TATA box and EREs, and assayed the ability of truncated and full length ERs both to mediate synergy *in situ* and to interact with co-regulatory proteins *in vitro*.

We report here that E_2 is essential for the synergistic activation of the reporter luciferase gene, with activation occurring most efficiently with the wild type (WT) receptor. Our results suggest that although the integrated effects of both AF-1 and AF-2 are required for the transcription ability of ERα, the AF-1 domain of ERα in the context of the full-length receptor is critical for mediating E_2-induced synergistic responses. AF-1 sub-domains, Box-1 (aa 41-64) and Box-2 (aa 87-108), are both essential for AF-1 mediated synergy. We found that the regions necessary for the synergistic response are also important for the recruitment of co-regulatory proteins. Moreover, we also show here that the p160 family of co-regulators, exemplified here by TIF-2, can interact with both the amino- and the carboxyl-terminal activation domains of ERα. This interaction could be important not only for intra-molecular interaction between the activation domains within an ERα molecule but also for the inter-molecular interaction between the receptors when bound to multiple ERE sequences in tandem. We propose that these interactions are critical for the receptor to synergistically activate responsive genes from tandem ERE sequences.

MATERIALS AND METHODS

Plasmids and expression vectors

The pBluescript KS (+) (pBS; Stratagene, La Jolla, CA) bearing ERα cDNA is described previously (Muyan et al., 2001). ERα truncation mutants were generated by PCR with the introduction of *Sal*I and *Bam*HI sites at the 5' and 3' ends, respectively. A Kozak consensus sequence was placed at the 5' end of each construct to ensure efficient translation. ABCD-ABCD single chain variant ER was generated through an *Nde*I restriction site, which encodes histidine and methionine, created by PCR at the 5'- or 3'-reading frame of the ABCD to generate two separate plasmids. The ABCD cDNA with the 5' *Nde*I restriction enzyme site was excised from one plasmid with *NdeI* and *Sca I* enzymes and inserted into the other plasmid containing the ABCD cDNA with the 3'*Nde*I site using the same enzymes. ΔBox1 and ΔBox2 ER variants were generated by deleting sections of the cDNA corresponding to amino acids 41-64 and 87-108, respectively, using PCR. The AF2m variant was generated by PCR, using ER-AF2 mutant cDNA (kindly provided by Dr. Donald McDonnell, Duke University, NC) that contains a three amino acid replacement (D538A, E542A and D545A) destroying AF-2 function of ERα (Tzukerman et al., 1994) as the template. A PCR generated fragment containing the AF-2 mutation was inserted in place of the wild type AF-2 to produce AF2m cDNA. The AF2mΔBox1 and AF2mΔBox2 were constructed by inserting *SalI–FseI* fragments of ΔBox1 and ΔBox2 into AF2m. The "null" mutant was engineered by replacing the *Hind III-BamHI* fragment of AF2m into the CDEF construct excised with the same restriction enzymes. All constructs with the exception of BCDEF contain a carboxyl-terminus hexahistidine-tag preceded by Arg-Gly-Gly linker to give flexibility, for facilitating immuno-detection and protein purification. The presence of histidine tag had no effect on *in vitro*

biochemical and *in situ* biological properties of the WT-ERα (data not shown). The DNA sequences in all constructs were verified by sequencing (Core Nucleic Acid Facility, University of Rochester). The cDNA constructs were excised from pBS-KS and inserted into the mammalian expression vector pM^2AH, as described earlier (Muyan et al., 2001). The pM^2AH contains strong Harvey mouse sarcoma-long terminal repeat as promoter.

Luciferase reporter constructs were generated by inserting the *Hind*III–*Nco*I fragments containing 1, 2, or 3 tandem EREs upstream of the vitellogenin B1 TATA box, obtained from CAT reporter vectors (Sathya et al., 1997), into pGL3-Basic luciferase reporter (Promega Corporation, Madison, WI). The TATA-luc reporter was constructed by excising the three EREs from 3ERE-TATA construct by *Xho*I digestion and religating the vector DNA. All plasmids were purified using Qiagen plasmid maxi prep for transfection studies (Qiagen, Carlsberg, CA).

Generation and production of GST-Cofactor fusion proteins were described previously (Muyan et al., 2001). p68 RNA helicase cDNA was obtained from a human testis Marathon Ready cDNA library (Clontech, Palo Alto, CA) using primers based on published cDNA sequence (Hloch et al., 1990) and cloned into the multiple cloning site of pGEX-2TK (Amersham Pharmacia Biotech, Piscataway, NJ).

In vitro transcription and translation and Gel mobility shift assays

In vitro transcription and translation reaction was performed from supercoiled pBS-KS constructs, using TNT reticulocyte-lysate system and T3 polymerase (Promega) as per manufacturers' instruction. Five μl of reaction products were resolved on a 10% SDS-PAGE and transferred onto PVDF membrane (Millipore Corporation, Bedford, MA) and probed with ER specific antibodies (HC-20 to ERα F domain from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, or EVG-F9 to A/B domain, a gift from Dr. Abdulgamed M. Traish, Boston University) with ECL+Plus detection system (Amersham Pharmacia Biotech), and used in gel shift analyses.

EMSA assay using in vitro synthesized ERs was carried out as described previously (Muyan et al., 2001).

Cell culture, transfections, Western Blotting and reporter assays

Mammalian cells were obtained from ATCC (Rockville, MD) and the media were purchased from Tissue Culture Support Center, Washington University, St. Louis, MO. COS-1 (African green monkey kidney cells) were maintained in DMEM high glucose medium, HeLa (epitheloid carcinoma of human cervix) and HepG2 (human hepatocellular carcinoma) cells were maintained in Eagle's MEM with non-essential amino acids and Earle's BSS. Both media were supplemented with 10% Fetal Bovine serum (Hyclone) and 0.5 % Penicillin and Streptomycin (Life Technologies, Inc.).

Western blot analysis, transactivation assay and immunocytochemistry were accomplished as described previously (Muyan et al., 2001).

Statistical analysis

A t-test was applied to logarithms of normalized luciferase values in the presence of estradiol for each receptor type (WT or variant ERα), to test the null hypothesis that the geometric mean of luciferase induction for the 2ERE construct was twice the geometric mean of the luciferase induction for the 1ERE construct, against the one-sided alternative that it was greater than twice the mean for the 1ERE construct. Similar one-sided t-tests were used to test whether the geometric mean for the 3ERE-construct was greater than 1.5 times the geometric mean for the 2ERE construct, or that it was greater than 3 times the mean for the 1ERE construct. A single, pooled variance estimate was used for all tests for a given receptor type, obtained by fitting a one-way analysis of variance model to the log transformed data for that receptor type.

RESULTS

Role of ERα A and B domains in estradiol-induced synergy

Structural analysis of ERα has led to the identification of a transcriptional activation function at the amino terminal A/B region (Kumar et al., 1987; Tora et al., 1989). To examine the role of A/B region in mediating synergistic activation of a reporter gene from three consensus ERE sequences in tandem, we constructed variants lacking either 30 amino acids at the N-terminus (BCDEF) or the entire A/B domain (CDEF) of ERα (Fig. 1A). Mammalian expression vectors bearing the cDNA for WT, BCDEF or CDEF were co-transfected into COS-1 cells with a reporter plasmid containing none (as control), one, two or three EREs upstream of a minimal TATA promoter that drives the firefly luciferase cDNA. Normalized luciferase activities in the absence and presence of 10^{-9} M E_2 from each reporter were compared to the basal activity from the reporter bearing no ERE in the absence of E_2, a value set to 1 (Fig. 1B). The relative luciferase activities induced by WT-ERα (WT) in the presence of E_2 were 1.3, 18.4 and 120.6 for 1, 2 or 3 EREs, respectively, indicating a synergistic pattern of augmentation ($p<0.001$). In the

absence of E_2, the luciferase values were 2.3, 4.8 and 6.7, respectively, suggestive of an additive increase in reporter gene induction in the absence of hormone.

The pattern of response in the presence of E_2 for BCDEF was similar to that of the WT, exhibiting synergy in the presence of two and three EREs. In the absence of E_2, the relative luciferase values were 1.0, 2.1 and 10.4 for one, two and three EREs, respectively, displaying an apparent synergy (Fig. 1B, Insert). This low but notable response could have resulted from increased ligand-independent activity (Kumar et al., 1987), increased sensitivity of BCDEF to trace amounts of E_2 present in the growth media, shown previously to affect ERα transcriptional activity (Mattick et al., 1997) or increased affinity to AF-2 dependent co-factors (see below). Although these results suggest that the N-terminal 'A' domain plays a role in "silencing" ERα transactivation in the absence of hormone, as recently reported (Metivier et al., 2000), this region is not critical for the observed synergistic pattern of activation at multiple EREs in response to E_2.

We next examined the role of the AF-1 function by deleting the entire A/B region of the receptor (CDEF) and measured luciferase induction in the absence and presence of E_2. Enhancement of luciferase activity was observed only in the presence of the hormone. The luciferase activities were 1.2, 2.7 and 5.7 for one, two and three EREs, respectively, an apparent additive response to the hormone ($p>0.05$) (Fig. 1D, 0.3 μg). The dramatic decrease in the extent of luciferase activity induced by CDEF was not due to low levels of the receptor variant expressed in cells. We detected similar levels of the WT-ERα, (ERα) BCDEF and CDEF by Western blotting (Fig. 1C). Furthermore, the decrease did not result from an altered intracellular distribution in that the variant receptor was localized in the nucleus, just as the WT receptor (Fig. 2C). To further exclude the possibility that the reduced activation of the reporter enzyme activity by CDEF somehow masked the synergy, we transfected cells with increasing amounts of the CDEF expression vector (Fig. 1D). Although the levels of luciferase activity rose with increasing concentrations of the CDEF variant, there was no alteration in the additive pattern of luciferase activity. These results suggest that the A/B region AF-1 function of ERα is critical for mediating a synergistic pattern of activation of the estrogen responsive reporter gene.

Role of E and F domains of ERα in synergistic response

It is well established that the E domain of ERα has a hormone dependent transcriptional activation function, AF-2 (Lees et al., 1989; Tora et al., 1989). The F domain is also thought to play a role in the transcriptional activity of ER and effectiveness of anti-estrogens (Montano et al., 1995). To assess the role of the carboxyl terminal domains in estrogen-induced synergistic response, we generated truncation mutants that lack the F, or both E and F domains of ERα. Fig. 2A shows the comparison of the relative luciferase activities induced by ABCDE and ABCD constructs. The pattern of E_2-induced luciferase activity is synergistic in response to ABCDE, however, the activity at three EREs is approximately 50% of that of the WT receptor (compare to Fig. 1B). This suggests that the F region is not essential for mediating the synergistic pattern of response, however, it augments the extent of synergy mediated by the WT receptor.

The removal of both E and F regions rendered the ABCD variant ineffective in altering the luciferase activity even in the presence of three EREs. It was possible that the ABCD variant is incapable of transactivating the reporter due to differences in the level of synthesis and/or intracellular distribution compared to the WT receptor. We confirmed that the level of synthesis of ABCD protein in transfected COS-1 cells was similar to WT-ERα by performing Western blotting (Fig. 2B) and ensured the nuclear localization of ABCD by immunocytochemistry using an antibody specific to the amino-terminal region of ERα (Fig. 2C).

Since ABCD was synthesized at comparable amounts and localized in the nucleus similar to ERα, a decrease in the DNA binding ability of the variant receptor could be responsible for the negligible transcriptional response. To examine this issue, we compared the DNA binding abilities of WT-ERα and ABCD synthesized *in vitro* (Fig. 3D). The addition of equal amounts of *in vitro* translation reaction containing WT-ERα retarded the electrophoretic migration of the labeled ERE (lane 7). The ER-ERE complexes were further impeded by the receptor-specific antibody (lane 9), but not non-immune serum (lane 8), indicating the specificity of interaction. In contrast, there was no detectable retardation of the labeled ERE by ABCD (lanes 1 and 2), unless the antibody was added in the reaction mixture (lane 3). In other experiments, where we used higher concentration of the ABCD protein translated *in vitro*, we were able to detect low amounts of the ERE-bound species and the addition of antibody to the A/B domain or to the carboxyl-terminal six-histidine tag produced a super-shifted band (data not shown). Similar results were reported previously (Fawell et al., 1990), where the weak binding of ABCD was attributed to the lack of the dimerization domain located in the E region (Fawell et al., 1990). Addition of the antibody is thought to stabilize weak interactions between the protein and DNA. Alternatively, the divalency of antibodies may tether the receptor subunits in a dimer configuration, producing a strong super-shifted band.

These data suggest that the ineffectiveness of the ABCD variant in inducing the reporter gene transcription derives from the lack of the E region required for dimerization and stable interaction with the ERE. If so, simulating dimerization should lead to activation by ABCD variant, if the variant possesses an independent transcription activity in mammalian cells. To pursue this rationale, we constructed a single chain ABCD-ABCD molecule by covalently linking two copies of the variant cDNA (Fig. 4A), using an approach we developed to produce a biologically active tethered receptor (Muyan et al., 2001). The ABCD-ABCD protein, just as the WT- ERα, quantitatively interacted with the ERE (lane 4-6, Fig. 2D) and was expressed at levels comparable to WT and ABCD (Fig. 2B) and localized in the nucleus (data not shown). However, the fusion variant, as ABCD, had no effect on the level of luciferase activity in the absence or presence of E_2 at any concentration tested (data not shown).

Although these results suggest that ABCD is incapable of inducing transcription of the reporter gene in transfected cells, transactivation by AF-1 may require the structural features of the E and F domain, as suggested by an earlier report that the amino terminal AF-1 is functional only in the context of the full length receptor (Tzukerman et al., 1994). To examine the role of AF-1 in the mode of transcriptional activation, we used the full length receptor with only minimal structural alterations necessary to prevent AF2 function. The AF-2 mutant (AF2m) contains the three amino acid replacements D538A, E542A and D545A that destroy AF-2 activity (Tzukerman et al., 1994). As shown in Fig. 3A, AF2m had no effect on the luciferase activity in the absence of E_2, but increased the enzyme activity 1.0, 2.9 and 10.9 fold for one, two and three EREs, respectively, when the cells were treated with 10^{-9} M E_2. Statistical analyses showed a significant synergy for three ($p<0.01$) but not for two EREs. These results indicate that receptor with only AF-1 function can mediate synergistic response to E_2 but to a lesser extent than WT, which synergizes with two EREs (Fig. 1A). The Null variant receptor (Null) containing AF-2 mutations and lacking the amino-terminal A/B domain produces no augmentation in the reporter enzyme activity in the absence or presence of E_2. Overall these results indicate that the enhancement of luciferase activity by AF2m is dependent upon the functionality of AF-1. The increase in the reporter enzyme activity by AF2m only in the presence of E_2 also suggests that ligand is required for AF-1 dependent activation. Since the enzyme activity induced by AF2m is substantially lower than that induced by the WT receptor, these results further indicate that both AF-1 and AF-2 are required to restore full receptor activity, in turn increasing the level of synergistic response in the presence of multiple EREs.

AF-2 mutated receptor requires both Box1 and Box2 sub-domains of AF-1 to mediate synergy

Since the A/B region was essential for synergistic response to E_2, we considered the roles of sub-domains within the A/B region previously shown to contribute to AF-1 activity. McInerney *et al* (1996) described two such regions termed Box-1 and Box-2, corresponding to amino acids 41-64 and 87-108, respectively, in the amino-terminus of the ERα. Box-1 was found to be responsible for partial agonistic activity of trans-hydroxytamoxifen. Box-2 was suggested to play a role in E_2-stimulated transcriptional activity. We recreated these deletions in the WT receptor and tested them for E_2-mediated synergistic response. While constructs had minimal effects on luciferase activity in the absence of E_2, the enzyme activities in the presence of E_2 for one, two and three EREs were 1.1, 4.3 and 14.8, respectively, for the Box-1 deletion, and 1.0, 7.4 and 37.5, respectively, for the Box-2 deletion (Fig. 3B), both of which show significant synergy ($p<0.001$) with trends similar to the WT receptor. These results indicate that either Box-1 or Box-2 is sufficient for ER mediated synergistic response to E_2 only in the context of a functional AF-2. We note that Box-1 or Box-2 deletion lowered the overall activity of the reporter compared to the WT receptor. The E_2-induced luciferase activities with the WT-ERα were 1.3, 18.4 and 120.6 (Fig. 1B). The Box-1 deletion also clearly had a greater effect than the Box-2 deletion. To test whether Box-1 or Box-2 has a dominant role in synergistic transcriptional activation, we deleted the Box-1 or Box-2 sub-domains in the context of the AF-2 mutation. As shown in Fig. 3B, the Box-1 deletion together with the AF-2 mutation results in a complete loss of activity. This suggests that the Box-1 sub-domain is required for AF-1 mediated transcriptional activity. When Box-2 was deleted in the AF-2 mutant, transcriptional activity was still evident from an increase in luciferase activity in the presence of E_2. However, the enhancement of transcription was not synergistic ($p>0.01$). This suggests that the Box-2 sub-domain is required to produce the synergistic pattern of luciferase induction. Since Box-2 cannot produce synergistic pattern of activation unless Box-1 is present to mediate transcription, it appears that Box-1 sub-domain is critical for transcriptional activation but both Box-1 and Box-2 are required for AF-1 mediated synergy.

Does cellular context affect AF-1 and AF-2 transactivation and synergy?

There is considerable evidence that the transactivation capacities of ERα AF-1 and AF-2 depend on cellular contexts (Tora et al., 1989; Tzukerman et al., 1994; Xing et al., 1995). To test whether there are cell-specific differences in ER-mediated synergy, we performed similar transfection experiments in HeLa or HepG2 cell. In

HeLa cells, the WT-ERα induced luciferase activities for one, two and three EREs were 1.4, 12.6 and 34.5, respectively in response to 10^{-9} M E_2 (Fig. 4, upper panel). Although the extent of luciferase activation was lower in HeLa cells compared to COS-1 cells, the pattern of transcriptional enhancement was synergistic for two and three EREs compared to a single ERE ($p<0.01$). Similar to COS-1 cells, the AF2 mutant receptor (AF2m) also induced a synergistic response at three, but not two, EREs in HeLa cells in the presence of E_2 ($p<0.01$) with a 12-fold increase, while ABCD alone had no affect the enzyme activity. CDEF mediated responses in HeLa cells were 1.5, 2.8 and 5.6 for one, two and three EREs, respectively, which is an additive increase in the enzyme activity. As observed for COS-1 cells, Box-1 and Box-2 domains of AF-1 are important for transcriptional response and synergy alone or within the context of AF-2 mutation (lower panel).

Similar results were obtained in HepG2 cells in which AF-1 is reported to have strong activation function (Lavinsky et al., 1998; Tzukerman et al., 1994). While ABCD alone did not alter the luciferase activity, the AF-1 function was also synergistic in the context of full length receptor. As with WT-ERα, AF2m increased the enzyme activity synergistically from tandem EREs in response to E_2 (data not shown).

These results show that although the cellular context contributes to the extent of transcriptional regulation by ERα in response to E_2, structural features of the receptor are primarily responsible for the synergistic pattern of transcription. Our results indicate that AF-1 is a critical domain to mediate the synergistic pattern of transcription in transfected mammalian cells, and that both AF-1 and AF-2 are required to act cooperatively to mediate the full extent of response.

Co-factor interactions with ERs

Both the amino-terminal AF-1 and the carboxyl-terminal AF-2 domains of ERα interact with a complex array of co-regulator proteins that mediate both the interactions between receptors and the basal transcription apparatus, and the remodeling of chromatin structure. Among the co-regulators, the p160 family of co-factors including SRC-1 (Onate et al., 1995), TIF-2 (Voegel et al., 1996) and AIB-1 (Anzick et al., 1997) have been shown to interact with ERα in an agonist-dependent manner (Cowley and Parker, 1999; Hall and McDonnell, 1999; McKenna et al., 1999). Similarly, TIF-1 (LeDouarin et al., 1995; Thenot et al., 1997) is involved in the hormone-dependent regulation of transactivation of responsive genes by ERα. Given the importance of co-factor interaction in the transcriptional ability of ERα, we also wanted to address whether the recruitment of co-factors by the ERE-bound-receptor in the absence and presence of E_2 is altered by the same mutations that affect the mode of transcription induced by receptors. To accomplish this, we performed EMSA using receptors synthesized *in vitro*. The amount of protein synthesized in the transcription/translation system *in vitro* is variable (Muyan et al., 2001). To compensate for this variation we normalized the amounts of the receptors based on function as we described previously (Muyan et al., 2001). In brief, we quantified the amount of radiolabeled DNA that was bound (shifted) by the receptors in EMSA when equal aliquots of reactions were used. We then adjusted the amount of reaction mixture to obtain an equivalent DNA binding. The reaction mixtures were then pre-incubated without or with 10^{-7} M E_2 and followed by an incubation with ^{32}P-end labeled ERE. The fragments of co-factors produced as GST fusion proteins were then added into reaction mixtures and samples were subjected to native polyacrylamide gel electrophoresis (Fig. 5).

