Properties of Overlapping EREs: Synergistic Activation of Transcription and Cooperative Binding of ER†

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ABSTRACT: We have designed a novel estrogen-responsive unit, overERE, which consists of two overlapping ERE separated by 5 bp (center-to-center). In gel retardation assays, this sequence forms a low-mobility complex that migrates like an estrogen receptor tetramer. The receptor−overERE complex was specific and was supershifted by anti-ER H222 antibodies. Dose response studies showed that the formation of the receptor tetramer−overERE complex was cooperative. Truncated receptors were used to assess the contribution of the receptor domains. Deletion of the E domain of the ER prevented the formation of an ER−tetramer complex, which reflects a novel function of this receptor domain. In transfection experiments, 17-β-estradiol activated transcription from an overERE-containing promoter 4−6 times better than from an ERE-containing promoter. This synergistic effect was observed using either the natural hormone (17-β-estradiol) or xenoestrogens (phenol red, chlordane). We conclude that two overlapping estrogen-responsive elements can elicit synergistic induction of transcription.

Nuclear receptors mediate the cellular actions of several hormones and effectors such as steroid hormones, T3, retinoic acid, and vitamin D (*1*, *2*). The superfamily of nuclear receptors includes, in addition to orphan receptors, the steroid receptors family and the family of receptors forming heterodimers with RXR (*3*, *4*). The responsive elements of the latter receptors generally consist of direct repeats of the AGGTCA half-site or variants of this site (*5*). Interestingly, the estrogen-responsive element is different in that it is usually palindromic and consists of inverted repeats of this half-site separated by 3 bp (*6*). This is also the structure of the responsive elements of the glucocorticoid receptor family (*7*), but in this case, the sequence of the half-sites is different.

Nuclear receptors have a common domain structure with two highly conserved regions: the central DNA-binding domain and the C-terminal hormone-binding domain (*8*−*14*). In the case of the estrogen receptor (ER), following hormonal stimulation, the ER binds to an estrogen-responsive element (ERE) as a homodimer, thus modulating gene expression (*15*−*18*).

The efficiency and the specificity of the regulation of a gene by a hormone depends not only on the sequence, the orientation, and the spacing of the responsive elements' half-sites but also on the number and location of the responsive elements (*19*−*21*). Indeed, a common feature of many hormonally regulated promoters is the presence of several adjacent responsive elements. In several cases these elements elicit synergistic induction of transcription (*22*, *23*). It is also believed that, in the case of some nuclear receptors and transcription factors, cooperative binding is observed on adjacent elements (*23*, *24*). The distance between these elements is critical. It is usually optimal when it is a multiple of helix turns and thus allows the binding of the receptors on the same side of the DNA helix (*20*, *25*). However, the distance can also be very long. It is assumed, then, that the looping of the DNA allows for receptor interaction and stabilization of the complex (*26*−*28*). It is also believed that the flexibility of the receptor protein is critical for such interactions to take place. However, it should be noted that, in some cases, cooperative binding of nuclear receptors on adjacent sites was not observed (*22*).

The aim of the present study was to ask whether two ERE on opposite sides of the DNA double helix could still elicit synergistic transcription. To minimize the contribution of DNA looping, we designed a sequence which consists of two EREs separated by 5 bp center-to-center (overERE). In this case the EREs are arranged in an overlapping manner so that each receptor dimer binds on an opposite side of the DNA. This design was possible because of the structure of the ERE (i.e., two half-sites separated by 3 bp). Furthermore, this structure is similar to that of a GRE, and we had previously shown that a sequence composed of two overlapping GREs was responsible for the regulation for the cAspAT gene by glucocorticoids (*29*).

In this study we show that such an overERE sequence is functional and cooperatively binds a tetramer of ER. Furthermore, the two overlapping EREs display a synergistic transcriptional activation. We also found that the E domain

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of the receptor is crucial for the formation of an ER tetramer−overERE complex. Thus, binding of two ER dimers on opposite sides of the DNA double helix enhances their transcriptional efficacy.

MATERIALS AND METHODS

Cell Culture. The human hepatoma cell line HepG2 (*30*), MCF-7 cells (human breast adenocarcinoma), and CMT cell line were maintained in Dulbecco's minimal essential medium (DMEM) without phenol red supplemented with 10% fetal calf serum (Gibco), 100 U/mL penicillin, 100 μL/mL streptomycin (Diamant), and 0.5 μg/mL fungizone (Squibb).