TIF-$2_{623-986}$ (TIF-2), TIF-$1_{638-851}$ (TIF-1), AIB-$1_{522-827}$ (AIB-1) or SRC-$1_{219-399}$ (SRC-1) at 1 μg co-factor/reaction effectively interacted with the E_2 complexed WT-ERα bound to ERE (Fig. 5A, lanes 6, 9, 12 and 15). This was reflected as a complete mobility shift in the E_2-ERα-ERE complex. The co-factors had little effect on the electrophoretic migration of the ERα-ERE complex in the absence of E_2 (Fig. 5A, lanes 5, 8, 11 and 14). The absence of any interaction of GST alone with the complex in the absence or presence of E_2 indicates that ERα specifically recruits the co-factors tested. We recently reported also that co-factor proteins can interact with ERs even in the absence of E_2, albeit with lower efficiencies than those observed with E_2-bound receptors (Yi et al., 2001). Indeed, WT-ERα recruited co-factors even in the absence of E_2 when we increased the concentration of co-factors about 10-fold (Fig. 5B, lanes 2, 5, 8 and 11). These effects were specific to the co-factors, as GST alone at the same concentration to those of the co-factors had no effect on the electrophoretic migration of the receptor (data not shown). These results indicate that although unliganded ERα can interact with the tested co-factors, binding of ERα to E_2 greatly increases the affinity of the receptor to co-regulators such that the E_2-ERα-ERE complex can effectively recruit co-factors when they are present at low concentrations (Yi et al., 2001). It is also evident that the electrophoretic mobilities of the ERα-ERE-CF complexes in the absence or presence of E_2 are not the same (compare lanes 2 and 3, 5 and 6, and 11 and 12 in Fig. 5B). This is likely the consequence of the conformational change in the receptors induced by the binding of E_2 and is consistent with the previous observations that E_2-induced structural changes in the LBD are required for the recruitment of AF-2 dependent co-factors (for a review see: McKenna et al., 1999).

The mutant receptors that lack either the Box-1 (ΔBox1; Fig. 5B) or the Box-2 (ΔBox2; data not shown) region were as effective as the WT in recruitment of co-factors. On the other hand, the AF2m receptor that lacks the AF-2 function was impaired in its interaction with co-factors (Fig. 5B). There was minimal, if any, retardation in the electrophoretic migration of ERE-bound AF2m in the absence or presence of E_2 when co-factors were present. Likewise, there was no observable interaction between the co-factors and the AF2 mutant that contains the Box-1 (AF2mΔBox1) or Box-2 deletion (AF2mΔBox2) (data not shown). These results indicate that the polypeptides of these co-factors containing nuclear receptor interacting domains (NIDs) specifically interact with the AF-2 region and suggest that the presence of functional AF-2 is critical for the receptor to recruit co-factors and for subsequent induction of transcription. These findings are consistent with previous observations that mutations in the helix 12 diminish the ability of the mutant receptor to interact with SRC-1 and to induce transcription (Danielian et al., 1992; Kalkhoven et al., 1998; Tzukerman et al., 1994).

Among the co-factors, p68 RNA helicase (p68) was shown to enhance ERα-induced transcription through a direct interaction with the amino-terminal region of the receptor (Endoh et al., 1999). In addition to p68, recent studies indicate that GRIP-1 (Webb et al., 1998) and the human homolog of TIF2 (Benecke et al., 2000) interacts qualitatively with the amino-terminal region of the ERα in GST pull-down assays. The GRIP-1/TIF-2 interaction with ER however, involves a region in the co-factor that is distinct from NID. The carboxyl-terminal region of GRIP1/TIF-2 encompassing a glutamine-rich domain apparently interacts with the Box-1 region of ERα (Webb et al., 1998). Considering that the amino-terminal region is a site of cofactor interaction, we wished to address whether structural features in the AF-1 domain critical for transcriptional synergy are also associated with the recruitment of co-regulatory proteins. To accomplish this, we examined the interactions of GST fusion full-length p68 and the carboxyl-terminal region of TIF-$2_{1125\text{-}1325}$ (Voegel et al., 1996), which contains the glutamine-rich carboxyl-terminal region (TIF2-CTR), with receptors synthesized *in vitro* using EMSA. We failed to observe a quantitative interaction of the ERs with p68 in the absence or presence of E_2 (data not shown). However, as we (Yi et al., 2001) and others (Benecke et al., 2000) showed previously, TIF2-CTR quantitatively interacts with WT-ERα (Fig. 6D, lanes 2 and 3). The extent of interaction of the co-factor at various doses tested with the ERE bound ER was the same whether or not the receptor was pretreated with E_2 (data not shown). These results indicate that TIF-CTR indeed interacts with the receptor independently from ligand, and suggest that TIF-2 is also a co-regulator for the ligand-independent AF-1 function, as suggested previously for GRIP-1 (Webb et al., 1999) and for TIF-2 (Benecke et al., 2000). TIF2-CTR is quantitatively recruited by the AF2m mutant ERα (Fig. 6D, lanes 5 and 6) similar to WT-ERα. This interaction is not observed in the CDEF variant (data not shown). Overall, these results indicate that the co-factor specifically interacts also with the amino-terminal AF-1 domain of ERα, each with distinct interacting surfaces

Our attempts to show a direct interaction with the truncated ABCD mutant or the ABCD-ABCD fusion receptor bound to the consensus ERE failed, while the CDEF truncated mutant effectively recruited the NID containing AF-2 dependent co-factors when pre-treated with E_2 (data not shown). Possibly recruitment of a co-factor to the amino terminal region requires that the receptor be structurally intact. This suggestion is also consistent with our observation that the functionality of AF-1 requires the structural integrity of the LBD of the receptor (Fig. 2).

To address whether the Box-1 or the Box-2 region of the AF-1 domain is responsible for the interaction with the co-factor, we used the Box-1 or Box-2 deletion mutants. Results revealed that the ΔBox-2 mutant still retains the ability to interact with TIF-2 (Fig 6D, lanes 11 and 12). This contrasts with the ΔBox-1 mutant, which showed little alteration in electrophoretic mobility when incubated with the co-factor whether or not the mutant receptor was pre-treated with E_2 (Fig. 6D, lanes 8 and 9). We note that in some experiments a low but detectable interaction of the TIF2-CTR occurred with the ΔBox-1 mutant. This suggests that although the Box-1 sub-domain of ERα is the primary target for the co-factor, sequences that flank the Box-1 also contribute to the optimal interaction.

Thus, it appears that the interaction of Box-1 with the members of the p160 family co-factors is critical for the transcriptional ability of ERα. Consistent with the previous observations (Webb et al., 1998; Benecke et al., 2000), these results indicate that TIF-2 interacts through distinct surfaces with both the amino-terminal and the carboxyl-terminal regions of ERα. These data imply that the recruitment of p160 proteins is critical for both AF-1 and AF-2 functions of ERα in the absence or presence of E_2. Thus, it appears that the ERα surfaces critical for transcription induction and synergy are also regions important for interaction with the relevant co-factors.

DISCUSSION

The homeodynamic regulation of estrogen action relies on transient fluctuations in hormonal levels that are sensed by target organs which respond to the hormone by altering local gene expression patterns (Buller and

O'Malley, 1976; Chan et al., 1978). Information necessary for the direction, degree, sensitivity and timing of the response is primarily encoded in the regulatory DNA elements of each responsive gene. Gene transcription in cells occurs in a milieu of proteins that include activators that bind to *cis*- acting elements, co-regulators, the general transcriptional machinery and the components of the chromatin within which the gene resides (McKenna et al., 1999). The fine-tuning of this multi-protein complex at a promoter is critical for the modulation of gene expression in a spatio-temporal manner in response to estrogens. The transcriptional results are, however, not the simple arithmetic addition of the independent effects of individual regulators, but can be the more-than-additive, i.e synergistic, effects of multiple regulators.

ERα is capable of regulating natural or experimental estrogen responsive genes synergistically if the regulatory site harbors multiple receptor binding sites, EREs (Chang et al., 1992; Klein-Hitpass et al., 1988; Martinez and Wahli, 1989; Massaad et al., 1998; Ponglikitmongkol et al., 1990; Sathya et al., 1997; Tora et al., 1989; Xing et al., 1995). ERα has six distinct functional domains, including an activation function AF-1 within the amino terminal A/B domain, a DNA binding domain C (Kumar et al., 1986), and a second activation function AF-2 within the carboxyl terminal hormone binding domain E (Lees et al., 1989; Tora et al., 1989) (see Fig. 1A). It is well documented that the amino terminal activation function AF-1 and the ligand dependent carboxyl-terminal AF-2, can independently and cooperatively act to enhance transcription in a cell and promoter specific manner (Beekman et al., 1993; Kraus et al., 1995; Tora et al., 1989; Tzukerman et al., 1994). Previous studies aimed toward the delineation of structural features of ER responsible for the synergistic activation of reporter genes utilized amino- and carboxyl-terminal truncation mutants of ERα containing only AF-1 or AF-2 (Klein-Hitpass et al., 1988; Tora et al., 1989; Xing et al., 1995). These truncated mutants not only exhibited cell specific differences in their activation levels, but also differed in their ability to synergistically activate estrogen response. The response depended both on the presence of other *cis*-acting elements in complex promoter regions and on the cell type. Assigning a role for a specific region in the receptor in synergistic activation is often complicated by the potential interaction between ER and upstream regulatory factors bound to complex promoter regions. For example, truncated *Xenopus* ER lacking the N-terminal AF-1 synergized with upstream activators even better than the wild type ER (Xing et al., 1995), suggesting that presence of other *cis* -acting elements at a promoter affect the synergistic properties of truncated ER. Also, the AF-1 function of human ER has been found to be highly specific to cell and promoter contexts and is active only in the context of a full length ER (Tzukerman et al., 1994).

The use of both full length and truncated ERs at a minimal TATA box promoter was, therefore, necessary to address the specific roles of ERα functional domains in the ER-mediated synergistic response at multiple ERE sites. We tested the relative levels of luciferase activation from the TATA promoter mediated by WT-ERα in response to E_2 in COS-1, HeLa and HepG2 cells. While the E_2 occupied WT-ERα induces synergistic activation of the reporter gene in each cell type, the extent of ER-mediated synergy in HeLa or HepG2 cells is lower than in COS-1 cells. This suggests that structural features of ERα and cellular factors collaborate to influence the extent of ER-mediated synergy. It is possible that the extent of transcriptional synergy depends on the presence of factors in COS-1 cells that are present at lower levels in HeLa and HepG2 cells.

Our observations indicate that E_2 is required for the synergistic induction of the reporter enzyme activity. Our results showing that even the "hormone-independent" AF-1 activity is evident only when E_2 is present highlights the dependence on E_2 for synergistic regulation of responsive genes bearing tandem ERE sequences. This is consistent with previous observations showing that there is no ligand independent activity of ERα in cultured cells (Berry et al., 1990; Mattick et al., 1997). The lack of synergy with the CDEF variant and restoration of synergy with the AF-2 mutant, albeit to a much lesser extent compared to WT receptor, indicate that the amino terminal A/B region or the AF-1 function is required to restore the synergy function. Thus, it appears that AF-1 is not only important for ER transactivation (Tzukerman et al., 1994) and repression by dominant negative mutants (Ince et al., 1995) but also, as we show here, for mediating synergy.

In the context of a mutated AF-2 function, we ascertained the roles of AF-1 sub-domains Box-1 and Box-2 in synergistic response to E_2 in transfected cells. The Box-1 deletion together with the AF-2 mutation results in a complete loss of activity, suggesting that the Box-1 sub-domain is required for AF-1 mediated transcriptional activity. When Box-2 was deleted, transcriptional induction was still observed, but it was not synergistic. This result identifies Box-2 as a sub-domain that is required for synergistic response but is not sufficient for mediating transcription.

Our results showing that structural features of ERα are critical for synergistic regulation of transcription suggest that ERα presents functional surfaces that are chemically and spatially complementary to target surfaces of co-activators (Carey, 1998). Indeed, we show here that the p160 family members, AIB-1, SRC-1 and TIF-2 as well as other nuclear receptor co-factor TIF-1 interact with the AF-2 domain of ERs in an agonist-dependent manner

through NIDs, as shown previously (Anzick et al., 1997; Eng et al., 1998; Hong et al., 1996; Kalkhoven et al., 1998; Kraichely et al., 2000; LeDouarin et al., 1995; Nishikawa et al., 1999; Onate et al., 1995; Routledge et al., 2000; Voegel et al., 1996). We show here that the unliganded ERα bound to an ERE can recruit these co-factors depending upon the amount of co-factor present. These results suggest that the high degree of regional flexibility, therefore relative positioning, of Helix 12 (Tanenbaum et al., 1998), could allow the receptors to interact with co-factors in the absence of ligand, albeit at a much lower efficiency. E_2 appears to increase the affinity of the receptor to co-factors dramatically such that even in the presence of low concentrations of co-factors, the ERα can recruit them quantitatively. Although primarily attributed to traces of estrogenic compounds in culture media (Berry et al., 1990; Mattick et al., 1997), hormone-independent co-factor binding to ERα could account also for the basal transcriptional responses to the receptor, the magnitude of which vary in a cell-specific manner (Tora et al., 1989; Tzukerman et al., 1990; Webster et al., 1988). More importantly, we show here that a distinct surface of TIF-2 (Benecke et al., 2000) and of GRIP-1 (Webb et al., 1998), which encompasses glutamine-rich sequence in the carboxyl-terminal region, both interact with the Box-1 region of the AF-1 domain of ERα in a ligand-independent manner. The ability of AF-1 and AF-2 of ERα to interact with different regions of the same co-activator both in the absence and presence of E_2 supports the suggestion that AF-1 also operates through binding to p160 proteins (Webb et al., 1998) and critical for the integrated function of AF-1 and AF-2 domains (Benecke et al.,, 2001).

Recent studies indicate that nuclear receptor–mediated gene expression is a dynamic event in which transcription of target genes is modulated cyclically as a self-regulatory process (Chen et al., 1999; Kraus and Kadonaga, 1998; Shang et al., 2000). It appears that agonist-occupied and ERE-bound ER recruits p160 family co-activators which in turn recruit p300 to the target gene promoter. The acetyltransferase activity of p300 acetylates histones, which leads to remodeling of chromatin and the recruitment of RNA Polymerase II and the induction of gene transcription. Following transcription, dissociation of p300 from and subsequent binding of CBP to the complex occur. CBP in turn acetylates co-regulators in the complex. This acetylation leads to the disruption of the p160 co-regulator-receptor complex and transcriptional attenuation. The complex subsequently dissociates from the ERE-bound receptor resulting in the termination of transcription and re-modeling of chromatin. A sequential cycle of association and dissociation of co-factor complexes through acetylation therefore appears to act as a molecular switch to control gene expression.

How is synergistic transcription regulated by agonist-occupied ERs that are bound to tandem ERE sequences? There are several plausible explanations, none of which are mutually exclusive. First, the process may involve an increase in the local concentrations of co-factors that interact with ER molecules bound to the tandem ERE sequences. Co-regulators with different histone modifying activities show different preferences for free histones as compared to nucleosomes and distinct targets within the histone substrates (Strahl and Allis, 2000). Higher concentrations of co-regulators at the promoter could lead to extensive modifications of local chromatin architecture leading to extended transcriptional initiation and re-initiation events. Second, an assortment of co-regulatory proteins, most of which have similar, overlapping functions, are involved in the formation of the transcription complex through direct or indirect interactions with ER. It is possible that different co-activators could interact with individual ER molecules bound to tandem EREs resulting in a heterogenous population of co-regulator complexes. This grouping of co-regulators could be particularly effective at recruitment of modifying proteins, nuclear receptor co-activator interacting protein (Chen et al., 1999), or complexes, as exemplified by TRAP/DRIP (Fondell et al., 1996; Rachez et al., 1999) that result in a delay in the disruption of the transcription complex. Third, the ability of a co-activator to interact with both the AF-1 and AF-2 domains of adjacent ERα, proteins, as exemplified here with TIF-2, could be critical for intra- and inter-molecular interactions of ER molecules. This notion is consistent with a recent observation indicating that TIF-2 can mediate functional interaction between the AF-1 and AF-2 domains of ERα to induce transcription (Benecke et al., 2001). This in turn could stabilize the transcription complex leading to a delayed dissociation of the complex and consequently to an extended transcriptional event.

It is apparent that estrogen-mediated transcriptional synergy is a complex multi-level event. Despite its complexity, further study of ER co-regulators in particular, and nuclear receptors in general, and their associated complexes in simple or composite responsive elements would yield penetrating insights into the mechanism of transcriptional synergy.

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FIGURE LEGENDS

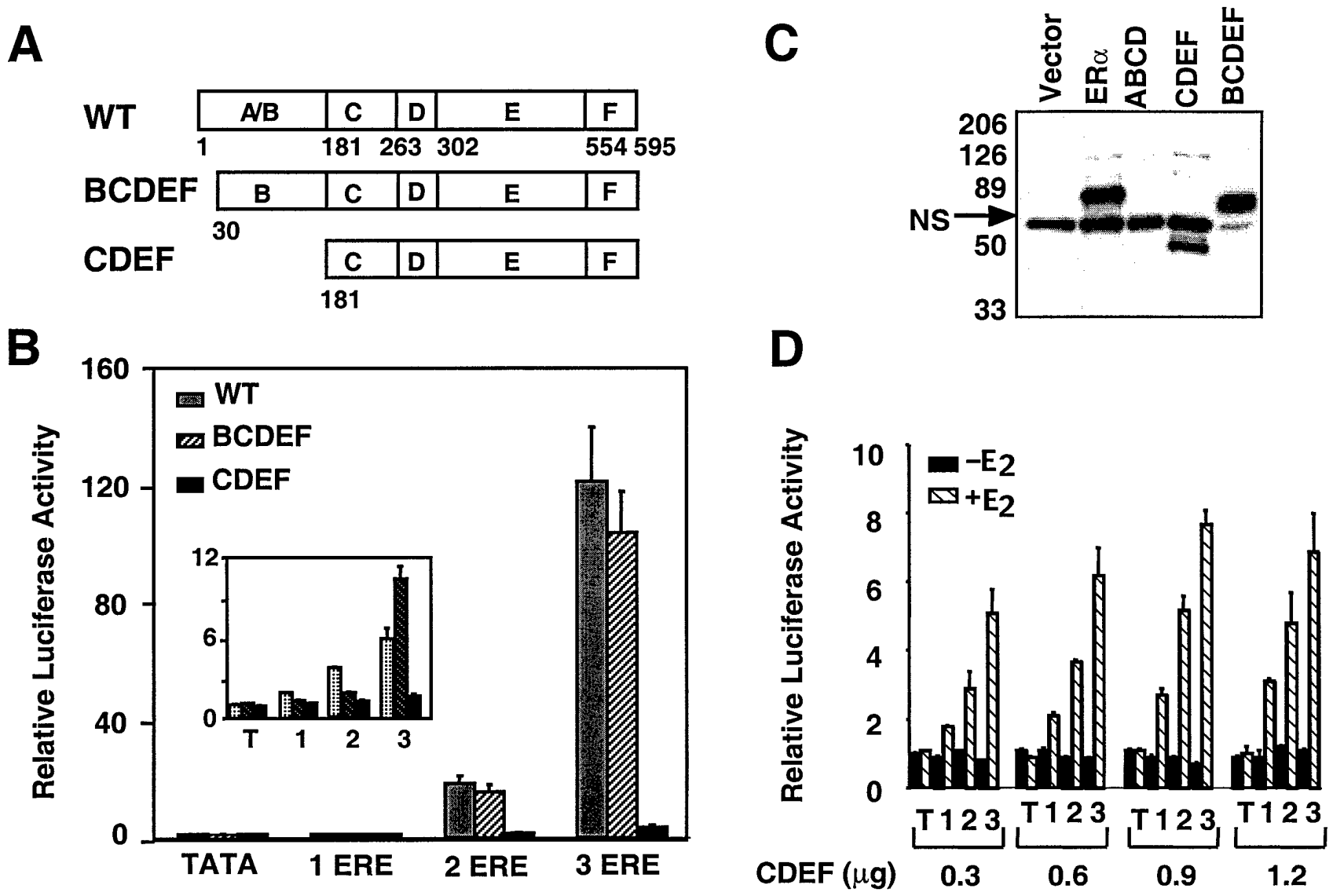
Figure 1. *The role of A/B domain of ERα in estradiol-induced synergy.* **A.** Schematic representation of ERα wild type (WT), and variants BCDEF and CDEF receptor. **B.** COS-1 cells were transfected with luciferase reporter containing none (TATA), one (1 ERE), two (2 ERE,) or three (3 ERE) tandem consensus EREs together with vector expressing ERα wild type (WT), variants BCDEF or CDEF and plasmid bearing β–galactosidase cDNA driven by CMV promoter as internal control. Cells were treated for 24 hours in the absence (Insert in which T, 1, 2, 3 indicate TATA, 1 ERE, 2 ERE and 3 ERE sequences, respectively), or presence of 10^{-9} M E_2. The extracts were assayed for luciferase and β-galactosidase activities and the normalized luciferase/β-galactosidase activities in response to receptor constructs were compared with the normalized activity for the TATA construct in the absence of E_2, set to 1. Shown are the mean ± SEM of three independent experiments performed in duplicate. **C.** Western blot analysis of proteins expressed in COS-1 cells. COS-1 cells transfected with pM^2-AH expression vector bearing no (vector), WT-ERα, (ERα), ABCD, CDEF or BCDEF cDNA in the absence of 10^{-9} M E_2. Thirty μg of total protein were loaded on a 10% SDS-PAGE, transferred onto PVDF membrane and probed with the HC-20 antibody directed against the F domain. Molecular weight markers are indicated to the left. NS refers to a non-specific protein detected in all lanes. Absence of a specific band in the ABCD lane indicates the specific recognition of the constructs with the HC-20 antibody. **D.** COS-1 cells were transfected with increasing concentrations (300 ng to 1.2 μg) of the expression vector bearing CDEF cDNA in the absence ($-E_2$) or presence of 10^{-9} M E_2 ($+E_2$).