Plasmids. Expression vectors for human ER and ER deleted fragments (HE13, HE15, HE19, HE21, HE38, HE70, and HE72) were previously described by S. Green et al. (*31*) and T. Ylikomi et al. (*32*). The plasmid ΔMTV-CAT (gift from Dr. R. Evans, San Diego, CA) was derived from the plasmid MMTV-CAT by the deletion of the sequence from position −190 to position −88 of the mouse mammary tumor virus long terminal repeat (*33*). A *Hind*III site, created at the deletion site, was used as a cloning site for all oligonucleotides used in this study. These oligonucleotides were also subcloned into the *Hind*III site of the Tk-CAT plasmid (gift from Dr. C. Forest, Meudon, France). The double-stranded oligomers [ERE, (ERE)2, overERE, and (overERE)2] have 5′ extensions that are compatible with a *Hind*III site. However, the restriction site is lost in the recombinant plasmid. The (ERE)2 sequence was obtained by the ligation of two ERE oligonucleotides into the *Hind*III site of the ΔMTV-CAT plasmid: sequence of ERE, strand A, 5′AGCTGCTCAGCT AGGTCA CTG CGACCT CTACT3′; strand B, 5′AGCTAGTAG AGGTCT GAG TGACCT AGCTGAGC3′; sequence of overERE, strand A, 5′AGCTGCTCAGCT AGGTCAGGTCGACCTGACCT CTACT3′; strand B, 5′AGCTAGTAG AGGTCAGGTCGACCT GACCT AGCTGAGC3′; sequence of (overERE)2, strand A, 5′AGCTGCTCAGCT AGGTCAGGTCGACCTGACCT C AGGTCAGGTCGACCTGACCT CTACT3′; strand B, 5′AGCTAGTAG AGGTCAGGTCGACCTGACCT G AGGTCAGGTCGACCTGACCT AGCTGAGC3′. (The ERE sites are underlined.) The GRE sequence that we used was derived from the promoter of the cytosolic aspartate aminotransferase gene promoter (*29*). It had the same transcription efficiency as a consensus GRE sequence. The luciferase plasmid (RSV-Luc) was purchased from Promega.

Transfection Experiments. Transfection experiments were performed as described by Massaad et al. (*34*). One day prior to the transfection, HepG2 cells (10^6 cells/10-cm dish) were seeded into the usual culture medium containing 10% fetal calf serum. Ten milliliters of fresh medium with 10% charcoal-treated serum were added to the cells 2−3 h prior to the transfection. The CAT plasmids (5 μg of DNA), the hER expression vector (5 ng and 5 μg), and the luciferase expression vector (1 μg) were introduced into the cells by the calcium phosphate coprecipitation technique followed by a glycerol shock. Following the glycerol shock, 10 mL of fresh medium containing 5% charcoal-treated serum was added to the cells. Sixteen hours later, serum-free medium was added, and the cells were then treated with the various hormones or drugs tested. After an additional 24-h incubation, cells were homogenized for chloramphenicol acetyltransferase (CAT) and luciferase assays.

A similar transfection protocol was used for MCF-7 cells. The cells were seeded at 10^6 cells/6-cm dish and transfected by the calcium-phosphate method (*35*). Furthermore, during the treatment with the various drugs, serum was not removed from the culture medium because it is essential for the survival of these cells.

Luciferase Assay. Luciferase activity was used to normalize the transfection efficiency in all culture dishes (*36*). It was assayed using a kit from Promega according to the manufacturer's instructions. Briefly, the transfected cells were washed twice with 5 mL of calcium and magnesium-free PBS and lysed in 500 μL of Reporter Lysis Buffer 1X (Promega) for 15 min. After a 5-min centrifugation, 20 μL of the supernatant was mixed with 100 μL of luciferase assay reagent (Promega) at room temperature. The luciferase activity was measured using a luminometer 30 s after the addition of the assay reagent.