Figure 2. *Role of E and F domains of ERα in synergistic response.* **A.** Schematic representation of ABCDE and ABCD variants. COS-1 cells were transfected and processed as described in legend of Fig. 1B. The results are the mean ± SEM from three independent experiments performed in duplicate. **B.** Western Blot analysis of proteins expressed in COS-1 cells. COS-1 cells were transfected with 1μg of expression vector bearing none (Vector), WT-ERα (WT), ABCDE, ABCD or ABCD-ABCD fusion variant cDNA. Processing of COS-1 cells for western blot analysis is described in Fig. 1C. The EVG-F9 antibody directed against the amino terminal region of ERα was used in detection of protein constructs. **C.** Intracellular localization of ER variants. COS-1 cells were transfected with 1μg of expression vector bearing WT-ERα (WT), ABCDE, ABCD or CDEF variant cDNA. After 24 hours, the cells were fixed and proteins were detected using ER-specific antibodies followed by FITC-conjugated secondary antibodies, and visualized using a fluorescent microscope, as described in Materials and Methods. DAPI staining was used to identify nuclei. As a control, in the last panel, cells were transfected with cDNA coding for only EF domain which localized in the cytoplasm. Antibodies used was either EVG-F9 for ABCD and ABCD or HC-20 for HC-20 for WT, CDEF and EF constructs. **D.** Construction and DNA binding analysis of the ABCD-ABCD fusion variant. Construction of ABCD-ABCD fusion receptor cDNA was accomplished as desribed in Materials and Methods. pBluescript vector bearing cDNA coding for the ABCD, ABCD-ABCD or the wild type ERα (WT) were transcribed and translated *in vitro* and used in a gel-shift analysis with [^{32}P] labeled consensus ERE as described in Materials and Methods. The receptor type used in the binding assay is indicated on the top of the gel. The position of free ERE, ER-ERE complex and ER-ERE-Ab supershifted complex are as indicated.

Figure 3. *AF-1 mediated response to estradiol is synergistic in the context of a full length ER.* COS-1 cells were transfected with luciferase reporter containing none (TATA), one (1ERE), two (2ERE) or three (3ERE) EREs along with a vector expressing the full-length AF2 mutant (AF2m) or the AF2 mutant CDEF (Null) variant. Stars in the schematics indicate the amino acid replacements (D538A, E542A and D545A). The extracts were assayed as described in legend of Fig. 1B. The relative luciferase activities from three independent experiments performed in duplicate are shown. Only 3-ERE response to the Null constructs in the absence ($-E_2$) or presence ($+E_2$) of 10^{-9} M E_2 is shown. **B.** The effects of AF-1 sub-domains of Box 1 and Box 2 deletions in the context of full-length receptor without or with AF-2 mutations on luciferase activity. Deletion of a region between aa 41-64 or 87-108, represented as black boxes, depictes the variant ΔBox1 or ΔBox2 receptors, respectively, in the context of full-length receptor without (ΔBox1 and ΔBox2) or with AF-2 mutation (AF2mΔBox1 and AF2mΔBox2). Transfection and processing of COS-1 cells for reporter enzymes as described in leg of Fig. 1B.

Figure 4. *ERα-mediated synergistic response to estradiol in HeLa cells.* HeLa cells were transfected with luciferase reporter containing none (TATA), one (1 ERE), two (2 ERE) or three (3 ERE) EREs along with the expression vector bearing WT-ERα, CDEF, AF2m or ABCD variants (**A**) or with Box1, Box2, AF2m-Box1Δ or TAF2m-Box1Δ variants (**B**). Cells were treated without (data not shown) or with 10^{-9} M E_2 for 24 hours. Luciferase activities were normalized for transfection efficiency and expressed as relative to the TATA construct. Results from three independent experiments performed in duplicate are shown.

Figure 5. *Recruitment of co-factors by WT-ERα and ER variants.* (**A**) The *in vitro* synthesized receptors were pre-incubated in the absence (-; 0.01% ethanol) or presence (+) of 10^{-7} M E_2 (E_2) followed by the addition of the end-labeled consensus ERE. Expressed in bacteria as GST fusion proteins, co-factors (CF) AIB-$1_{522-827}$ (AIB-1), TIF-$1_{638-851}$ (TIF-1), TIF-$2_{623-986}$ (TIF-2), SRC-$1_{219-399}$ (SRC-1) or GST alone (GST) were then added into reactions in the amount of 1 μg/reaction (+). (**B**) Interaction of co-factors with the WT and the mutant receptors. The amount of co-factor used was 2.5 μg/reaction in the absence (-) and 0.25 μg/reaction in the presence (+) of 10^{-7} M E_2. (**C**) The recruitment of $TIF_{1125-1325}$ (TIF2-CTR) produced as a GST fusion protein by WT and mutant receptor in the absence (-) or presence (+) of 10^{-7} M E_2. The amount of co-factor used was 2 μg/reaction. In all panels, reactions were resolved on 4% native polyacrylamide gels and a representative autoradiogram of at least two independent experiments is shown. In Panel B, C and D free ERE is not shown.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WT
BCDEF
CDEF
Relative Luciferase Activity
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120
80
40
0
12
6
0
T
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2
3
TATA
1 ERE
2 ERE
3 ERE
C
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ERα
ABCD
CDEF
BCDEF
206
126
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NS
50
33
D
Relative Luciferase Activity
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6
4
2
0
-E2
+E2
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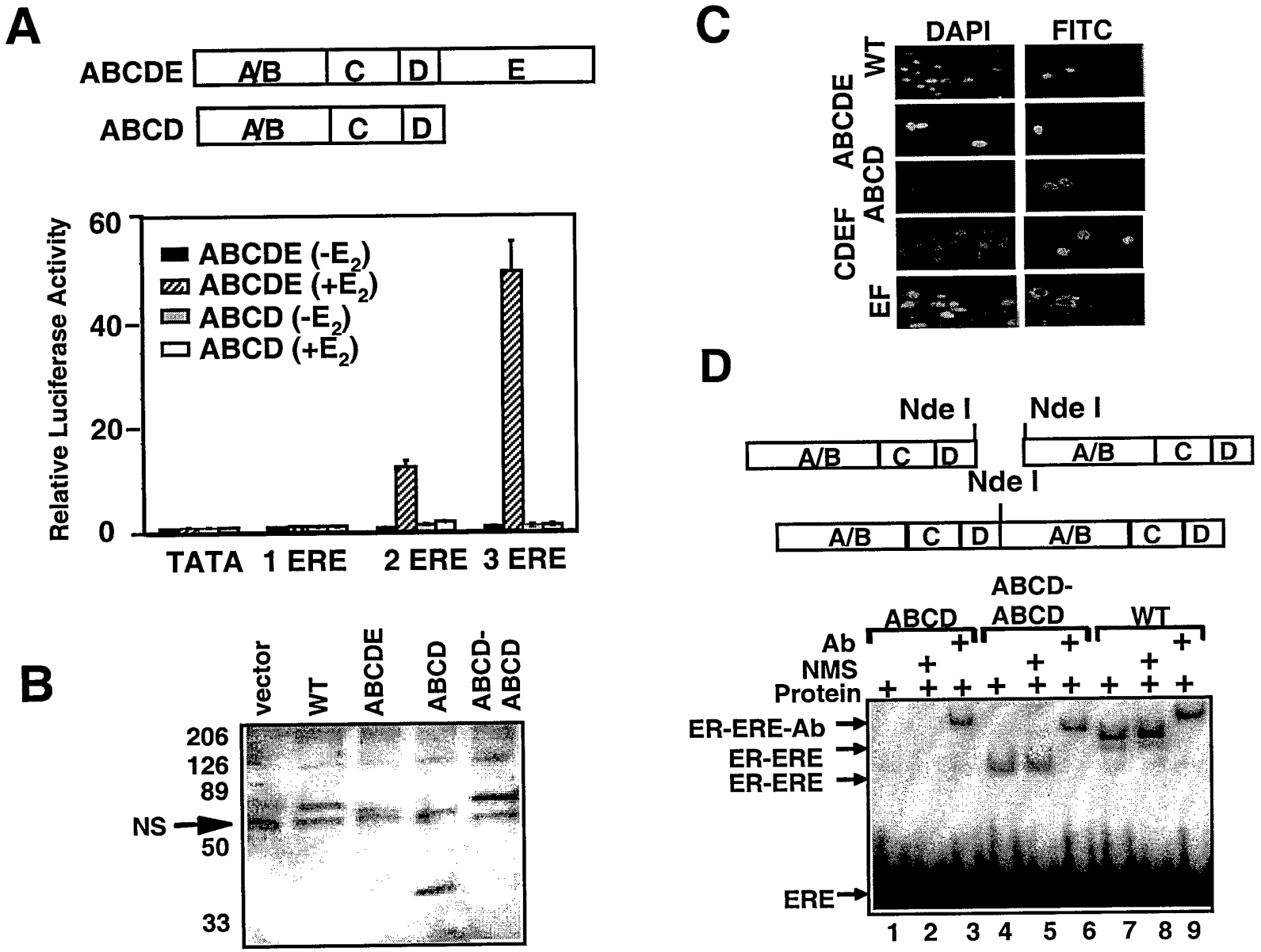
0.9
1.2

Figure 1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Relative Luciferase Activity
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ABCDE (+E2)
ABCD (-E2)
ABCD (+E2)
TATA
1 ERE
2 ERE
3 ERE
B
vector
WT
ABCDE
ABCD
ABCD-ABCD
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126
89
NS
50
33
C
DAPI
FITC
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ABCD
CDEF
EF
D
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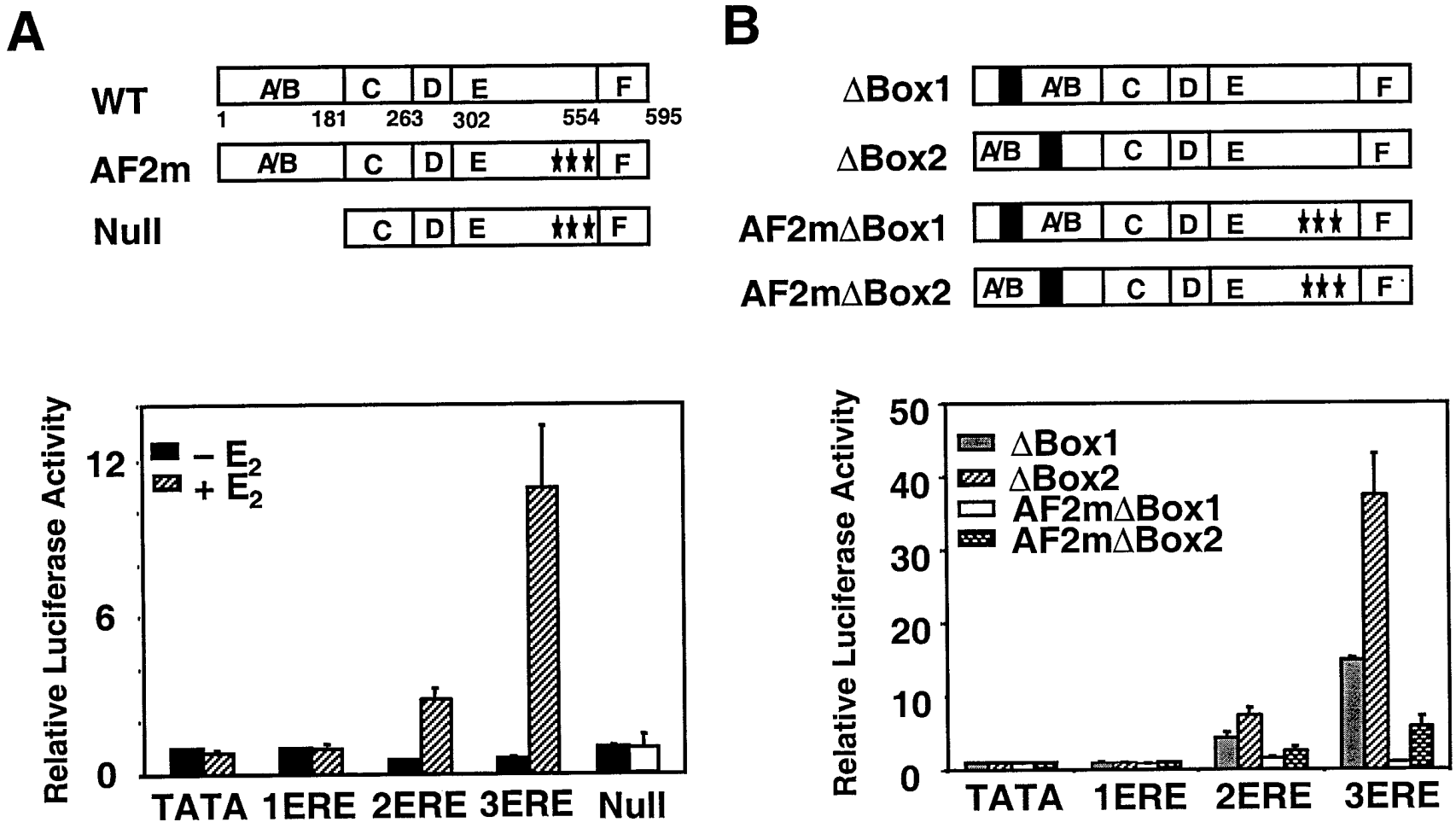
Figure 2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263
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ΔBox2
AF2mΔBox1
AF2mΔBox2
Relative Luciferase Activity
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6
0
– E2
+ E2
TATA
1ERE
2ERE
3ERE
Null
50
40
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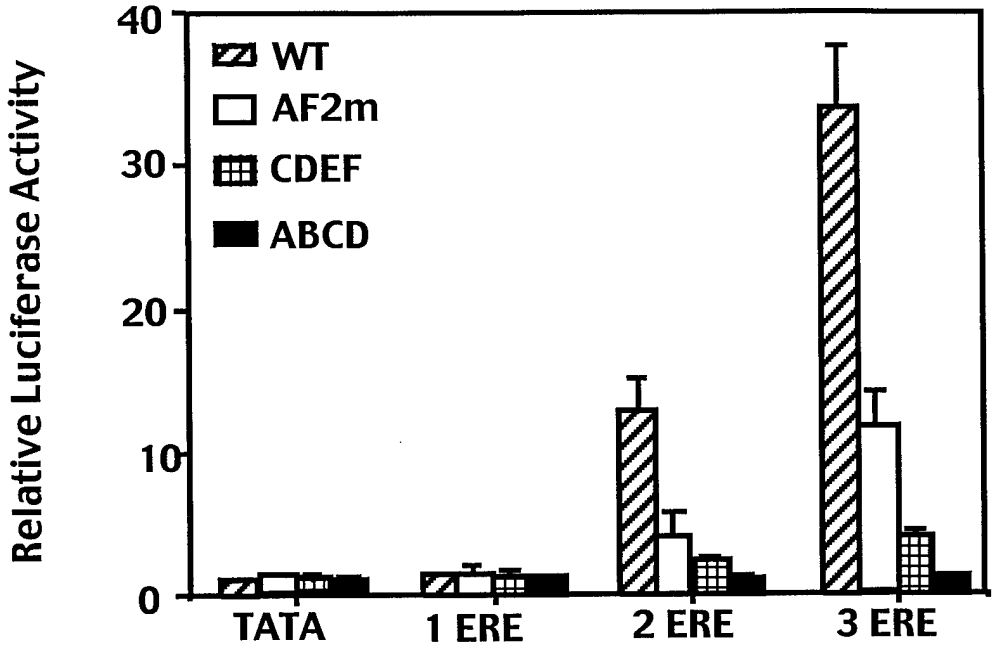
TATA
1ERE
2ERE
3ERE

Figure 3

A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CDEF
ABCD
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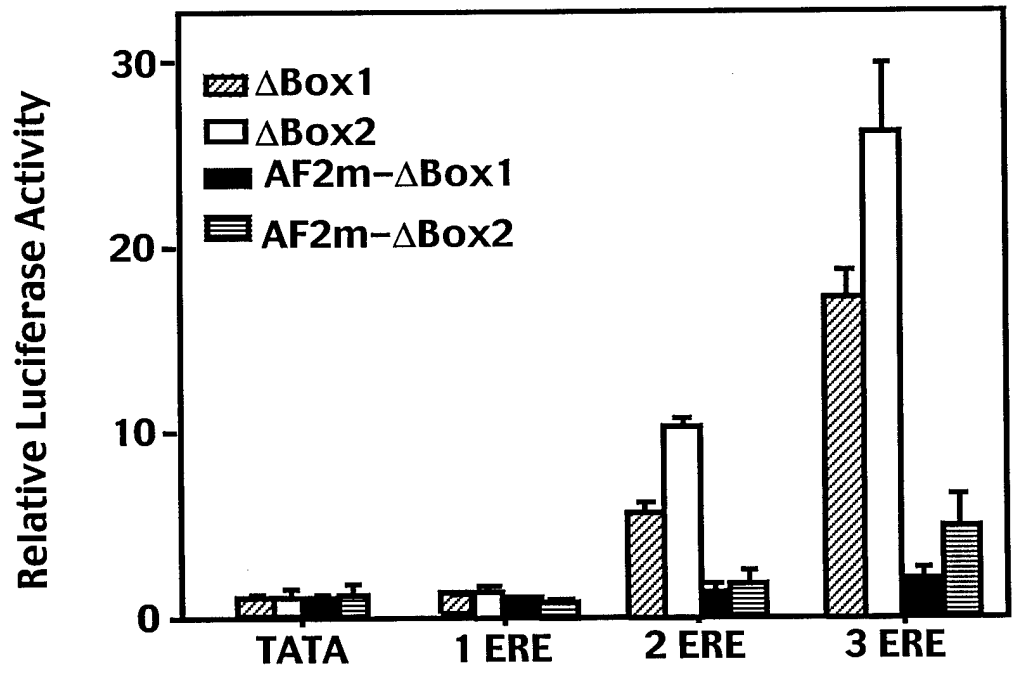
1 ERE
2 ERE
3 ERE

B


Relative Luciferase Activity
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20
10
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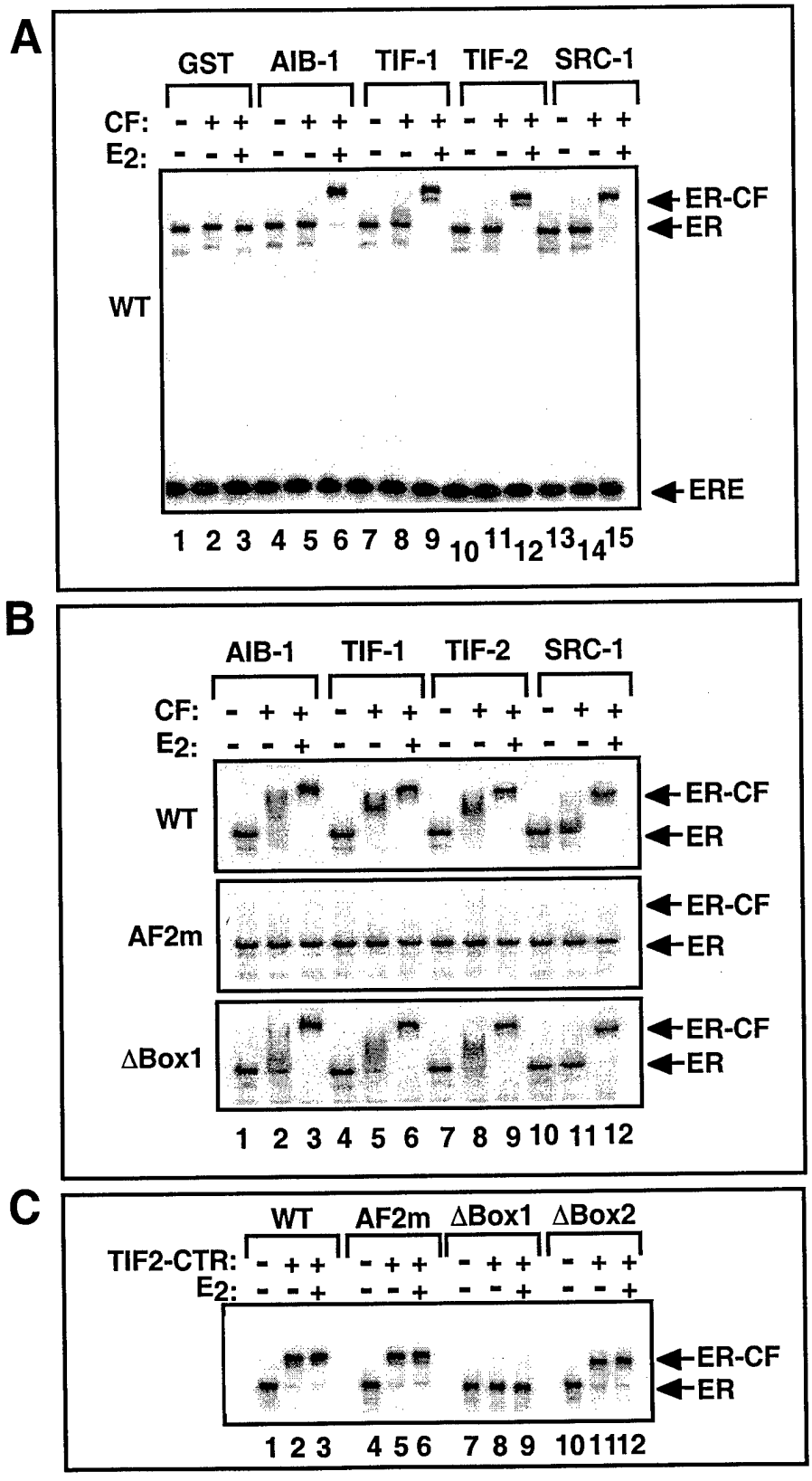
ΔBox2
AF2m–ΔBox1
AF2m–ΔBox2
TATA
1 ERE
2 ERE
3 ERE

Figure 4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TIF-1
TIF-2
SRC-1
CF:
E2:
WT
AF2m
ΔBox1
ER-CF
ER
1 2 3 4 5 6 7 8 9 10 11 12
C
WT
AF2m
ΔBox1
ΔBox2
TIF2-CTR:
E2:
ER-CF
ER
1 2 3 4 5 6 7 8 9 10 11 12

Figure 5

ERE AND ER LIGAND INDUCED STRUCTURAL CHANGES ARE INDEPENDENT DETERMINANTS FOR THE RECRUITMENT OF CO-FACTORS AND TRANSCRIPTIONAL RESPONSES BY ERα AND ERβ

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Estrogen signaling is mediated by ER α and β. ERs are converted from an inactive form to a transcriptionally active state through conformational changes induced by ligand and ERE sequences. It is unknown whether integrated influences of ligand and ERE determine the final ER conformation. This would present altered functional surfaces to specific co-regulators that could provide ERs with a diverse regulatory repertoire. We observed that ERα and ERβ use the same nucleotides for DNA contacts and bind to EREs with similar affinity and preferences independent from ligands. Although EREs and ligands induce conformational changes in ERs, these changes are not integrated to provide the receptors with novel functional features. We found that ERE determines the receptor affinity and thereby the relative amount of receptor binding, while the nature of ligand dictates the interaction of ligand-dependent co-factors by altering the affinity of ERs to co-factors. We also found that preferential interaction of a ligand-dependent co-factor through a distinct surface with ERα occurs independently from ligands, but the extent of interaction is dependent upon the ERE sequence. In transfected cells, ERα is more transcriptionally active than ERβ. The identity of ERE sequence, however, determines the potency of transcription when ERs bind to an agonist. Antagonists prevent ERs from inducing transcription independently from ERE sequences. Thus, ERE and ligand induced structural changes are independent determinants for the recruitment of co-factors and transcriptional responses by ERs. The ability of ERα to differentially recruit a co-factor through distinct interacting surfaces could contribute to ER subtype-specific gene responses.

INTRODUCTION

The estrogen responses are mediated by estrogen receptors (ER) α and β. ERs are members of a superfamily of nuclear receptors that function as ligand-modulated transcriptional regulators. Although ERα and β share high structural homology in their DNA binding domain (DBD), the amino- and carboxyl-terminal regions of the receptors that possess ligand-independent and -dependent activation functions, AF-1 and AF-2 respectively, are less conserved (52). While ERα and ERβ display similar biochemical characteristics (36, 37), distinct structural features that account for different transcriptional responses to ligands in a promoter and cell dependent manner also indicate functional differences between the receptor subtypes (14, 22, 35, 49, 61, 80).

The elements of estrogen-responsive gene transcription involve a multi-step regulation in which ER is converted from an inactive form to a transcriptionally active state. This regulation is initiated by a conformational change in ER upon estrogen binding, dissociation from chaperone proteins, and dimerization (18). The ER then binds to a palindromic DNA motif, estrogen responsive element (ERE), recruits co-factors and alters transcription (45). The minimal, or the core consensus ERE, is a 13 base pair inverted repeat, 5'-GGTCAnnnTGACC-3' (14, 49). Estrogen responsive genes, however, contain single or multiple copies of EREs that deviate from the consensus sequence by one or more nucleotides. Although these EREs confer estrogen responsiveness mediated by ERα, they are less potent enhancers of transcription than the consensus ERE (12, 15, 26, 44, 63). EREs are modulators of the conformation of the DNA binding domain (DBD) of ER (17, 55, 66, 67, 84), as shown for numerous transcription factors and nuclear receptors (42). A single nucleotide change in the consensus ERE, for example, requires the formation of new interconnected hydrogen bonds between the response element and the DBD of ERα, thereby altering the conformation of the region (66, 67).

In addition to estrogens, ER also binds anti-estrogenic compounds that act as agonist and/or antagonist. While the pure anti-estrogens, ICI 182,780 (ICI), are effective antagonists, tamoxifen (TAM), or its active metabolite trans-4-hydroxytamoxifen (4-OHT), displays mixed agonist/antagonist properties depending upon promoter and cell-context (20, 28, 29). Anti-estrogens sterically hinder correct alignment with the interacting surfaces of the carboxyl-terminal ligand binding domain (LBD) of ERs and alter the conformation of the region. Recent studies indicate that the LBDs of ERα and ERβ, despite the poor amino acid homology, display similar tertiary and quaternary architecture (9, 62). Although this accounts for the similar binding affinity of ligands to both receptors (35), differences in the amino acid sequences between the LBDs of ERs are also responsible for subtype-specific alterations in the conformation of LBD induced by anti-estrogens (62). Studies also suggest that interactive conformational changes occur among the receptor domains upon ligand binding (43, 75, 84).

Thus, it is likely that integrated influences of the ligand and the ERE could determine the final conformational state of ER. Since ER-mediated transcriptional responses are dependent upon the nature of ERE, ligand, promoter and cellular context, different conformation of ERα and ERβ influenced by ERE sequences and ER-ligands would present altered functional surfaces to specific co-regulators. This mechanism would then provide the receptors with a diverse regulatory repertoire in a gene- and cell-specific manner.

In this report, we address how various ligands cause alterations in the conformation of ERβ and ERα that influence the receptor affinity and specificity for binding with ERE sequences, and, in turn, the ability of DNA-

bound and ligand-occupied receptors to recruit co-factors. We show here that the extent of AF-2 dependent co-factor recruitment by ERα or ERβ is altered by both ER-ligands and ERE sequences. When ER is liganded with E_2, the extent of co-factor recruitment is primarily affected by the ERE sequence which determines the affinity, and thereby the relative amount, of receptor binding. 4-OHT or ICI occupation of the receptors prevents co-factor interactions with the receptors independent from the identity of ERE sequences. The recruitment of both AF-1 and AF-2 dependent co-factors through different domains, exemplified by TIF-2, occurs in a receptor subtype-specific manner. In transfected mammalian cells, we found that ERα is a more potent transcription activator in response to an agonist than ERβ, regardless of ERE sequence. However, the identity of ERE sequence determines the potency of transcription when ERs bind to E_2. This was also the case for 4-OHT or ICI when both compounds display partial agonist activity. When 4-OHT or ICI acts as pure antagonist, it prevents ERs from inducing transcription independently from ERE sequences. These results collectively indicate that ERE and ligand induced structural changes are independent determinants for the recruitment of co-factors and transcriptional responses by ERs. Moreover, we suggest that the ability of ERα to differentially recruit a co-factor through distinct interacting surfaces could contribute to ER subtype-specific gene responses.

MATERIALS AND METHODS

Plasmids

The human wild-type (WT) ERα cDNA provided by the late Dr. Angelo Notides and was inserted into pBluescript II KS (+) (pBS-KS). An expression vector bearing human ERβ cDNA was a gift from Dr. Simak Ali. This ERβ cDNA encodes a 477 amino acid ERβ. The extended sequence encoding the additional amino-terminal 53 amino acids was generated using PCR from human placental DNA (Sigma-Aldrich, St. Louis, MO) with primers based on the published sequence of the full length ERβ (58) and inserted in frame into the pBS-KS bearing the parent "short" ERβ cDNA. The resultant "long" ERβ, referred to here as the WT-ERβ, was then sequenced. Receptor cDNA was excised from pBS-KS and inserted into a mammalian expression vector (pM^2-AH) as described previously (54).

Fragments of AIB-1 containing nuclear interacting signature motifs within the region encompassing residues 522-827 (3), residues 219-399 of SRC-1 (59), and 623-986 of TIF-2 (79) were obtained by PCR and inserted into pGEX-2TK (Amersham Pharmacia Biotech, Piscataway, NJ). The residues 1125-1325 of TIF-2 contain glutamine-rich region that is shown to interact with ER (4) was also obtained with PCR. Fragment of TIF-1 containing residues 638-851 was amplified with PCR from a human testis cDNA library (Clontech Laboratories, Palo Alto, CA) with primers based on the published sequence (41, 73).

GST-cofactor fusion proteins were expressed in *E. coli* BL21(DE3) cells and purified using GST Purification Modules as recommended (Amersham Pharmacia Biotech., Piscataway, NJ). Protein contents in eluates were estimated using Bio-Rad Protein Assay Kit (Bio-Rad Labs, Hercules, CA). Equal aliquots GST-fusion proteins were resolved by SDS-PAGE and visualized by Coomassie staining of the gel.

Electrophoretic Gel mobility shift assay (EMSA)

Both strands of oligomers (Sigma-Genosys,The Woodlands, TX, or Integrated DNA Technologies, Inc, Coralville, IA) were annealed and [^{32}P]-end labeled as described (15). The end-labeled DNA (0.125 nM) was incubated with 0, 2.5, 5, 10, 15, 20 nM of human recombinant ERα or ERβ expressed in baculovirus-infected insect cells (Panvera, Madison) in a binding buffer in a total volume of 10 μl. The concentrations of recombinant ERs provided by the supplier were based on the quantitation of [^{3}H]-estradiol-receptor complexes using a hydroxyapatite assay. The binding buffer contains final concentrations of 40 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.1% NP-40, 5% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 μg/μl BSA, 0.1 μg/μl poly (dI-dC) (Midland Certified Reagents, Midland, TX). Reactions were incubated on ice for 1 hour and loaded onto an 8% native PAGE. The dried gel was visualized and quantified by PhosphoImager (Molecular Dynamics, Sunnyvale, CA). In assessing the effects of ligands on the binding specificity and affinity of the receptors, varying concentrations of receptors were pre-incubated without (0.01% ethanol) or with a saturating concentration (10^{-6} M) of 17β-estradiol (E_2; Sigma-Aldrich, St. Louis MO), 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) or ICI 182,780 (ICI; Tocris, Ballwin, MO) for 15 min on ice. This was followed by incubation with 0.125 nM end-labeled ERE for 30 min. Reactions were resolved as described above. For the effects of ERE sequences on ligand bound ERs, 10^{-7} M 16α-[^{125}I]-iodo-3, 17β estradiol (2200 Ci/mmol, NEN Life Sciences) was incubated with 15 nM receptors in the absence or presence of varying concentrations of radioinert E_2, 4-OHT or ICI for 15 min on ice. Unlabeled 0.125 nM oligonucleotides containing ERE sequences were then added into the reaction mixtures for an additional 30 min. Reaction mixtures were processed as described (35).

The affinity of ER for various EREs was determined by competition assays. The concentration of unlabeled oligonucleotides that contain various ERE sequences required to reduce ER complex formation with the labeled optimal ERE (p17) by 50% (IC_{50}) is proportional to the affinity of ER for the unlabeled oligonucleotide by the equation (10) $K_i = IC_{50}/(1+F/K_d)$, where K_i is the equilibrium constant for the unlabeled oligonucleotide and K_d is the equilibrium dissociation constant for the labeled p17. F is the concentration of labeled p17 in the assay. When the competition was performed with unlabeled p17, $K_i=K_d$.

For the recruitment of co-factors, 15 nM of the receptors were pre-incubated in the absence (0.01% ethanol) or presence of varying concentrations (10^{-9} to 10^{-6} M) of E_2, 4-OHT or ICI for 15 min on ice. The end-labeled consensus ERE was then added and reactions were incubated for an additional 30 min. This was followed by the addition co-factors in the amount of 1 μg/reaction for 30 min. Reactions were then resolved on 6 % non-denaturing PAGE.

Missing Nucleoside Hydroxyl Radical Assay (HRA)

Single stranded oligonucleotides were labeled at the 5' end with [γ-^{32}P] by polynucleotide kinase, gel purified and annealed with unlabeled complementary strand. Double stranded DNA was 3'-labeled using by DNA polymerase I Klenow fragment by incorporation of [α-^{32}P] dGTP. The labeled DNA, randomly cleaved by hydroxyl radicals as described (24), was incubated with ER on ice for 30 min. The amount of ER used was adjusted to bind

the majority of the end-labeled ERE oligomers. ER bound EREs were separated from free EREs by 5% native PAGE. Radioactive bands containing bound and free ERE were excised from the gel. The EREs were eluted, precipitated and dissolved. Equal concentrations of bound and free EREs were subjected to 18% sequencing gel electrophoresis. Maxam-Gilbert G-specific sequencing reactions were performed simultaneously. It should be noted that identification of critical residues at the 5' end of both strands when the oligomers were 5' end-labeled was difficult. This was also the case for the 3' end resolution of oligomers that were 3'-end labeled. The difficulty most likely arose from the length of the oligomers used in HRA (64). Using the same ERE oligomers labeled at either end, however, allowed us to obtain a corroborative assessment of critical residues.

Partial proteolysis of ERα and ERβ

Preparation of radiolabeled ERα and ERβ: Supercoiled pBS-KS (+) bearing none, as control, or receptor cDNA was transcribed/translated using a rabbit reticulocyte translation system with 2 μl L-[^{35}S]Methionine (1175 Ci/mmol; NEN Life Sciences, Boston MA) as directed (Promega, Madison, WI). Equal aliquots of reaction mixtures (5 μl of 50 μl reactions) were subjected to electrophoresis under reducing conditions on 10% SDS-PAGE. Bands corresponding to ERα and ERβ were excised from the gel and counted to estimate receptor concentrations.

Partial Proteolysis of ERα and ERβ: To assess the conformational changes induced by ER-ligands, equal molar concentrations of receptors were incubated without (0.01% ethanol) or with 10^{-6} M of E_2, 4-OHT or ICI for 10 min at room temperature (RT). The reactions were then subjected to 0, 1, 2.5, 5, 10 and 25 μg/reaction trypsin or chymotrypsin in a total of 10 μl reaction mixture for 10 min at RT. Reactions were terminated by the addition of 2 X sample buffer containing 5% β-mercaptoethanol, boiled for 5 min and subjected to 8-16% gradient SDS-PAGE.

To probe the conformational changes of ER ligand-ER complexes upon binding EREs, 15 nM ERs were incubated without or with 10^{-6} M E_2, 4-OHT or ICI for 15 min at RT. 0.125 nM end-labeled ERE was added into the reaction and incubated further for 15 min at RT. Reaction mixtures were then subjected to 0, 1, 2.5, 5, 10 and 25 μg/reaction trypsin or chymotrypsin, or 0, 2.5, 5, 10, 25 and 50 μg/reaction Endoproteinase Glu-C (Calbiochem, La Jolla, CA) for 10 min at RT. Samples, in a total volume of 10 μl, were immediately subjected to 8% non-denaturing PAGE.

Cell culture and Transfection

Maintenance and transfection of COS-1, CHO, HeLa and Hep G2 cells were described previously (54). Reporter plasmids contain none or two copies of EREs in tandem, with 38bp center-to-center distance, located upstream of a promoter. The promoter we utilized was a simple TATA box or thymidine kinase that drives the firefly luciferase cDNA as the reporter enzyme. Following transfection, the cells were incubated in fresh medium with or without E_2, 4-OHT or ICI for 24h. An expression vector carrying the β-galactosidase cDNA driven by the CMV promoter was also co-transfected as the control for transfection efficiency in the amount of 200 ng. Processing of cell lysates for the reporter enzymes was described previously (54). Results are the mean±SEM of three independent experiments in duplicate. In all transfections, normalized luciferase values are presented as the fold-change over the enzyme activity induced by the parent expression vector bearing no cDNA in the absence of E_2 (data not shown).

RESULTS

ERE sequences that bind ERβ with high affinity

The core ERE sequence is a 13 base pair palindrome, 5'-GGTCAnnnTGACC-3'. ERα binds this sequence efficiently and mediates gene transcription *in situ* in response to estrogen (33). However, only a few of the most highly responsive genes have this palindromic sequence. Most estrogen-regulated gene sequences vary from the core by one or more nucleotides (15, 26). Additionally, many genes have commonly appearing nucleotides that flank the core sequence. Guided by sequences present in natural settings, we previously designed and tested a series of ERE oligomers for binding affinity to ERα by using EMSA and binding competition assays (15). Results indicated that sequences with variations in the core retain effective binding affinity for ERα if they have appropriate flanking sequences. We then proposed rules that predict whether an ERE-like sequence confers high enough ER affinity to be a functional ERE.

Although the DBDs of ERβ and ERα are highly homologous, a recent study suggests that ERα and ERβ display different binding patterns for natural EREs having one or more nucleotide changes from the core sequence (26). This implies that the binding affinity and specificity of ERs to various EREs may not be the same. To examine whether the rules developed for ERα also serve as a useful guide for the binding of ERβ to ERE-like sequences, we measured affinity to a series of EREs varying in sequence (Fig. 1). The DNA substrates consist of a test sequence with one, two or three nucleotide changes (boldfaced) from the core ERE embedded (brackets) within a larger oligomer with no ERE features (background oligomer). The binding ability of ERs to EREs (summarized in Fig. 2B) was assessed by EMSA.

We first examined how one or more nucleotide substitutions in the core affect ER binding. ERβ interacts in a concentration dependent manner with the p13 that contains the Vitellogenin A2 core (Fig. 2A). Increasing formation of ERβ-ERE complex (Bound) was correlated with a decrease in the labeled ERE that remained unbound (Free). A single change in the core (13d1) reduced ERβ binding about ten-fold (Fig. 2A). Binding was undetectable when two nucleotide changes were introduced (13d2). The decreased binding of ERβ to p13d1 was independent from the identity of the nucleotide or the position of substitution (data not shown).

We also tested whether specific flanking nucleotides could increase/restore the receptor binding. As a first approximation, we used the flanking nucleotides of the Vitellogenin A2 gene ERE. Placement of an A residue at position -7 and a T at position +7 without (p15) or with one nucleotide substitution (p15d1) increased the ERβ binding compared to that with p13 and p13d1 two- and ten-fold, respectively. The same flanking substitutions also restored binding by compensating for two substitutions within the core (p15d2). These results indicate that the flanking sequences are important for binding (Fig. 2A). Binding of the receptors to p15 with three nucleotide changes in the core (p15d3) was negligible (data not shown). A G residue at the 5' end with a C placement at the 3' end of the core (15GC) also enhanced the ERβ binding. A C residue (15CG) or a T (15TA) was not as effective as the A or G (Fig. 2B). Similar results were obtained for ERα (data not shown). These results indicate that the identity of the nucleotides immediately flanking the core can be critical for effective binding.