CAT Assay. The CAT activity was determined using the two-phase assay developed by Neumann et al. (*37*). Briefly, 60 μL of cellular extract, heated at 65 °C for 10 min, were incubated with 1 mM chloramphenicol, acetyl CoA (0.5 mM), and 0.5 μCi [^{3}H]-acetyl CoA (NEN product no. NET-290 L) at 37 °C for 30 min. The solution was then transferred to a minivial and layered with 4 mL of Econofluor (NEN product no. NEF 969). After vigorous mixing, the two phases were allowed to separate for at least 15 min, and the radioactivity was then counted in a scintillation counter. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not unreacted acetyl-CoA, can diffuse into the Econofluor phase. For these experiments, blanks were obtained by assaying CAT activity in cells that have undergone the same treatment in the absence of a CAT plasmid.

Cell Extract Preparation. CMT cells were transfected as described by Ishikawa et al. (*38*). Briefly, the cells were plated at 2×10^6 cells/10-cm dish. Twenty-four hours later, the cells were washed twice with phosphate-buffered saline and 500 μL of trypsin solution was added. The cells were incubated for 10 min at room temperature, and 20 μg of purified plasmid containing 400 μg of DEAE dextran and 0.1 mM chloroquine was added. The cells were incubated for 4 h followed by a DMSO shock for 2 min. One hour prior to harvesting, 17-β-estradiol (10^{-7} M) was added to the cells. The cells were washed twice with chilled phosphate-buffered saline and collected in the binding buffer (20 mM Tris-HCl (pH 7.5), 2 mM DTT, 20% (v/v) glycerol and 0,4 M KCl). Whole-cell extract (WCE) was prepared by freezing the cells at −80 °C, thawing them over ice, and centrifugating at 10 000*g* for 15 min at 0 °C in a Sigma centrifuge. The supernatant was conserved at −80 °C.

ER was also expressed in SF9 cells using pBlueBac-His baculovirus expression system purchased from Invitrogen corporation. In some preparations, we have observed partial proteolysis, as documented by Western blots, which result in the appearance of two dimeric complexes in EMSA.

ER was also prepared using a transcription and translation coupled reticulocyte lysate system (TNT-Promega). Briefly, 4 μg of ER expression vector was incubated with TNT lysate, T7 RNA polymerase, an amino acids mixture (1 mM), and

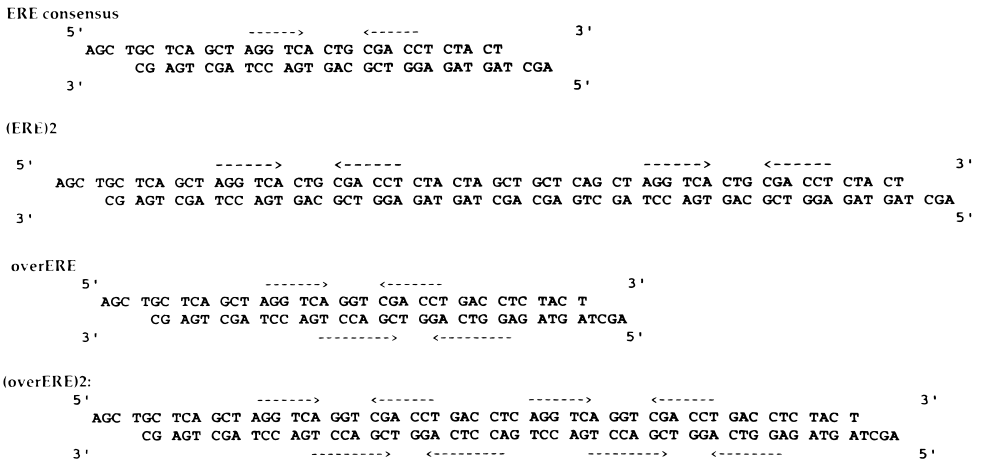
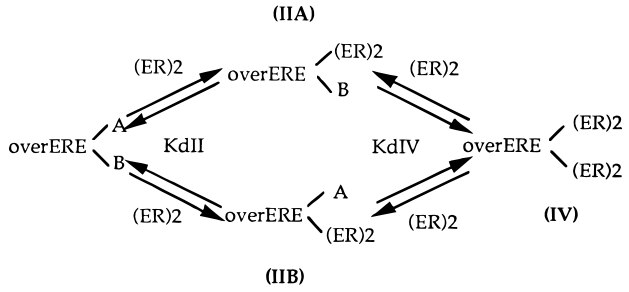


FIGURE 1: Sequences of the oligonucleotides used in this study. Arrows represent ERE half-sites.

a ribonucease inhibitor (40 UI). The reaction was incubated for 2 h at 30 °C.