We also examined whether nucleotides that are two residues distant from the core sequence further affect receptor binding to p15. We placed a 5' C two nucleotides away from the core, i.e., at position -8, and a 3' G at position +8, reflecting the Vitellogenin A2 gene ERE, CAGGTCAnnnTGACCTG-3'. Results revealed that the binding of ERβ to p17 with one (p17d1, data not shown) or two (p17d2, data not shown) nucleotide substitutions displayed a binding specificity and affinity (see below) similar to those for p15, p15d1 and p15d2. However, we also found that ERβ binds to p15d1TA with lower efficiency than to the identical sequence containing an additional 5' C and a 3' G residues at position –8 and +8, respectively, (p17d1TA; data not shown). Thus, bases that are two residues away from the core sequence can have an impact for binding of ERβ if the core ERE has a variation from the core. Overall, we found that ERβ binds similarly to the same range of ERE sequences, as does ERα. ERβ, as ERα, binds to 5'-C(A/G)GGTCAnnnTGACC(T/C)G-3' sequence with an optimal efficiency.

We also assessed the relative affinity of ERβ to various ERE sequences in comparison with ERα by competitive displacement assays. EREs were tested for the ability to compete with the optimal ERE sequence (p17) for ERα and ERβ binding. Results demonstrated that while the binding affinity (K_d) of ERβ for p17 was 5.6 ± 0.4 nM, one (p17d1) or two nucleotide substitutions (p17d2) reduced the K_ds of the receptor to 11.3 ± 3.4 and 101.2 ± 5.3 nM, respectively. Although both ERs bound to the ERE sequences with indistinguishable specificity, the affinity of ERα to EREs was two-fold higher than that of ERβ. K_d values of ERα for p17, p17d1 and p17d2 ERE sequences were 2.9 ± 0.7, 6.3 ± 1.5 and 53.5 ± 4.2 nM, respectively.

In addition to the natural hormone E_2, ERs also bind to compounds that act as partial agonists (4-OHT), or antagonists (ICI 182,780). Since the nature of the ligand is critical for the extent of transcriptional responses from ERE driven promoters (32), we also addressed whether ligands alter the affinity and specificity of ERα and ERβ

binding to various ERE sequences. We pre-incubated the receptors with a saturating concentration (10^{-6} M) of E_2, 4-OHT or ICI. We found that ligands had minimal, if any, effect on the binding pattern or affinity of ERs to EREs (data not shown). These results, as shown previously for ERα (20, 53), demonstrate that ligands do not have an impact on the ability of the receptors to bind to an ERE.

Although the affinity of ERβ for EREs is lower than that of ERα independent from ligands, the similar DNA binding specificity suggests that the same range of ERE-like sequences binds to both receptors. However, since the binding affinity of ERα to an ERE correlates with the extent of transcription (7, 12, 44, 56, 70), a lower binding of ERβ could be one of the underlying mechanisms for the differences in the transcriptional strength of the receptors (14, 52).

ERα and ERβ utilize the same nucleotides for binding to an ERE

Previous studies indicated that ERα makes contacts with one face of the palindromic sequence in adjacent major grooves of DNA (67, 68). The interactions are mediated by the binding of the first zinc-finger motif of each DBD that makes base-specific contacts within the major groove of the DNA helix, while the second zinc-finger motif forms a dimer interface between the two DBDs. These interactions determine the specificity of the response element recognition. Highly conserved amino acid sequence identity of the two DBDs together with a similar binding specificity to EREs, as we showed here, suggests that the ERα and ERβ employ similar contacts with EREs. However, our results also indicate that ERβ binds to an ERE with a lower affinity than ERα. Since reduced binding affinity to non-consensus EREs results from the formation of alternative patterns of intermolecular contacts between DNA and ER (67), it is also possible that ERβ makes different nucleotide contacts when binding to an ERE compared to ERα. To address this issue, we used HRA to detect differences in binding contacts between the two receptors. Since the affinities of both ERs to p17d2 are substantially lower than that for p17, we also examined whether contact sites are altered when ERs bind to a non-consensus ERE.

HRA assesses the contribution to protein binding of each member of a base pair independently of all other nucleotides in a linear double-stranded DNA molecule (24). This method is based on the expectation that if a base important for binding were missing in a particular DNA molecule, the protein binding affinity would be adversely affected. For this assay, hydroxyl radical treatment is used to remove a single nucleoside from DNA. This generates DNA fragments containing fewer than one randomly placed one-nucleotide gap per fragment, allowing the analysis of DNA-protein interaction at single-nucleotide resolution.

The receptors were incubated with 5' or 3' (data not shown) end labeled and gapped test oligomers. The ER bound EREs were separated from unbound EREs by non-denaturing PAGE. Radioactive bands containing the bound and free ERE were excised from the gel, eluted and were then subjected to denaturing PAGE analysis (Fig. 3). A low intensity, or missing, band in the lane containing ERE bound ERs (lanes 5 and 6), or conversely a high intensity band in the lane containing free ERE (lanes 4 and 7), identifies a nucleoside important for the formation of the ER-ERE complex. The intensities of individual DNA bands were quantified by PhosphoImager. Shown in Fig. 3 is the TGACC half-arm of the bottom strand. A high ratio of free to bound ERE is represented as long horizontal bars, and approximates the strength of contacts with ERs. The crystal structure of the DBD of ERα indicates that the G at position +3, and the T and A at position +2 and +4, respectively, make multiple contacts with the region (67, 68). We also observed that both ERα and ERβ have a strong interaction with the G at position -3 and the T and A at position -2 and -4, respectively, of the TGACC half-arm of the bottom strand of the consensus ERE. Similarly, the G at position +3 and surrounding T and A contacted the receptors in the TGACC half-arm of the upper strand (data not shown). The G residues at positions -5 and -6 on the GGTCA half-arm of the upper strand together with flanking bases A and C at position -7 and -8 also showed contacts with both receptors (data not shown). These results demonstrate that both ERα and ERβ make contacts with the same nucleosides in the consensus ERE.

When two nucleotide substitutions were introduced into the consensus sequence (p17d2), the contact sites were altered only in the TGACC half-arm of the bottom strand. Changing the T to an A at position -2 did not have significant effects on contacts. The G at position -3 remained critical for interaction with both ERα and ERβ. Strong interaction was lost when the A at position -4 was substituted with a C. The C at position –5 and the flanking C and T then became the critical bases for contacts, further emphasizing the role of flanking sequences in the binding of receptors to non-consensus EREs.

Thus, despite differences in affinities, ERα and ERβ utilize the same nucleotides for binding to an ERE. Base contact sites are altered when the receptors interact with a non-consensus ERE. These results reaffirm our conclusion that the ER subtypes interact with EREs similarly. Furthermore they suggest that the decreased DNA affinity of ERs for non-consensus EREs result from the formation of alternative patterns of intermolecular contacts.

ER-ligand and/or ERE induced conformational changes in ER

ER-ligand induced conformational changes in the ER. Ligand binding results in substantial alterations in the conformation of ERα manifested as changes in receptor hydrophobicity, epitope exposure and protease sensitivity (4, 21, 23, 69, 83). Limited proteolysis has been a useful structural probe for investigating the effects of ligand on ER conformation (4, 34, 69). To examine how different ligands alter protease accessibility of ERα and ERβ, we pre-incubated equal molar concentrations of [^{35}S]-methionine labeled receptors synthesized *in vitro* in the absence (Control, 0.01% ethanol) or presence of 10^{-6} M E_2, 4-OHT and ICI. They were then subjected to partial proteolysis with varying concentrations of chymotrypsin or trypsin. Reactions were resolved on an 8-16 % gradient SDS-PAGE.

As shown for ERα (34, 69) and recently for ERβ (34), proteolysis of ERβ with increasing concentrations of chymotrypsin (Fig. 4) resulted in proteolytic fragments with distinct electrophoretic migration. In the absence of ligand, proteolysis of the full length ERβ (M_r of 60 kDa, lane 1), produced four distinct fragments that migrate with M_r of 35, 33, 31 and 27 kDa, corresponding to βC1, βC2, βC3, βC4, respectively, (lanes 2-6). When the receptor was occupied with E_2 (lanes 7-12), the βC1 and βC2 (lanes 11 and 12) were more resistant to the same concentration of protease than in the absence of ligand (lanes 5 and 6). This was reflected in a slower disappearance of βC1 and a delayed appearance of βC4. The electrophoretic migration patterns of proteolytic fragments of ERβ complexed with 4-OHT (lanes 13-18) were similar to those of ERβ bound to ICI (19-24). However, the degree of enzyme sensitivity of these fragments differed from those observed for ERβ both in the absence or presence of E_2. Quantitative appearance of βC3 and subsequent further proteolysis to βC4 occurred at lower concentrations of protease. Moreover, we also observed that the appearance of βC3 occurs at a lower enzyme concentration when ERβ was bound to 4-OHT (lane 16) in contrast to ICI (lane 22). Results were similar with exposure to trypsin (data not shown). These results indicate that each ligand is capable of inducing a distinct conformation in the receptor.

Digestion of the full length ERα (M_r of 65 kDa, lower panel, lane 1) produced three distinct fragments, αC1, αC2 and αC3 that migrated at 35, 33 and 30 kDa, respectively, in the absence of ligand (lanes 2-6). When the receptor was occupied with E_2 (lanes 7-12), the αC1 and αC2 (lanes 11 and 12) showed more resistance to the same concentrations of protease. Similar resistance of αC1 and αC2 was also observed at the penultimate concentration of protease when ERα was bound to 4-OHT (lane 17) or ICI (lane 23) compared to the receptor in the absence of ligand (lane 5). In contrast to E_2-bound receptor (lane 12), the highest concentration of chymotrypsin produced identical proteolytic fragments in the absence (lane 6) and the presence of 4-OHT (lane 18) and ICI (lane 24). Consistent with previous observations (4, 34, 69), these results indicate that ER-ligands also induce distinct conformational changes in ERα.

ERE-induced conformational changes in ligand occupied ER: In addition to ligands, EREs also allosterically modulate receptor conformation upon binding (17, 55, 66, 67, 84), as shown for numerous transcription factors and nuclear receptors (42). Reports have indicated that a single nucleotide change in the consensus ERE causes the formation of new interconnected hydrogen bonds between the response element and the DBD of ERα, thereby altering the conformation of the region (66, 67). Previous studies also suggest that DNA induced changes are transmitted to both the carboxyl- and the amino-termini of the receptor (43, 75, 84). It is therefore likely that a dynamic interplay between ERE sequence and ligand determines the final conformational state of the receptor. To examine whether various ERE sequences alter the conformation of ERs in the absence or presence of ligands, we initially used [^{35}S]-methionine labeled receptors synthesized *in vitro* and unlabeled ERE followed by SDS-PAGE as described above. We found, however, that a significant portion of ERs synthesized *in vitro* is incapable of binding DNA. In the presence of a heterogenous population of receptor species, the interpretation of effects of ERE and ligands on ER conformations using labeled receptors was, at best, difficult.

As an alternative approach, we employed a gel shift-proteolysis assay (84) using non-labeled ERs and labeled ERE. The assay combines EMSA as a detection approach for functional ER-ERE interaction and partial proteolysis as an approach for the analysis of the conformation of the ERE bound ER complexed with an ER ligand. Experimental paradigms to detect conformational changes in the absence (as described above) or the presence of an ERE, as we are employing here, are not the same, although both approaches probe ER structure using proteolytic digestion. The former allows the detection of the entire proteolytic population of ER species that contain differentially digested fragments. This is because the labeled methionine residues are distributed throughout the ER. The latter assesses DNA-induced conformational changes in ER fragments that remain bound to DNA. This is because the label is in the DNA. If an ERE is capable of inducing conformational changes in ERs, proteolysis should produce different fragmentation based on the identity of the ERE.

To address this issue, end-labeled p17, p17d1 or p17d2 were incubated with equal amounts of ERs. This was followed by exposure of the ER-ERE complexes to various concentrations of chymotrypsin. Reaction mixtures

were then subjected to 8% non-denaturing PAGE (Fig. 5). Chymotrypsin treatment of the ERE-ERα or -ERβ complex (lanes 1-6) resulted in proteolytic fragments having faster electrophoretic migration with increasing concentrations of the enzyme. Although at lower concentrations of the enzyme there were no discernable differences in the digestion products, the impact of ERE sequence on the extent of proteolytic fragmentation was clearly visible when high concentrations of chymotrypsin were used. The unliganded ERα or ERβ bound to the consensus ERE (p17; upper panel) showed more resistance to the protease compared to ERs bound to ERE sequences containing one (p17d1; middle panel) or two (p17d2; lower panel) nucleotide substitutions. This was reflected in the pronounced (p17d1 and p17d2) and early (p17d2) appearance of C2 and C3 for ERα and C3 and C4 for ERβ. These results demonstrate, as shown for other DNA binding proteins including ERα (17, 42, 55, 66, 67, 84), that EREs alter the conformation of both receptors.

We also wanted to examine whether ligands can further influence the ERE-induced conformational changes. If a ligand induces a distinct fragmentation pattern in ERs that are bound to different EREs compared to unliganded receptors, this would suggest that ERE and ligand alter the conformation of ERs in an integrated, interactive manner. We observed that exposure of ERE bound ERα or ERβ in the presence of 10^{-6} M E_2, (lanes 7-12), 4-OHT (lanes 13-18) or ICI (lanes 19-24) to varying concentrations of chymotrypsin produced proteolytic patterns that were indistinguishable from those of the unliganded receptors (lanes 1-6). Thus, it appears that ligands do not produce altered protease accessibility in the ER-ERE complex compared to the unliganded ER. However, since ERs are not labeled in this gel shift-proteolysis assay, a similar digestion pattern does not rule out the possibility of interactive conformational changes induced by both ERE and ER ligand. This is because the proteolytic fragments represent ER species that remain bound to the ERE, and may or may not contain regions that are influenced by both ERE and ER ligand.

To ensure that binding to an ERE did not lead to dissociation of ligand from either ERα or ERβ prior to protease exposure, consequently similar proteolytic fragmentation, we pre-incubated the receptors with [^{125}I]-E_2. Radio-iodinated ligand remained bound to the receptor both in the absence and presence of ERE under the same conditions described above. Moreover, the labeled ligand was quantitatively removed by competition binding with increasing concentrations of inert E_2, 4-OHT and ICI, the extent of which was independent from the identity of ERE sequences (data not shown).

To further confirm that the similar pattern of proteolysis of the unliganded or liganded receptors bound to ERE sequences is not due to the protease used, both ERs were subjected to varying concentrations of Endoproteinase Glu-C. Although the exposure of ERs to the protease produced a distinct fragmentation pattern from that observed with chymotrypsin, the proteolysis pattern of the unliganded or liganded ER species was the same whether ERs bound to p17, p17d1 or p17d2 (data not shown).

Since ER ligands have no affect on the binding specificity or affinity of ER to ERE sequences, nor do EREs affect ligand interactions with ERs, these data collectively suggest that interactive conformational changes induced by both the ligand and ERE are minimal. This implies that conformational changes induced by ERE and ligands are primarily confined to the corresponding functional domains.

The recruitment of co-factors by ligand-ER-ERE complexes

If, as our data suggest, ERE and ligand do not induce interactive conformational changes in ERs, the nature of co-factor interaction with the receptors in response to ligands should not be affected by the ERE sequence. Studies have indicated that ERs interact with a complex array of co-regulator proteins that mediate the interactions between receptors and the basal transcription apparatus, and the remodeling of chromatin structure (8, 13, 19, 50). Among the co-regulators, SRC-1, TIF-1, TIF-2 and AIB-1 have been shown to interact with the LBD in an agonist-dependent manner by GST-pull down, by yeast and mammalian two-hybrid analysis (3, 16, 25, 30, 34, 41, 57, 59, 65, 79). To further examine whether ERE and ligand can induce interactive conformational changes in ERs that affect the nature of co-factor interaction with the receptors, we utilized EMSA. Since it appears that ER and co-factor interactions are sensitive to ligand concentrations (34, 65), we initially addressed how various concentrations of ligands affect the recruitment of co-factors by ER and whether or not different ERE sequences have an impact on these interactions. Equal molar concentrations of ERα and ERβ were pre-incubated without or with E_2, 4-OHT and ICI ranging from 10^{-9} to 10^{-6} M. This was followed by incubation of the reaction mixtures with ^{32}P-end labeled ERE. The polypeptides of co-factors, as GST fusion proteins, were then added into reaction mixtures in the amount of 1 μg/reaction. After the reaction, samples were subjected to non-denaturing gel electrophoresis. As shown in Fig. 6A and B, the ERE-bound ERα or ERβ (lane 2) was quantitatively supershifted by the TIF-$2_{623-986}$ (TIF-2) in the absence of ligand (lane 3). This interaction was independent from the identity of ERE sequence in that the pattern of interaction was the same whether the receptors bound to p17, p17d1 or p17d2 (upper, middle and lower panels, respectively). However, the extent of TIF-2 interaction with both receptors was correlated with the affinity,

hence the relative amount, of receptor binding to EREs. Binding of ERα to E_2 over a range of hormone concentration increased the proportion of receptor that bound TIF-2 (lanes 4-7). The co-factor retarded the migration of the entire population of ER-ERE complexes in the presence of 10^{-8} M E_2 (lane 5). This retardation was specific to TIF-2 as GST alone did not alter the migration of the receptors (data not shown). Significantly, binding of the receptors to increasing concentrations of either 4-OHT (lanes 8-11) or ICI (lanes 12-15) abolished TIF-2 interaction with the receptors bound to p17, p17d1 or p17d2.

The quantitative binding of TIF-2 with the unliganded ERα and ERβ, irrespective of the identity of ERE sequences, is surprising given the fact that recruitment of TIF-2 by ER has been reported to be ligand-dependent (3, 16, 25, 30, 34, 41, 57, 59, 65, 79). To address whether the amount of TIF-2 used contributes to the extent of interaction with the unliganded ER, concentrations of TIF-2 ranging from 0.1 to 1 μg/reaction were incubated with ER-ERE complexes in the absence or presence of 10^{-7} M E_2 (Fig. 6C and D). While the unliganded ER interacts with TIF-2 when the co-factor is present at high concentrations (lanes 4 and 5), E_2 increased the affinity of ER dramatically to TIF-2 such that the co-factor bound even at the lowest concentration tested (lane 7). This resulted in the complete retardation of the ER-ERE complex in the presence of E_2.

As with TIF-2, TIF-$1_{638-851}$ (TIF-1), AIB-$1_{522-827}$ (AIB-1) and SRC-$1_{219-399}$ (SRC-1) were also recruited by the unliganded ERα and ERβ bound to p17 (Fig. 7A and B; lane 3), p17d1 or p17d2 (data not shown). Increasing concentrations of E_2 enhanced the ability of the receptors to interact with the co-factors. This was reflected in the complete mobility shift of the E_2-ER-ERE-cofactor complex at 10^{-8} M E_2 (lane 5).

All co-factors in a concentration dependent manner interacted also with the ERE-ER complexes in the absence of ligands (Fig. 7C and D; lanes 1-5). The presence of 10^{-7} M E_2 increased the interaction of co-factors with both ERα and ERβ by augmenting the affinity of receptors for co-factors (lanes 6-10). Although the extent of interactions of ERα and ERβ with the co-factors was similar, discernable differences were also noted in the binding patterns of E_2-ER-ERE-cofactor complexes. While the TIF-1, and to a lesser extent TIF-2 (Fig. 6C), binding to E_2-ERα-ERE complexes showed a gradual decrease in the electrophoretic mobility of the complex, the interaction of equal molar concentration of ERβ with TIF-1 or TIF-2 under the identical conditions was essentially "all-or-none" as assessed by mobility. Although the underlying reason is not clear, one likely explanation is that the affinities of these co-factors could be different for ERβ than for ERα. Nevertheless, these results suggest that the mode of interaction among co-factors could differ depending upon the ER subtypes.

Thus, these results indicate that the AF-2 dependent co-factor interactions with both ERα and ERβ is similarly affected by ligands independently from the identity of ERE sequence. The data suggest that the integrated influences of ERE and ligand on conformational states of ERα and ERβ do not provide a means by which either receptor could present distinct functional surfaces to different AF-2 dependent co-regulators that could lead to a selective gene activation.

Studies based on alterations in epitope accessibility of ERα upon binding to an ERE sequence suggest that ERE induced conformational changes are transmitted to the amino-terminal region (84). Moreover, the partial agonistic effect of tamoxifen coupled ERα, but not ERβ, from consensus ERE driven promoters, is modulated through the amino terminal AF-1 (6, 48, 51). These observations raise the possibility that the integrated influences of ERE and ligand could affect the interaction of AF-1 dependent co-factor recruitment. Among the co-factors, GRIP-1 (81) and the human homolog TIF-2 (5), through distinct interacting surfaces other than signature nuclear receptor interacting domains (NID), are shown to interact with the amino-terminal ERα independently from ligands in GST-pull down assays. ERβ, on the other hand, fails to interact quantitatively with GRIP-1 (81).