Electrophoretic Mobility Shift Assay (EMSA). Oligonucleotides were hybridized and labeled using the Klenow fragment of DNA polymerase I. The assay was done essentially as described by Cao et al. (*39*). Binding reactions were carried out in 20 μL of buffer containing 20 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA, 10% (vol/vol) glycerol, 3 μg of bovine serum albumin/μL, 100 mM NaCl, 0.3 ng of radiolabeled purified DNA probe, and 1 μg of dIdC. In the case of baculovirus nuclear extracts, Nonidet P40 (Sigma) 0.1% was added to the medium in order to minimize the binding of accessory proteins to the ER. ER in the amounts indicated in the figure legends was added last. After incubation at room temperature for 15 min, the reaction mixtures were loaded on a pre-electrophoresed (100 V/12 cm, 30 min) 6% polyacrylamide gel (acrylamide/bisacrylamide, 29:1) containing 0.25× Tris-borate-EDTA, and electrophoresis was continued for 90 min (200 V/12 cm). The gels were then dried and autoradiographed. In supershift experiments, the H222 monoclonal antibody was incubated with the receptor during the binding reaction. To quantitate the retarded complexes, the bands were excised from the gel and counted in a scintillation counter. The densitometric studies were performed on the NIH Image software.

Equations To Calculate the Cooperativity Ratio. The equations reported by Tsai et al. (*24*) have been adapted for the overERE unit. We propose a model for the interaction of the ER with the overERE unit.



Complexes II and complex IV are respectively formed predominantly by a dimer or a tetramer of ER bound to the overERE unit.

$$\mathrm{KdII} = \frac{(\mathrm{overERE})(\mathrm{ER2})}{(\mathrm{IIA})} = \frac{(\mathrm{overERE})(\mathrm{ER2})}{(\mathrm{IIB})} = \frac{2(\mathrm{overERE})(\mathrm{ER2})}{(\mathrm{II})}$$

Since the overERE unit is formed of two identical ERE:

$$(\mathrm{IIA}) = (\mathrm{IIB}) = {}^{1}/_{2}(\mathrm{II})$$

$$\mathrm{KdIV} = \frac{(\mathrm{II})(\mathrm{ER2})}{2(\mathrm{IV})}$$

$$\text{Cooperativity ratio} = \frac{\mathrm{KdII}}{\mathrm{KdIV}} = \frac{4(\mathrm{overERE})(\mathrm{IV})}{(\mathrm{II})^2}$$

Complexes IV and II were determined by cutting the corresponding bands out of the gel and counting the radioactivity in a scintillation counter.

RESULTS

Binding of ER to the overERE Sequence. We have designed a novel sequence (overERE) formed of two overlapping ERE sequences. This was possible because a classical ERE consists of two half-sites separated by 3 bp. We selected these nucleotides and adjacent nucleotides in order to obtain two overlapping identical ERE (Figure 1).

To determine whether this sequence could bind the estrogen receptor, we performed EMSA using the ERE (Figure 2A) and the overERE (Figure 2B) sequences. In these assays, we have used DEAE-dextran purified extracts of cells infected with a baculovirus ER expression vector. In the case of the ERE sequence, two bands were observed (complex II); these bands probably correspond to the dimeric form of the ER with different states of proteolysis. In the case of the overERE, three bands were observed (Figure 2B): bands 1 and 2 are identical to those seen with the ERE sequence. An additional abundant low-mobility complex was observed with the overERE sequence (complex IV). This complex may contain additional molecules of ER. The addition of the H222 antibody directed against the C-terminal end of the ER supershifted these 3 bands (Ab-II and Ab-IV). These results indicate that the overERE sequence binds the estrogen receptor. Competition with a 1000-fold excess of unlabeled overERE totally abolished the binding of the

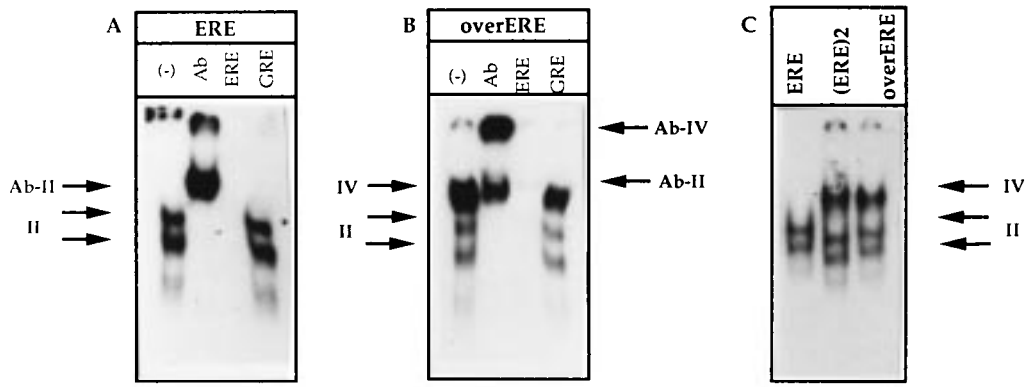


FIGURE 2: EMSA using ER-enriched baculovirus extracts. Baculovirus extracts (4 μL) were incubated with either the radiolabeled ERE (A) or the overERE (B) sequence in the absence (−) or the presence (Ab) of the H222 antibody, or an excess of ERE oligonucleotide (ERE) or GRE oligonucleotide (GRE). [II] corresponds to the dimeric ER complexes and [IV] to the putative tetrameric complex. [Ab-II] corresponds to supershifted complex [II]; [Ab-IV] corresponds to supershifted complex [IV]. Competition with ERE or GRE was done using a 1000-fold excess of unlabeled oligonucleotides. (C) The radiolabeled ERE, (ERE)2, or overERE probes were incubated with 4 μL of ER-enriched baculovirus extracts.

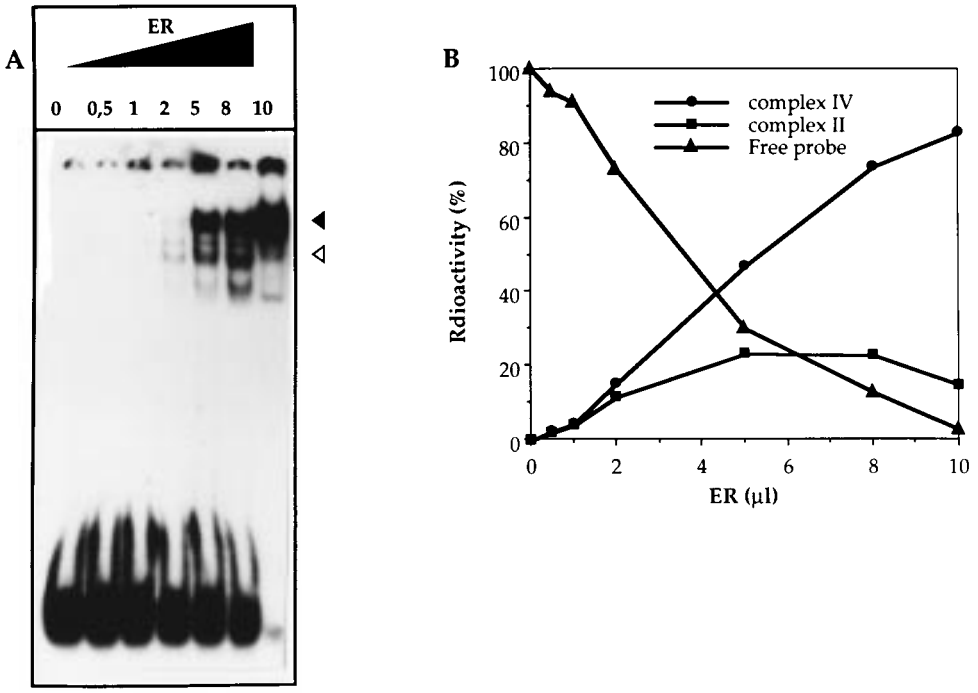


FIGURE 3: Cooperative binding of the ER to the overERE sequence. (A) The radiolabeled overERE was incubated with increasing amounts of ER-enriched baculovirus extracts (0.5−10 μL). The empty arrow indicates the position of the dimeric complex. The full arrow indicates the position of the tetrameric complex. (B) The bands were excised from the gel and counted. Results are expressed as the percent of radioactivity included in the band over total radioactivity: (●) complex (IV), (■) complex (II), and (▲) free probe. The figure shows a representative experiment which was repeated three times.