To examine whether ERE and ligand affect the nature of AF-1 dependent co-factor interaction with ERs, the polypeptide of the glutamine-rich (Q) region of TIF-2 containing residues 1125-1325 (TIF2-Q) was produced as a GST-fusion protein. This region has previously been shown to interact with the AF-1 domain of ERα in a ligand independent manner in GST pull-down assays (81). The recruitment of TIF2-Q by ERs occurred independently from ligands, the extent of interaction, however, was preferential for ERα. ERα bound to p17 (Fig. 8A, upper panel) effectively and quantitatively recruited TIF2-Q in the absence or presence of various concentrations (10^{-9} to10^{-6} M) of E_2 (lanes 4-7) 4-OHT (lanes 8-11) or ICI (lanes 12-15), while ERβ interacted with the co-factor minimally (Fig. 8A, lower panel). Increasing concentrations of the co-factor led to a gradual retardation and the eventual complete shift of the ERα-p17 complex (Fig. 8B, upper panel) in the absence (lanes 1-5) or presence of 10^{-7} M E_2 (lanes 6-10), 4-OHT or ICI (data not shown). However, TIF2-Q supershifted only about 10% of the ERβ bound to p17 at the highest concentration tested whether or not E_2 was present (Fig. 8B, lower panel). The extent of interaction of the co-factor was correlated with the amount of ERs bound to ERE with one (p17d1) or two nucleotide (p17d2) substitutions in the core (data not shown).

Thus, these results indicate that ERE and ER ligand induced conformational changes are not integrated to provide the receptor with novel functional properties, and that ERE and ligand are independent determinants for the recruitment of co-factors by ERs.

The effects of ERE sequences and ER-ligands on ER-mediated transcriptional responses

If, indeed, AF-2 dependent co-factor recruitment by both ERα and ERβ is affected by ERE sequences and ER-ligands acting independently, the extent of E_2-induced transcriptional responses mediated by either ER subtype should primarily be dependent upon the ERE sequence. To test this prediction, we transfected mammalian cells with an expression vector bearing the ERα or ERβ cDNA together with a reporter plasmid containing none or two EREs in tandem upstream of a minimal TATA box or the complex thymidine kinase (TK) promoter. Both promoters drive the *firefly luciferase* cDNA as the reporter. The transfection efficiency was normalized with the β-galactosidase activity from a co-transfected expression vector bearing the enzyme cDNA. E_2 at 10^{-9} M concentration dramatically augmented the luciferase activity induced by ERα in the absence of the hormone from the reporter plasmid bearing two optimal EREs (2x17) compared to the reporter plasmid bearing no ERE sequence (TATA) in transiently transfected COS-1 (Fig. 9A), CHO or HeLa (data not shown) cells. Although ERα augmented the reporter enzyme activity from two variant EREs (2x17d2) in the absence or presence of E_2, the extent of transcription was substantially lower than that from the consensus EREs. Similar results were observed also for ERβ which displayed significantly lower transcriptional activity compared to ERα in COS-1 (Fig. 9A), CHO or HeLa cells (data not shown), as shown previously (14, 22, 35, 49, 61, 80). Although the magnitude of transcriptional responses was lower compared to the responses from the TATA box constructs, the results were similar when we used the TK promoter (data not shown).

To examine whether 4-OHT and ICI can alter the pattern of transcriptional responses from the EREs in a promoter and cell-context dependent manner, we tested their effects on ER-induced transcriptional responses in transiently transfected COS-1, CHO and HeLa cells. We found that neither ER affected the luciferase activity from either promoter construct when cells were treated with various concentrations (10^{-11}-10^{-5} M) of 4-OHT or ICI. Both compounds, as expected, effectively antagonized the E_2-induced enzyme activity in response to either ER (data not shown).

HepG2 cells have been used as a model system in examining the partial agonist activities of ER-ligands (47). In these cells, similar to E_2, 4-OHT or ICI in a concentration-dependent manner (Fig. 9B; shown is 10^{-7}, 10^{-8} and 10^{-6} M for E_2, 4-OHT and ICI, respectively), augmented ERα-induced luciferase activity from the TATA box or TK promoter (data not shown) bearing EREs in tandem. As observed for E_2-mediated induction, the extent of luciferase activity induced by 4-OHT or ICI was also correlated with the affinity of ERα to the ERE sequence. Although ERβ-induced enzyme activity was minimal in response to all three compounds in this cell line, the transcriptional induction from different ERE sequences showed a pattern similar to that observed for ERα (data not shown).

Thus, these results collectively indicate that the integrated influences of ERE and ER-ligand do not provide ERs with altered functional features responsible for a differential regulation of estrogen responsive genes in an ERE, ER ligand, promoter- and cell-context dependent manner. These data corroborate our conclusion that the nature of ERE sequence determines the potency of transcriptional responses to either ER when bound to E_2 or an agonist. This is also consistent with numerous observations that non-consensus ERE sequences are less potent transcriptional enhancers than the consensus ERE in response to E_2 (12, 44, 63). Moreover, since the interaction of the AF-2 dependent co-factors with the AF-1 domain (81) occurs in a receptor-specific manner, our results further suggest that structural differences between the AF-1 domains of ERs reflect the functional differences between ER subtypes. These are manifested here as differences in the extent of transcriptional responses.

DISCUSSION

The recognition of an ERE sequence by the estrogen receptors is central to transcription of estrogen responsive genes. Although the consensus ERE is a palindrome, most of the responsive genes contain sequences that vary from the consensus by one or more nucleotides. Our results indicate that the characteristics of non-consensus EREs that effectively predict the relative binding affinity of ERα (15, 26) are equivalently relevant for ERβ. These data indicate that ERE sequences in responsive genes are not a predictor for receptor specificity.

Although both receptors show a similar binding specificity to EREs, ERα binds to an ERE with an approximately two-fold higher affinity than ERβ. Moreover, the affinity for an ERE sequence to both receptors was correlated with the number of nucleotide variations from the consensus. We determined that a single nucleotide substitution within the core reduced both ERα and ERβ binding, whereas two nucleotide changes abolished the interaction. Furthermore, substitution of certain nucleotides immediately flanking the consensus or variant core

could substantially improve receptor binding. The nature of nucleotides positioned two bases away from a variant core sequence can also have an impact on the binding of the receptors. For example, both ERα and ERβ bind to 5'-C(A/G)GGTCAnnnTGACC(T/C)G-3' with an optimal efficiency, as we proposed previously for ERα (15). One or two nucleotide substitutions within this ERE, however, reduced the binding affinities of the receptors two- and 20-fold, respectively.

These observations allow reliable predictions of ER binding affinity to EREs and effectiveness of an ERE *in vivo*. For example, the Vitellogenin B1 ERE2, 5'-GGTCAccaTGCCC-3', and the human pS2 ERE, 5'-GGTCActgTGGCC-3', have one change from the consensus sequence (shown in bold). These EREs should bind to ERs with a reduced affinity and mediate an estrogen response to a lesser extent compared to the consensus ERE. Indeed, studies indicate that the binding affinity of ERα to the optimal ERE is two-fold higher than to either B1 or pS2 ERE, and that transcriptional response of either ERE is about three-fold less than that of the consensus ERE (44, 56). The Vitellogenin B2 ERE sequence contains two nucleotide substitutions in the core, 5'-**A**GTTAggcTGACC-3' (shown in bold), a sequence that both receptors should fail to bind. However, the presence of the critical flanking residues 5'-AAGTTAggcTGACCT-3' (underlined) allows ERα to bind, albeit with a 20-fold lower efficiency compared to binding to the Vitellogenin A2 ERE (44). Transcriptional responses from the Vitellogenin B2 ERE are also substantially lower compared to the consensus ERE (44). The binding and hormone responsiveness of other natural EREs are also consistent with these predictions (15), producing a useful guide to ERE activity, and an increased understanding of ER-DNA interaction.

We show here that both ERα and ERβ utilize the same nucleotides to bind to ERE sequences. We also found that introduction of two nucleotide substitutions into the consensus ERE lead to asymmetrical changes in the contact sites. Re-arrangement of contact sites in response to substitutions include bases that flank the core repeat further emphasizing the importance of flanking sequences in the binding of the receptors to a non-consensus ERE.

Protease sensitivity assays have been widely used to characterize the ligand-induced conformational changes in ERα (2, 31, 34, 40, 47, 69) and ERβ (34). Here we found that ERs unliganded or liganded with E_2, 4-OHT, or ICI, displayed different sensitivities to partial proteolysis. While E_2 bound ERs were hypo-sensitive to the proteases, 4-OHT or ICI occupied ERs were more prone to proteolysis at lower concentrations of the enzymes. Since the proteolysis assay relies on access to cleavage sites that possess conformational flexibility found between the tightly folded domains (11), these data indicate that each ligand tested induces different conformations in both ERs.

Similarly, protease exposure of both ERs bound to the consensus or non-consensus EREs with one or two nucleotide changes produced different fragmentation patterns as assessed by EMSA. This indicates that each ERE sequence induces a different conformation in ERs. Consistent with the previous studies (42), our results also indicate that EREs, like ligands, are potent modulators of ER conformation.

Do ERE and ER ligand induced conformational changes occur in an integrated manner? Previous studies suggested that DNA-induced changes are transmitted to both the amino- and the carboxyl-termini of ERα as assessed by the alterations in epitope accessibility (43, 75, 84). Consistent with studies (1, 60), we found that ligands have no effects on the binding specificity or affinity of ER to EREs, nor do EREs affect ligand interactions with ERs. We also found that ligands do not produce different fragmentation patterns of ERs bound to EREs. These results, together with our findings that ligands do not alter the nature of co-factor interactions with ERs bound to various ERE sequences, suggest that although both the ERE and ER-ligand induce conformational changes in ERs, these changes are not integrated to produce distinct functional properties.

Studies showed that SRC-1, TIF-1, TIF-2 and AIB-1 interact with the AF-2 domain of ERs in an agonist-dependent manner through NID in GST pull down assays, and yeast and mammalian two hybrid systems (3, 16, 25, 30, 34, 41, 57, 59, 65, 79). Consistent with those, our results further indicate the following: First, the unliganded ER-ERE complexes can recruit co-factors depending upon the amount of co-factor present. The high degree of regional flexibility, therefore relative positioning, of Helix 12 (72), could allow the receptors to interact with co-factors in the absence of ligand, albeit at a much lower efficiency. Although primarily attributed to traces of estrogenic compounds in culture media (6, 46), true hormone-independent co-factor binding could account also for the basal transcriptional responses to ER, the magnitude of which vary in a cell-specific manner (74, 77, 82) as we also observed here. Second, the effects of E_2 on conformational changes of the LBD are critical for the efficient recruitment of co-factors. This was manifested as a dramatic increase in the receptor affinity for co-factors upon binding of E_2. Third, 4-OHT and ICI impede the interactions of both ERα and ERβ with co-factors in a concentration-dependent manner by inducing conformations in the LBD that are distinct from those of the unliganded receptor or of the receptor bound to E_2. Fourth, the effects of E_2, 4-OHT and ICI on the pattern of AF-2 dependent co-factor interactions with both receptors are independent from the identity of ERE sequences. Fifth,

distinct surfaces of a co-factor, as observed here and previously for TIF-2 (5) and GRIP-1 (81), can preferentially interact also with the ERα in a ligand-independent manner. The extent of interaction is, however, dependent upon ERE sequences. Since the ERE sequence is critical for the affinity, therefore the relative amount of receptor binding, the last two observations imply that the magnitude of co-factor recruitment is ultimately determined by the amount of receptor bound to an ERE. Our results that the extent of transcription induced by both ERs in the absence or presence of an agonist is correlated with the affinity of the receptor to a given ERE sequence support this conclusion. Our results are also consistent with numerous observations that non-consensus ERE sequences are less potent transcriptional enhancers than the consensus ERE in response to E_2 (12, 44, 63). We also observed, as shown previously (14, 22, 35, 49, 61, 80) that ERα is a more potent transcription activator than ERβ independently from the ERE sequence, ER-ligands, promoter- and cell-context. Since the interaction of the AF-2 dependent co-factors with the AF-1 domain, as shown here and previously (81), occurs preferentially with ERα, our results further suggest that structural differences in AF-1 domains of ERs could reflect the functional divergence between ER subtypes.

Since it appears that ERE and ligand induced conformational changes are not integrated to provide ERs with distinct functional features, how then does 4-OHT or ICI display partial agonist activity in a promoter and/or cell context dependent manner? The partial agonistic effect of tamoxifen bound ERα, but not ERβ, from consensus ERE driven promoters, is apparently modulated through the amino terminal AF-1 (6, 27, 39, 48, 51). Recent studies suggested that *in situ* interaction of co-repressor proteins, including SMRT and N-CoR (39), with ERα could explain the ability of tamoxifen to display partial agonistic activity. In contrast, we found, as shown previously (71), that SMRT can interact quantitatively with ERE-bound ERα or ERβ whether ERs are unliganded or liganded with E_2, 4-OHT or ICI (data not shown). Although it remains to be determined, interaction of co-repressors, or yet unknown co-regulators, alone or within a complex, specifically with tamoxifen bound ER in a cellular environment could indeed be required for the partial agonist activity of an antagonist.

In summary, our results indicate that the extent of ligand, or AF-2, dependent co-factor recruitment by both ERα and ERβ is altered by two factors acting independently: ER-ligands and ERE sequences. Following agonist binding, interaction of co-factors with each of the receptor subtypes is primarily affected by the ERE sequence which determines the affinity, therefore the relative amount, of receptor binding. Since ERα binds to EREs with a higher affinity compared to ERβ, the differences in the extent of transcriptional responses between the receptors (14, 22, 52, 76) must, at least in part, depend upon the amount of co-factor recruited. The interference of co-factor interaction with both ERs by partial agonist or antagonist, on the other hand, occurs independently from the ERE sequences. The extent of ligand-independent co-factor recruitment by ERs is dependent upon the ERE sequence and, as in the case of TIF-2, ER subtype. Distinct transcription activation domains, AF-1 and AF-2, of ERα have been shown to act independently and in concert to regulate estrogen responsive gene transcription in a ligand, promoter and cell specific manner (4, 38, 74). Since AF-1 is a critical domain for the transcriptional strength of ERα and ERβ (76, 78) and since AF2-dependent TIF-2 can also be recruited by ER-subtype preferentially and independently from ligands, the ability of ERα to interact with distinct surfaces of a co-factor could be critical for an *intra*-molecular interaction within ERα and an *inter*-molecular interaction between ERα molecules when bound to tandem EREs (81). This mechanism could allow the receptor to effectively regulate natural estrogen responsive genes in which ERE sequences are dispersed among other hormonal or non-hormonal response elements.

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FIGURE LEGENDS:

Fig. 1. **Test ERE Sequences.** *A*, The base oligomer sequence that surrounds the ERE test sequence. Braces enclose the region into which the test sequence is inserted. *B*, 5' to 3' of the upper strand of a test ERE sequence. *Underlining* indicates the extended bases that surround the 13 base perfect palindromic sequence (p13). *Boldfaced type* indicates base changes in the p13 sequence. Center nucleotides are shown in *lowercase type. C*, Numeration of bases in the optimal ERE sequence depicting the *vitellogenin A2 gene* ERE. The bases are numbered according to their distance from the center nucleotide A (0).

Fig. 2. **Binding of ER to ERE sequences.** *A*, The binding ability of ERβ to various ERE sequences was assessed by electrophoretic gel mobility shift assay. 0.125 nM ^{32}P-end labeled ERE was incubated with 0, 2.5, 5, 10, 15 and 20 nM of recombinant human ERβ on ice for 1h and subjected to 8% nondenaturing gel electrophoresis. Gels were dried, and exposed to PhospoImager. Free and Bound indicates the unbound and ER bound ERE oligomers, respectively. *B*, Summary of comparative analysis of ER binding to various ERE sequences. Depicted is the quantitative analysis of percent bound ERE. The data are the mean of three independent experiments. For simplicity, the SEM, which was less then 10% of the mean is not shown.

Fig. 3. **Identification of critical nucleosides of ERE sequences for interaction with ER.** The missing nucleotide hydroxyl radical assay was used to identify the critical contact sites in ERE sequences that are utilized by ERα and ERβ. The receptors were incubated with the 5' end-labeled and gapped optimal ERE without (p17; upper panel) or with two nucleotide substitutions (p17d2; lower panel) that are highlighted. The ER-bound EREs were separated from the free ERE by non-denaturing PAGE. Radioactive bands containing the bound and free ERE were excised from the gel and eluted. DNA was then subjected to denaturing PAGE analysis. A low intensity, or missing, band in the lane containing ERα (lane 5) or ERβ (lanes 6), or conversely a high intensity band in the lane containing free ERE (lanes 4 and 7), identifies a nucleoside important for the formation of the ER-ERE complex. This was assessed by the Maxam-Gilbert G-specific sequencing reaction (lanes 3 and 8). The lane 1 and 2 represent the uncut DNA and DNA subjected to hydroxyl radical treatment in the absence of any protein, respectively. The intensities of individual DNA bands were quantified with PhosphoImager. The ratio of free to bound DNA at each base was plotted for the half-arm of the CCAGT sequence or the non-consensus CCCGA sequence (changes are in bold) in the bottom strand of ERα (left column) or ERβ (right column). A high ratio, represented as long horizontal bars, approximates the strength of nucleoside contact with the receptors. Arrows indicate A residues deciphered by the Maxam-Gilbert G-specific sequencing reaction. A representative autoradiogram of three independent experiments is shown. Bars are the mean of three independent experiments. The SEM, which was less than 15 % of the mean, is not shown for simplicity.

Fig. 4. **The effects of ER-ligands on the pattern of partial proteolysis of radiolabeled ERs.** [^{35}S]-methionine labeled equal molar concentrations of ERβ and ERα synthesized *in vitro* were incubated without (0.01% ethanol) or with 10^{-6} M E_2, 4-OHT or ICI at room temperature for 10 min. Reactions were then subjected to 1, 2.5, 5, 10 and 25 μg/reaction chymotrypsin for 10 min at RT. Reactions were analyzed by 8-16% gradient SDS-PAGE and visualized by Phosphoimager. The positions of molecular weight markers in kDa are shown on the left. The estimated M_r of the full length ERβ is 60 kDa and the proteolytic fragments that migrate with M_r of 35, 33, 31 and 27 kDa, correspond to βC1, βC2, βC3, βC4, respectively, are shown on the right. The full length ERα migrates with a M_r of 65 kDa. The proteolytic fragments of αC1, αC2 and αC3 migrate with M_r of 35, 33 and 30 kDa, respectively. Shown is a representative experiment from three to five independent determinations.

Fig. 5. **The effects of ERE sequences and ER-ligands on the pattern of partial proteolysis ERs by proteases.** Recombinant ERα (left panels) and ERβ (right panels) were incubated on ice without (ethanol 0.01%; Control, lanes 1-6) or with 10^{-6} M E_2 (lanes 7-12), 4-OHT (lanes 13-18) or ICI (19-24) for 15 min. Equal molar concentrations of [^{32}P] end-labeled p17 (upper panel), p17d1 (middle panel) or p17d2 (lower panel) oligonucleotides were then added into reaction mixtures and incubated on ice for additional 15 min. This was followed by exposure of the ligand-ER-ERE complexes to 0, 1, 2.5, 5, 10 and 25 μg/reaction chymotrypsin. Following termination, the reactions were immediately analyzed by 8% non-denaturing PAGE. Note that due to lower affinity of both receptors to p17d2, images of p17d2 bound ERs were enhanced several folds for comparable intensities. Shown is a representative experiment from at least three independent determinations.

Fig. 6. **The effects of ER ligands and ERE sequences on the recruitment of AF-2 dependent TIF-2 by ERα**

and ERβ. Equal molar concentrations of recombinant ERα (*A*) and ERβ (*B*) were incubated on ice without (ethanol 0.01%; Control, lane 2 and 3) or with 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M of E_2 (lanes 4-7), 4-OHT (lanes 8-11) or ICI (12-15) for 15 min. Equal molar concentrations of [^{32}P] end-labeled p17 (upper panel), p17d1 (middle panel) or p17d2 (lower panel) oligonucleotide were then added into reaction mixtures and incubated on ice for additional 15 min. This was followed by the addition of 1 μg/reaction TIF-$2_{623-986}$ (TIF-2) fragment produced as GST fusion protein into the reactions. Samples were subjected to 6% non-denaturing gel electrophoresis. Gels were dried and autoradiographed. Free DNA is not shown. Lane 1 represents DNA in the absence of any protein. *C* and *D*, ERα (*C*) and ERβ (*D*) were pre-incubated in the absence (Control, ethanol 0.01%) or the presence of 10^{-7} M E_2 for 15 min followed by the additional incubation with [^{32}P] end-labeled p17 on ice for 15 min. GST fusion TIF-$2_{623-986}$ (TIF-2) was added in the amount of 0 (lane 1 and 6), 0.125, 0.25, 0.5, 1 μg (lanes 2, 3, 4, 5 and 7, 8, 9, 10, respectively). The reaction was further incubated for 30 min. Samples were resolved on 6% non-denaturing gels. Shown is a representative image from three independent experiments. Free DNA is not shown. ER and ER-CF represent ER-ERE complexes in the absence or presence of cofactors (CF), respectively. It should be noted that due to lower binding affinity of both receptors to p17d2 compared to p17 or p17d1, images of p17d2 bound ERs were enhanced several folds.