ER to the overERE. In contrast the addition of a 1000-fold excess of a GRE sequence did not affect the binding. These results indicate that the interaction between the ER and the overERE sequence is specific. Identical results were obtained using purified recombinant ER (data not shown).

Since the overERE sequence is composed of 2 EREs, we hypothesized that the low-mobility complex observed with the overERE could result from the binding of two dimers of ER. To support this conclusion, we have performed EMSA using an oligonucleotide containing two adjacent ERE which forms a complex comprising four ER molecules (*22, 23*) (Figure 2C). The migration of this complex is similar to that of the low-mobility complex observed with overERE. As we will show in the next section, this complex results from the cooperative binding of two ER dimers, and we will thus refer to it as ER tetramer−overERE complex. Furthermore, interference methylation assays have shown that the integrity of all four half-sites of the overERE sequence was required for the formation of this complex (data not shown).

ER Binds Cooperatively to overERE. Binding cooperativity of the ER to the overERE was assessed by EMSA using the radiolabeled overERE sequence and increasing amounts of ER. As shown in Figure 3A, the high-mobility complexes corresponding to a receptor dimer bound to overERE appeared first at low protein concentrations (complex II, empty arrow). An increasing receptor concentration enhanced progressively the formation of the low-mobility complex (complex IV, full arrow). The amounts of complexes II and IV and free DNA were evaluated by determining the radioactivity present in each band, and the values

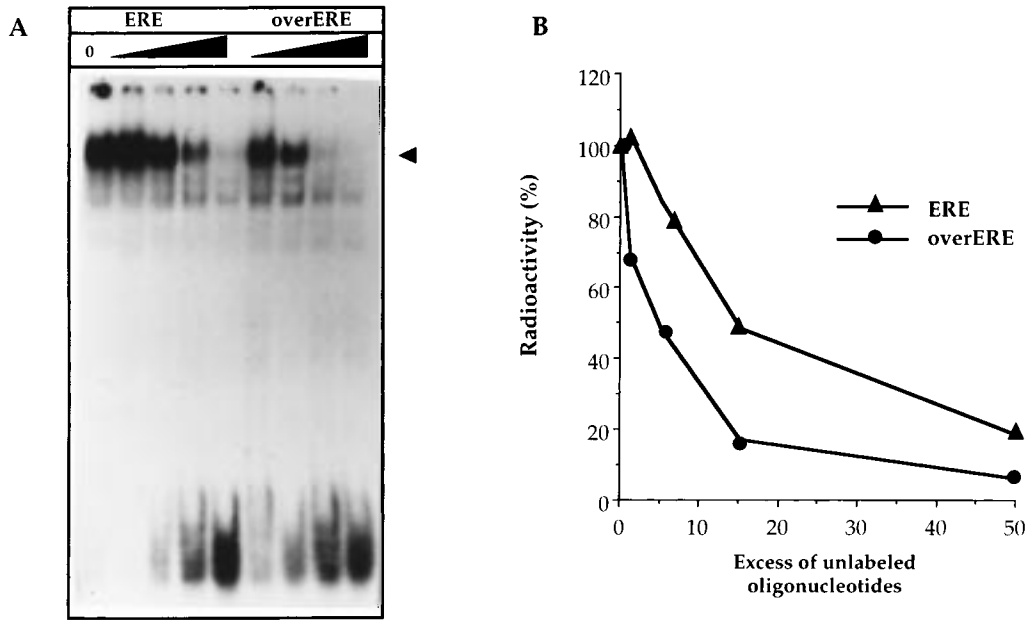


FIGURE 4: Affinity of the ER to the overERE sequence. (A) The radiolabeled overERE sequence was incubated with 4 μL of ER-containing baculovirus extracts. Increasing amounts of either unlabeled ERE or overERE were added (1×, 5×, 15×, 50×). The full arrow indicates the position of the tetrameric complex. The bands were cut off of the gel and counted. (B) The ratio of the radioactivity included in the band over total radioactivity was calculated and the results were expressed as percent of control: (▲) competition with unlabeled ERE and (●) competition with unlabeled overERE.

were plotted in Figure 3B. As shown in this figure, the amount of DNA in complex II increases first, but never exceeds 20% of the total DNA. In contrast, complex IV is only present at higher concentrations of the receptor, when it becomes rapidly the predominant form. By using the data presented in Figure 3B and the equations in Materials and Methods, we determined the relative dissociation constants (Kd) of complexes II and IV, and the ratio KdII/KdIV for this experiment and for two other similar experiments. The average of the ratio for the three values is 15.8 ± 4.7. Thus, the second ER dimer bound with higher affinity when the neighboring overlapping sequence is occupied. These binding characteristics suggest that the ER binds cooperatively to the overERE sequence.

To compare the apparent affinity of binding of the ER to the ERE and overERE sequences, we have performed gel retardation assays with radiolabeled overERE and increasing amounts of either unlabeled ERE or overERE. As shown in Figure 4A, the addition of an excess of the overERE oligonucleotide prevented the complex formation with a 3-fold higher efficiency than the ERE oligonucleotide.

Functionality of overERE. We next examined whether the overERE was functional in the context of the ΔMTV promoter. This promoter consists of a deleted long terminal repeat of the mouse mammary tumor virus lacking the fragment −190/−88, which contains the hormone regulatory sequences (*33*). The CAT activity of cells transfected with this plasmid was not regulated by 17-β-estradiol (data not shown).

We have cotransfected the HepG2 cells with either the ΔMTV-ERE-CAT, the ΔMTV-(ERE)2-CAT containing two adjacent copies of ERE, or the ΔMTV-overERE-CAT plasmids and increasing amounts of the ER expression vector (0 ng, 100 ng) (Figure 5A). 17-β-Estradiol (10^{-7} M) was added to the medium 24 h after transfection. The transcriptional activity of ΔMTV-ERE-CAT increased and reached a plateau at 30 ng of transfected ER expression vector. In the case of ΔMTV-(ERE)2-CAT, the pattern was similar to that of ΔMTV-ERE-CAT but the transcriptional activity was twice higher, while in the case of ΔMTV-overERE-CAT, transcriptional activity was 3−4 times the one obtained by the ΔMTV-ERE-CAT. These results indicate that two EREs linked in tandem have an additive effect, while two overlapping EREs display a synergistic transcriptional activity.

The possibility that promoter elements from the long terminal repeat of ΔMTV-CAT could contribute to the properties of the overERE was tested by subcloning the ERE and overERE sequences upstream of a different promoter, the thymidine kinase promoter (Tk). As shown in Figure 5B, the transcriptional activation achieved by overERE was 4−5 times greater than that of ERE. Again, these results show a synergism between the two overlapping ERE, independent of the promoter context. Similar results were also obtained in a different cell line MCF-7 (not shown).

Since the two overlapping ERE displayed a synergistic effect and the 2 adjacent sequences elicited an additive transcriptional activation, at least under the conditions used in this study, we designed a new sequence, $(overERE)_2$, which is formed of two overERE sequences linked in tandem separated by 21 bp (center-to-center). The transcriptional activation elicited by the $(overERE)_2$ sequence was similar to that displayed by the overERE sequence (data not shown). This observation suggests that a tetramer of ER bound to overERE is sufficient to maximally induce transcription at least under those conditions.

Effect of Xenoestrogens. The ER is activated by natural estrogens, as well as by other natural compounds, and by several chemical compounds with estrogen-like activities, xenoestrogens. We compared the responsiveness of ERE- or overERE-containing promoters to that of various classes of xenoestrogens.