Fig. 7. **The effects of ER ligands and ERE sequences on the recruitment of AF-2 dependent co-factor by ERα and ERβ.** Shown is the interaction of p17 bound ERα (panel A) and ERβ (panel B) in the absence (lane 2 and 3) or the presence of 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M of E_2 (lanes 4-7), 4-OHT (lanes 8-11) or ICI (12-15) with 1 μg/reaction of TIF-$1_{638-851}$ (TIF1), AIB-$1_{522-827}$ (AIB-1) and SRC-$1_{219-399}$ (SRC-1) produced as GST fusion proteins. Panel *C* and *D* depict the interaction of 0 (dashed, lane 1 and 6), 0.125, 0.25, 0.5, 1 μg/reaction (lanes 2, 3, 4, 5 and 7, 8, 9, 10, respectively) GST fusion TIF1, AIB1 and SRC with p17 ERE bound ERα (panel *C*) or ERβ (panel *D*) in the absence (Control, ethanol 0.01%, lanes 1-5) or presence of 10^{-7} M E_2 (lanes 6-10). Incubation, processing and resolving of samples were identical to those described in legend of Fig. 6. Shown is a representative image from three independent experiments. Free DNA is not shown. ER and ER-CF represent ER-ERE complexes in absence or presence of cofactors (CF), respectively.

Fig. 8. **The effects of ER ligands and ERE sequences on the recruitment of the glutamine-rich region of TIF-2 by ERα and ERβ.** The interaction of $TIF2_{1125-1325}$ (TIF2-Q)-GST fusion protein with p17 bound ERα or ERβ (Panel *A*) in the absence (Control, lane 3) or presence of 10^{-9} to 10^{-6} M E_2 (lanes 4-7), 4-OHT (lanes 8-11) or ICI (lanes 12-15). Interaction of various concentrations of TIF2-Q with ERα and ERβ (panel B) in the absence (Control, lanes 1-5) or presence of 10^{-7} M E_2 (lanes 6-10). Lanes 1 and 5 represent the ER-ERE complex in the absence of TIF2-Q. Incubation, processing and resolving of samples were the same to those described in legend of Fig. 6. Free DNA is not shown. ER and ER-CF represent ER-ERE complexes in absence or presence of cofactors (CF), respectively.

Fig. 9. **The effects of ERE sequences and ER-ligands on transcriptional responses induced by ERs.** (A) COS-1 cells were transiently transfected with 300 ng expression vector bearing cDNA for ERα or ERβ, together with 500 ng reporter plasmid. The reporter plasmid contained either no EREs (TATA) or two copies of the optimal ERE (2x17) or of a variant ERE containing two nucleotide substitutions (2x17d2) in tandem located upstream of a simple promoter, TATA box, driving the luciferase gene. Transfection efficiency was monitored by co-expression of 200 ng reporter plasmid bearing the β-galactosidase gene. Cells were treated without (no ligand) or with 10^{-9} M E_2 (E_2) for 24h. (B) Shown are the effects of 10^{-7} M E_2, 10^{-8} M 4-OHT or 10^{-6} M ICI on ERα-induced transcriptional responses from the TATA, 2x17 or 2x17d2 reporter plasmid in transiently transfected HepG2 cells. The data represent the mean ± SEM of three independent experiments in duplicate.

A Background Oligomer

5′-CCCGCGAGATAT{ }GAGATTCCTTA-3′

3′-CGCTCTATA{ }CTCTAAGGAATATA-5′

B Test Sequence

GGTCAgagTGACC	p13
CGTCAgagTGACC	p13d1C6
G**C**TCAgagTGACC	p13d1C5
GG**G**CAgagTGACC	p13d1
GGT**G**AgagTGACC	p13d1G3
GGTC**T**gagTGACC	p13d1T2
GG**G**C**T**gagTGACC	p13d2
AGGTCAgagTGACCT	p15
TGGTCAgagTGACCA	p15TA
CGGTCAgagTGACCG	p15CG
GGGTCAgagTGACCC	p15GC
AGG**G**CAgagTGACCT	p15d1
TGG**G**CAgagTGACCA	p15d1TA
AGG**G**C**T**gagTGACCT	p15d2
AG**CG**C**T**gagTGACCT	p15d3
CAGGTCAgagTGACCTG	p17
CAGG**G**CAgagTGACCTG	p17d1
CTGG**G**CAgagTGACCAG	p17d1TA
CAGG**G**C**T**gagTGACCTG	p17d2
CAG**CG**C**T**gagTGACCTG	p17d3

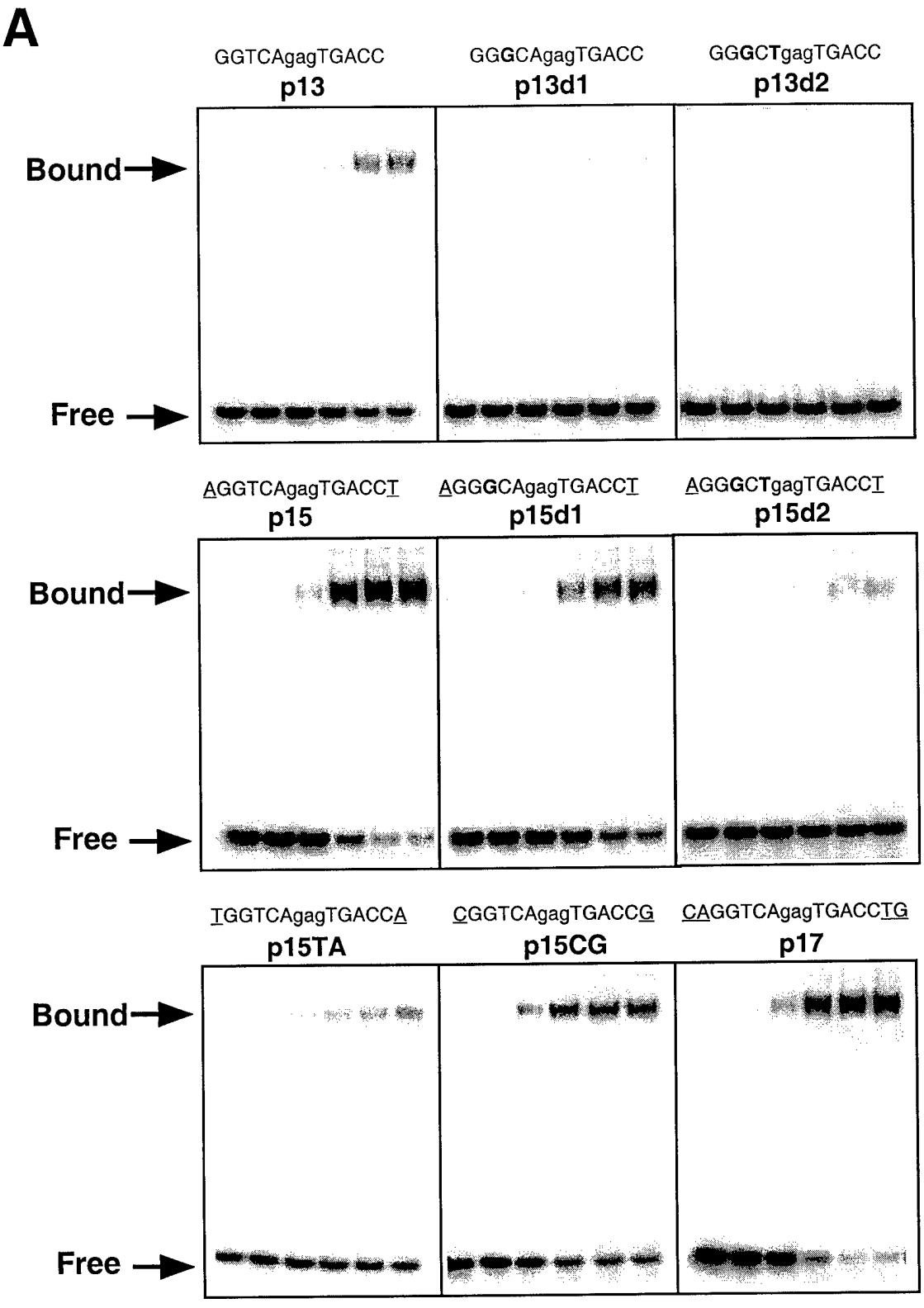
C Base Position

CAGGTCAgagTGACCTG

-8 -7 -6 -5 -4 -3 -2 -1 0 +1 +2 +3 +4 +5 +6 +7 +8

GTCCAGTcacACTGGAC

Figure 1.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p13d2
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Free
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p15
AGGGCAgagTGACCT
p15d1
AGGGCTgagTGACCT
p15d2
Bound
Free
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p15CG
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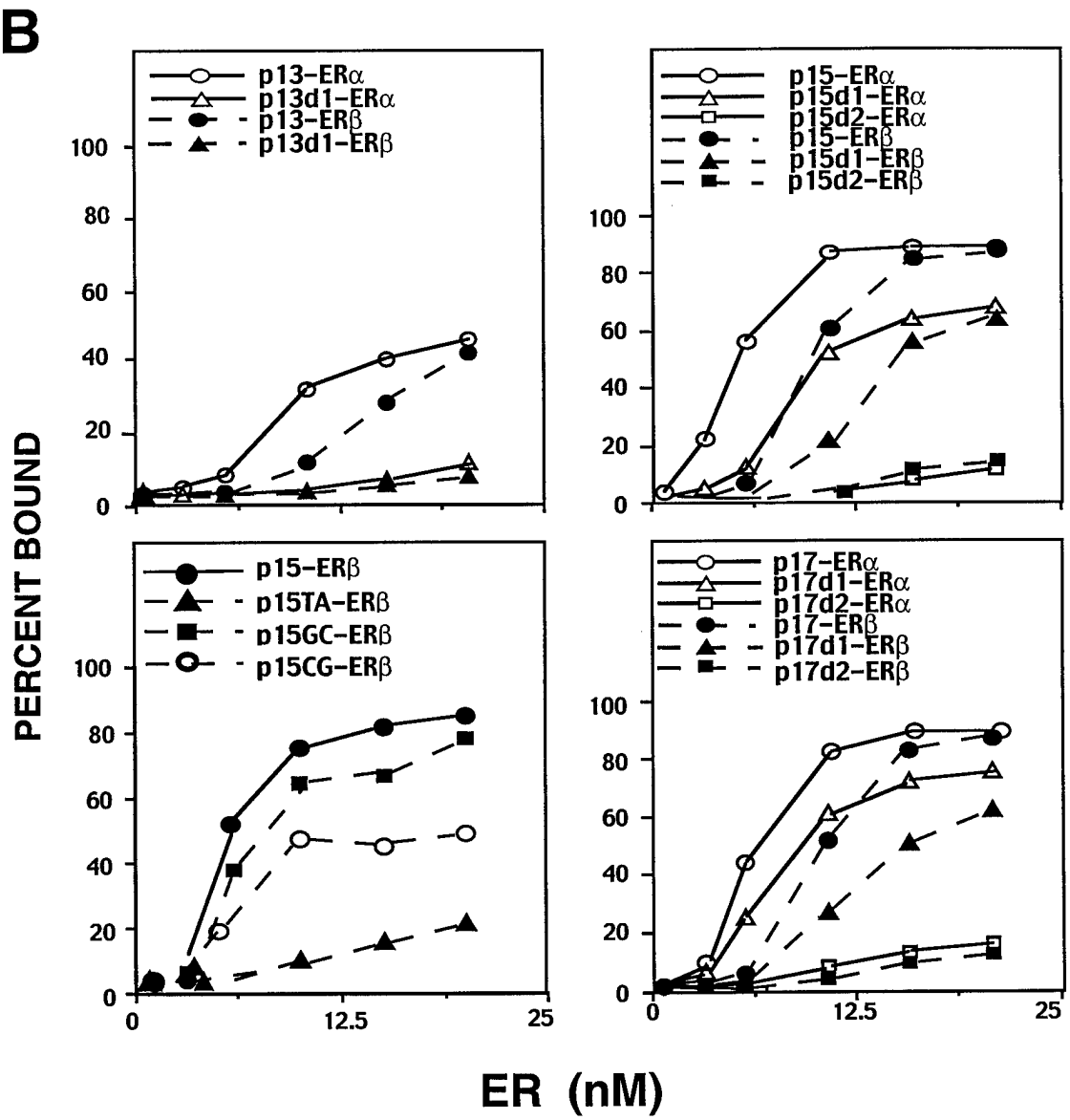
p17
Bound
Free

Figure 2A.


B
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p13-ERβ
p13d1-ERβ
p15-ERα
p15d1-ERα
p15d2-ERα
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p15d1-ERβ
p15d2-ERβ
p15-ERβ
p15TA-ERβ
p15GC-ERβ
p15CG-ERβ
p17-ERα
p17d1-ERα
p17d2-ERα
p17-ERβ
p17d1-ERβ
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PERCENT BOUND
ER (nM)
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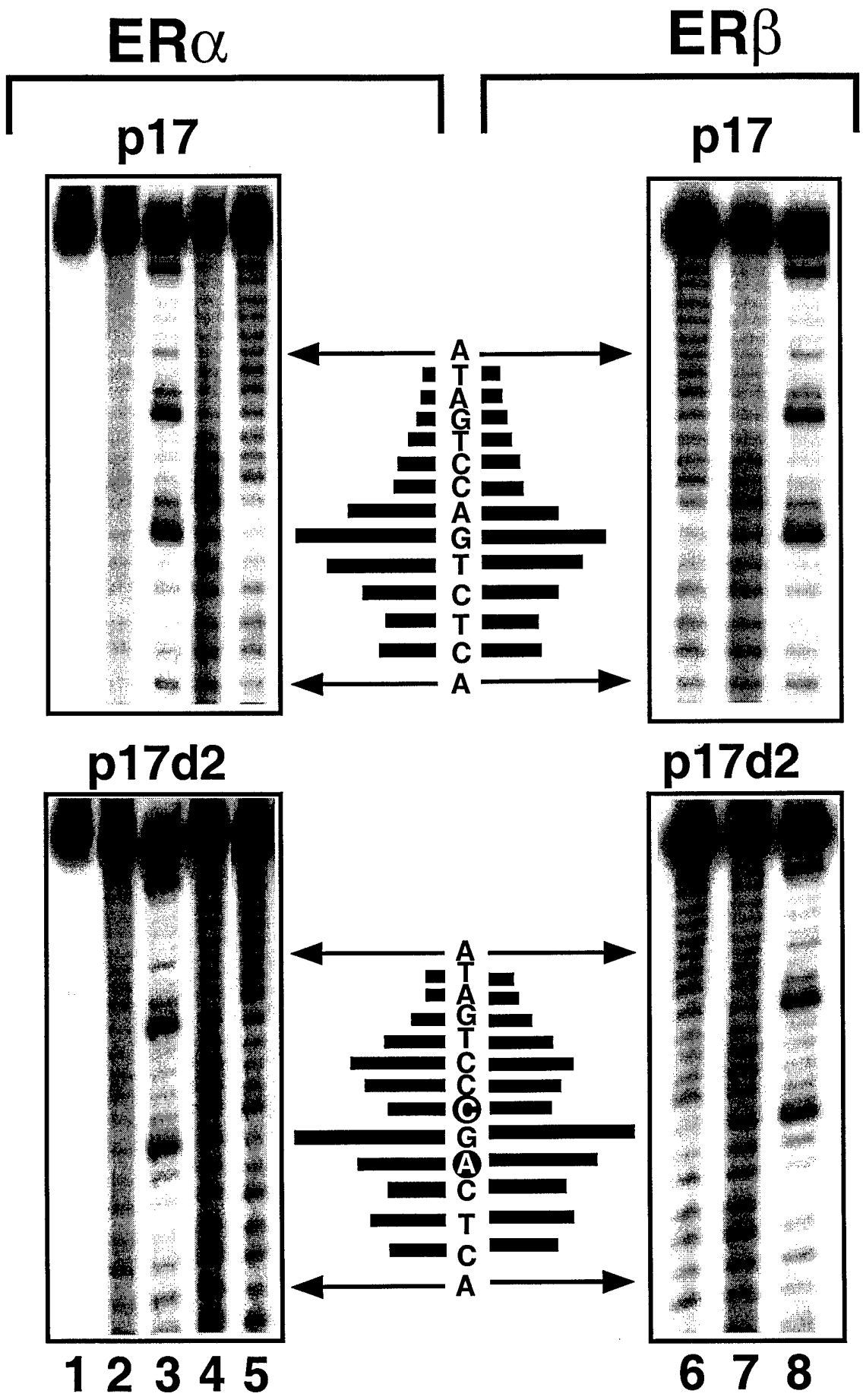
20
40
60
80
100
12.5
25

Figure 2B.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ERβ
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p17
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T
A
G
T
C
C
A
G
T
C
T
C
A
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p17d2
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C
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C
T
C
A
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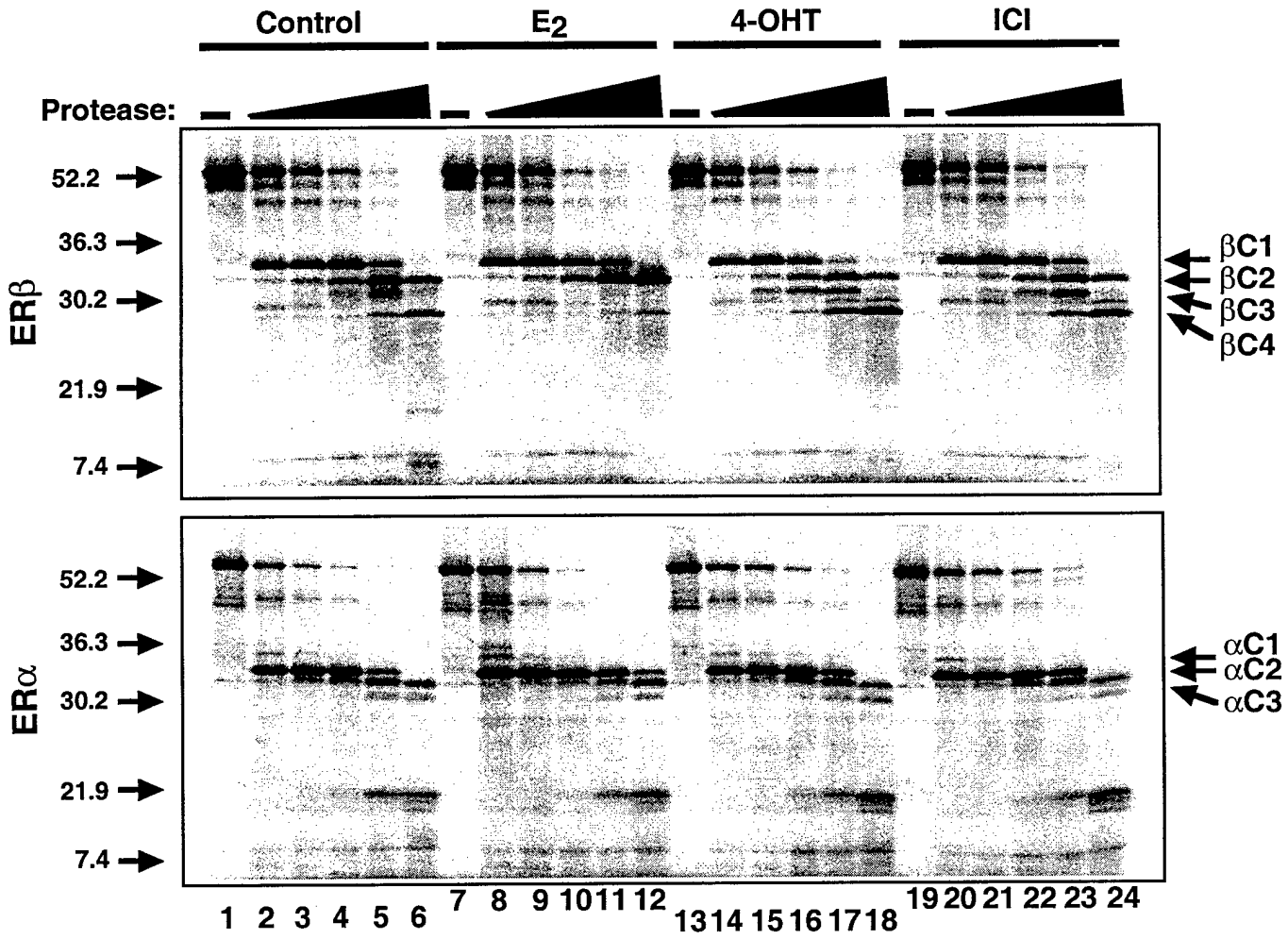
6 7 8

Figure 3.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4-OHT
ICI
Protease:
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36.3
30.2
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7.4
ERβ
βC1
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βC3
βC4
52.2
36.3
30.2
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αC2
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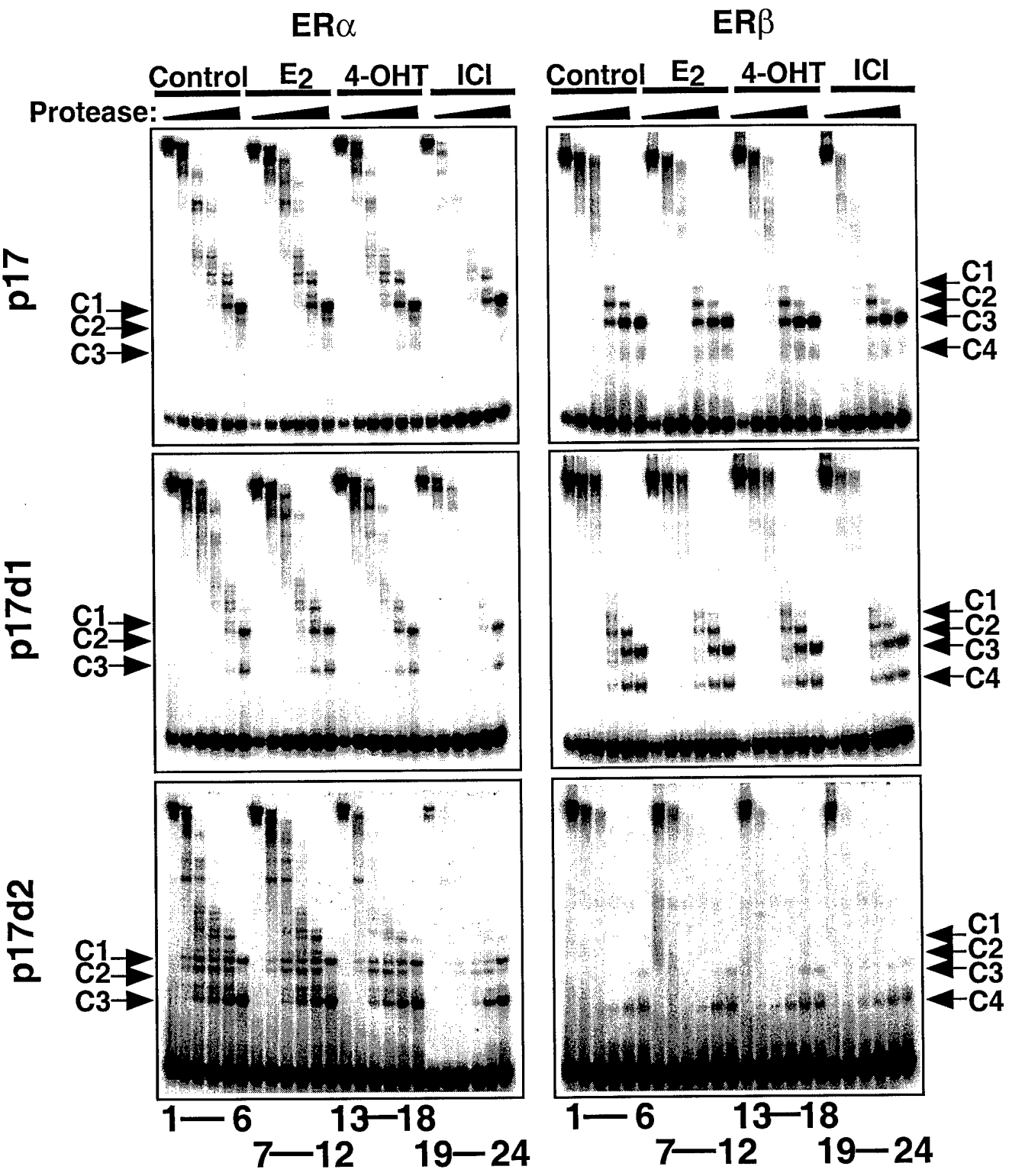
Figure 4.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ERβ
Control
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4-OHT
ICI
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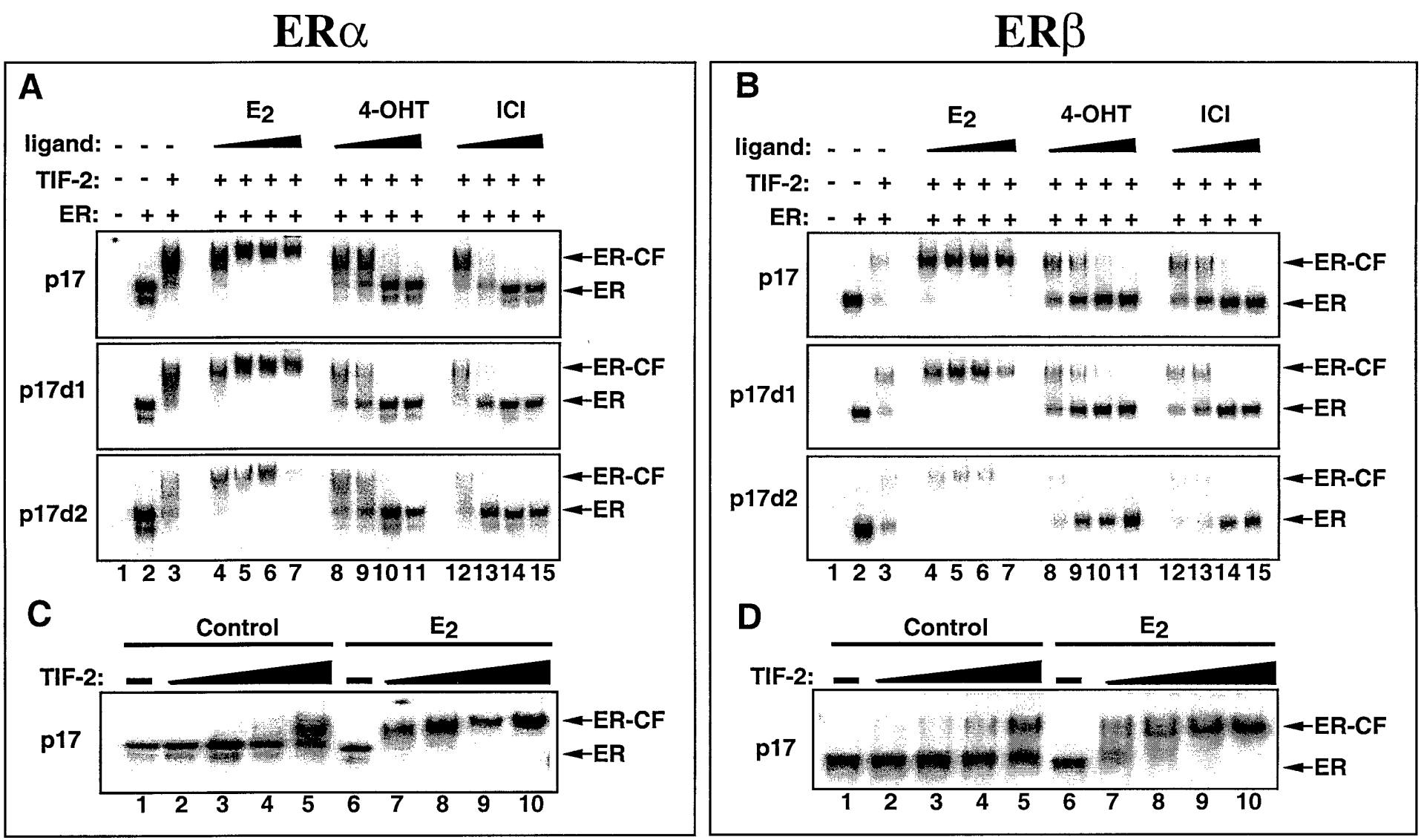
19—24

Figure 5.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ERβ
A
B
C
D
ligand:
TIF-2:
ER:
E2
4-OHT
ICI
Control
p17
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p17d2
ER-CF
ER
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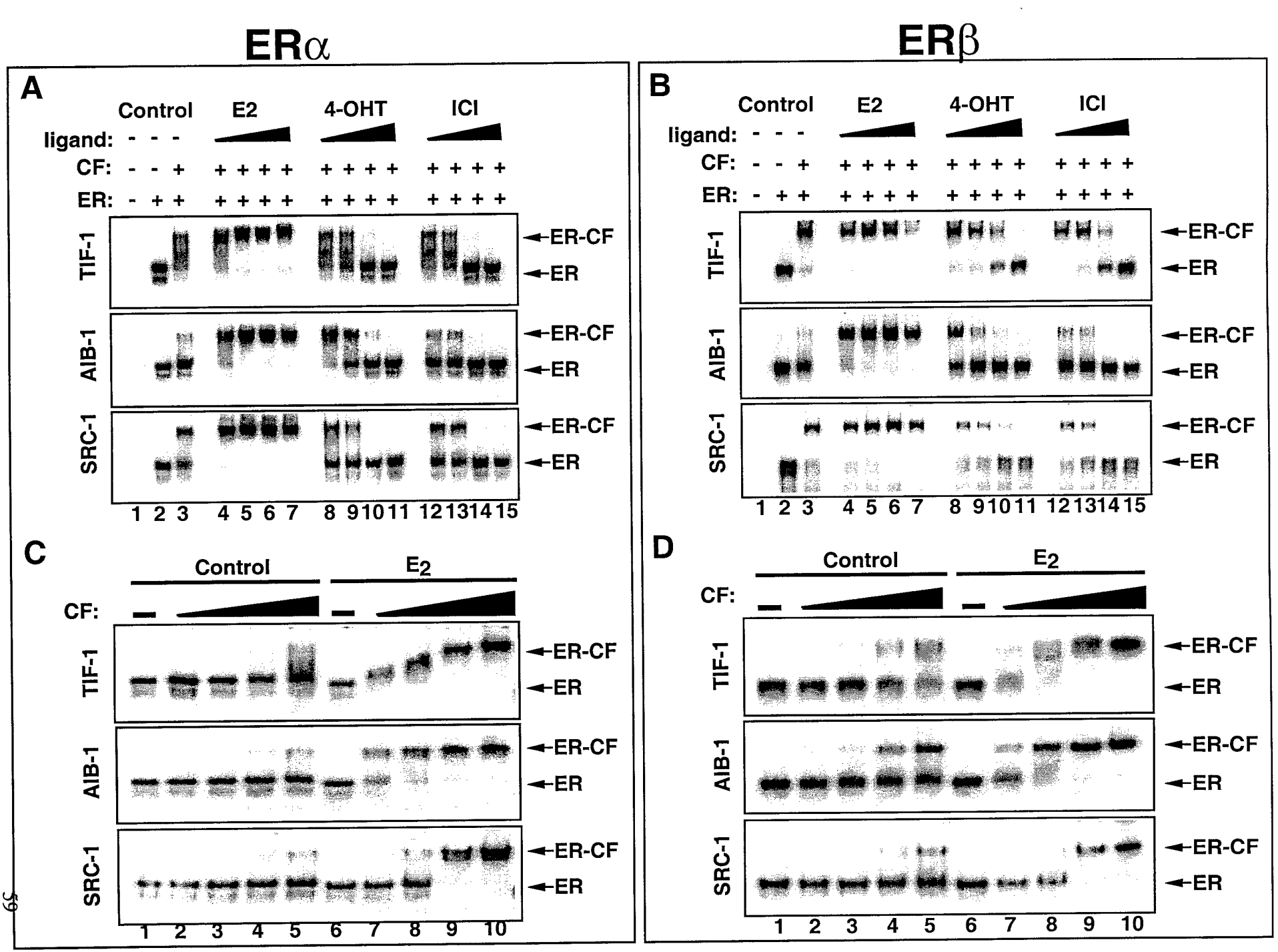
1 2 3 4 5 6 7 8 9 10

Figure 6.


ERα
ERβ
A
B
C
D
Control
E2
4-OHT
ICI
ligand:
CF:
ER:
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SRC-1
ER-CF
ER
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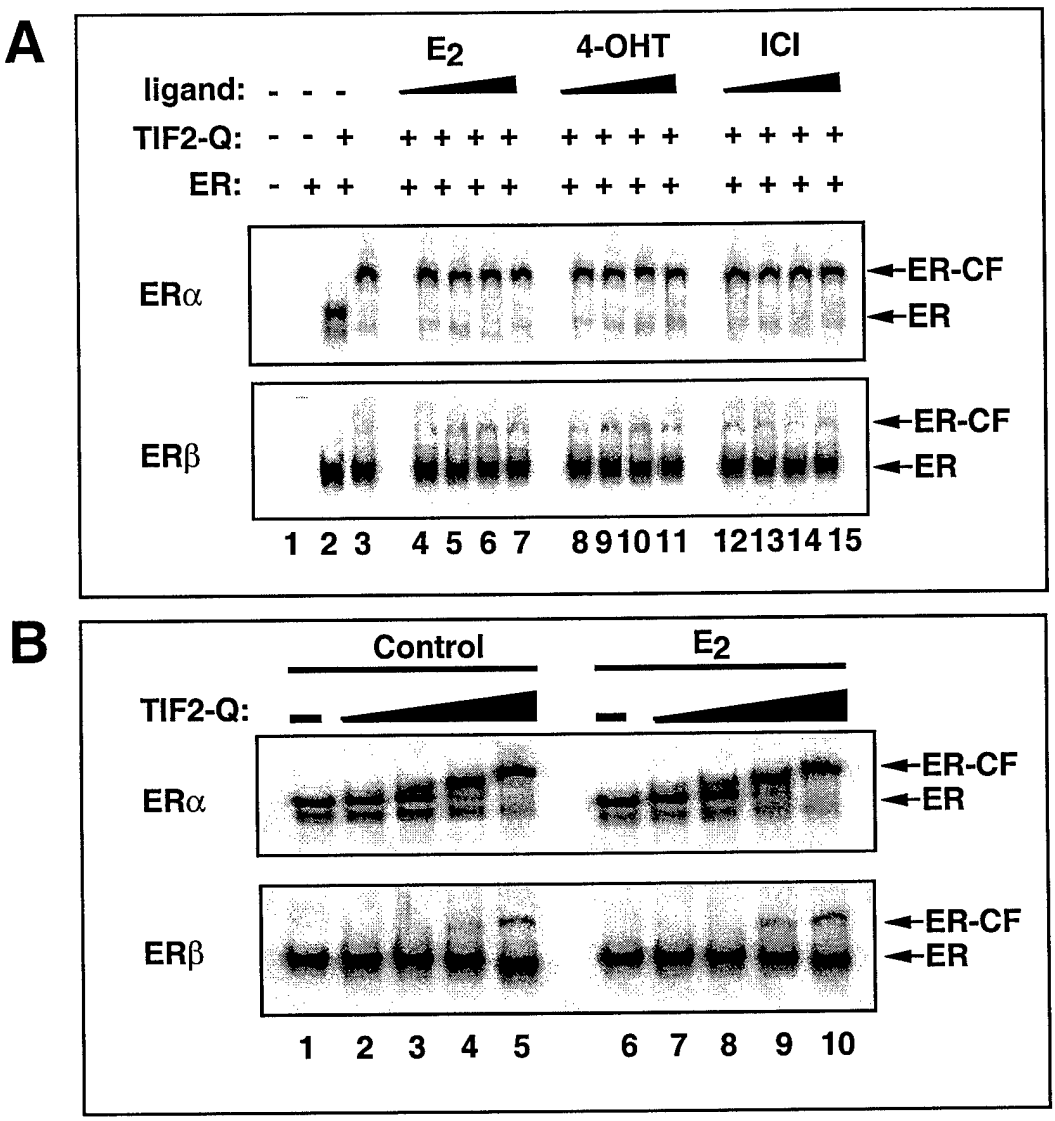
E2
1 2 3 4 5 6 7 8 9 10

Figure 7.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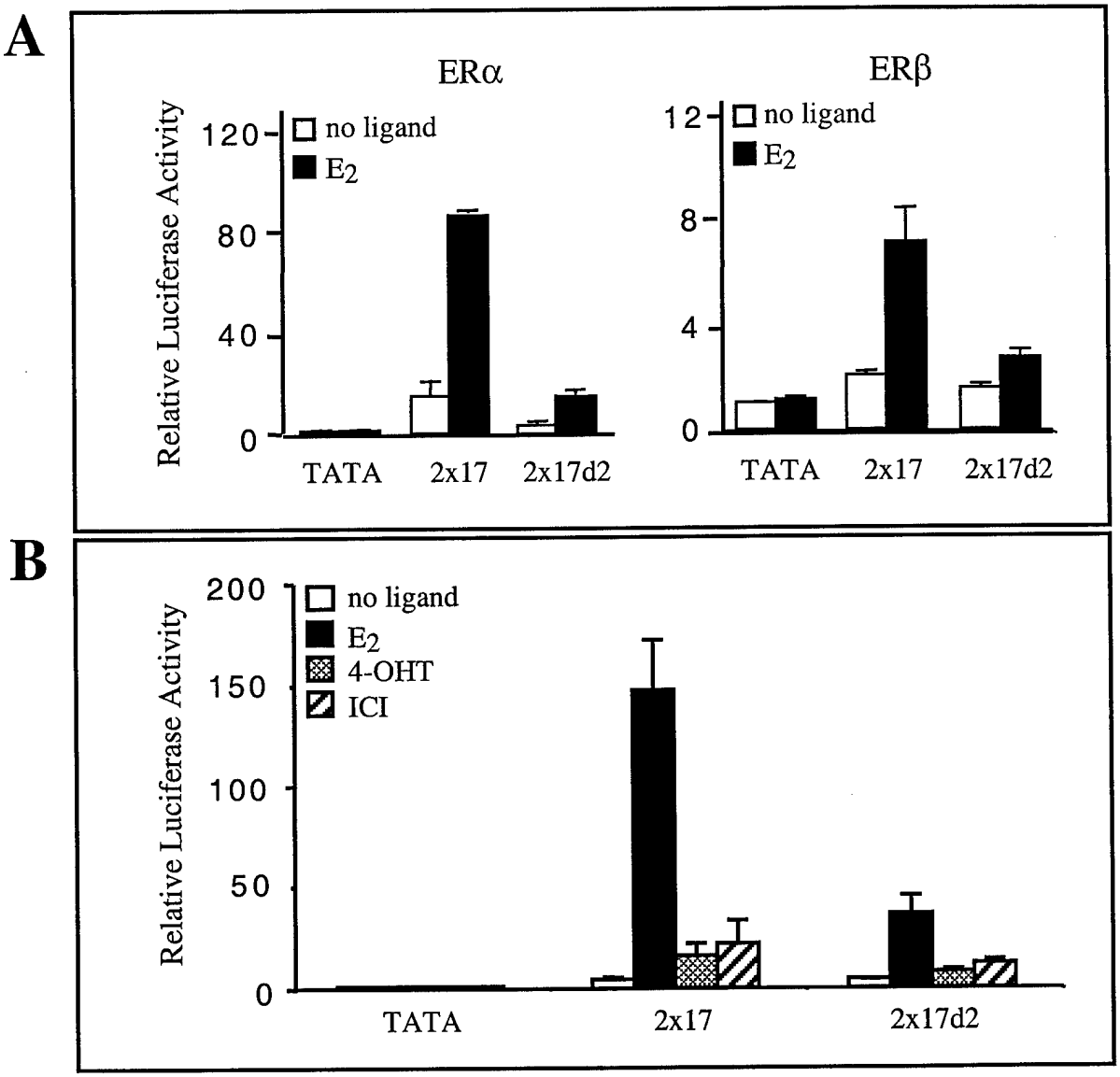
E2
4-OHT
ICI
ligand: - - -
TIF2-Q: - - + + + + + + + + + + + +
ER: - + + + + + + + + + + + + +
ERα
ER-CF
ER
ERβ
ER-CF
ER
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
Control
E2
TIF2-Q:
ERα
ER-CF
ER
ERβ
ER-CF
ER
1 2 3 4 5 6 7 8 9 10

Figure 8.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ERβ
Relative Luciferase Activity
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80
40
0
no ligand
E2
12
8
4
0
TATA
2x17
2x17d2
B
200
150
100
50
0
4-OHT
ICI

Figure 9