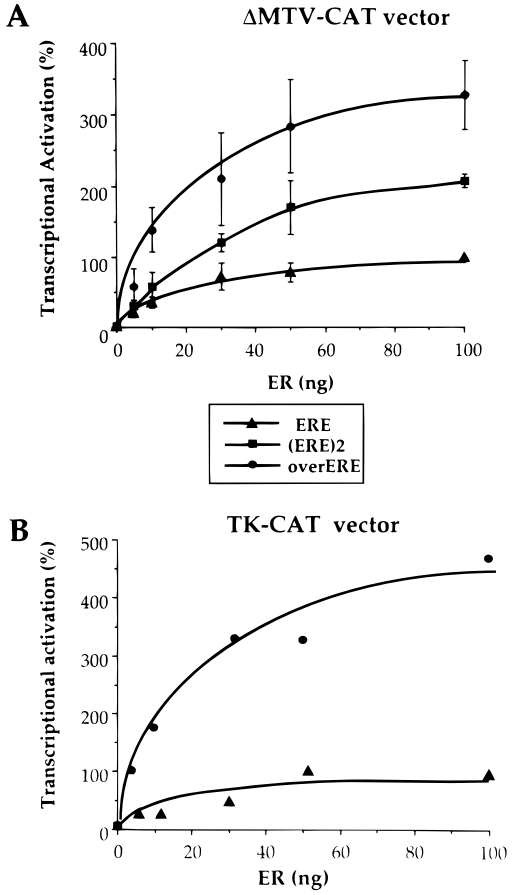


FIGURE 5: Functionality of the overERE sequence. (A) HepG2 cells were transiently transfected with either the ΔMTV-ERE-CAT (▲), or the ΔMTV-(ERE)2CAT (■), or the ΔMTV-overERE-CAT (●) plasmid and increasing amounts of ER expression vector (0−100 ng). Sixteen hours after the transfection, 17-β-estradiol (0.1 μM) was added. Each point is the mean ± SEM of four separate determinations; 100% is the maximal activation elicited by the ΔMTV-ERE-CAT plasmid and corresponds to 23700 ± 1900 arbitrary units. (B) HepG2 cells were transiently transfected with either the Tk-ERE-CAT (▲), or the Tk-overERE-CAT (●) plasmid and increasing amounts of ER expression vector (0−100 ng). Sixteen hours after the transfection 17-β-estradiol (0.1 μM) was added. Each point is the mean ± SEM of three separate experiments; 100% is the maximal activation elicited by the Tk-ERE-CAT plasmid and corresponds to 47000 ± 3800 arbitrary units.

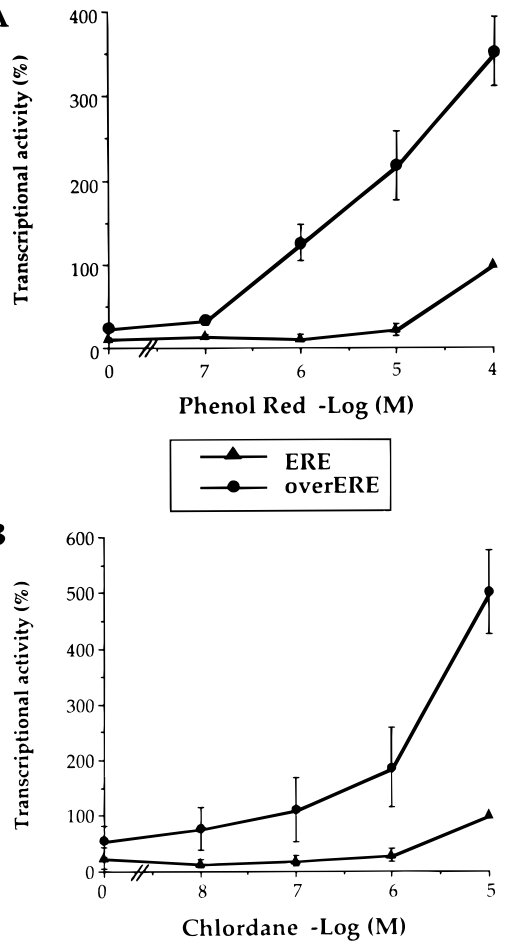


FIGURE 6: Effect of xenoestrogens. HepG2 cells were transiently transfected with either the ΔMTV-ERE-CAT (▲), or the ΔMTV-overERE-CAT (●) plasmids and 50 ng of the ER expression vector. Either phenol red (A) or chlordane (B) was added at the indicated concentrations; 100% is the maximal activation elicited by the ΔMTV-ERE-CAT plasmid and corresponds to 25200 ± 1600 arbitrary units. The results are the mean of 3 independent experiments.

Phenol red, a well-known dye that mimics estrogen activity, was added at different concentrations (Figure 6A). The transcriptional activation elicited by the overERE sequence was 4−5-fold higher than that elicited by the ERE sequence at the same concentration of phenol red added. As a consequence, when using the ERE construct, we were able to detect the estrogenic effect of phenol red at a concentration of 10^{-4} M, while the overERE sequence allowed us to detect a significant estrogenic activity of phenol red at a lower concentration (10^{-6} M).

We tested the estrogenic activity of a polychlorinated biphenyl compound (chlordane) using the same test described for phenol red (Figure 6B). Chlordane activated the transcription of the ERE-CAT plasmid at a concentration of 10^{-5} M. In contrast, we were able to detect the estrogenic activity of this molecule at a concentration of 10^{-7} M when we transfected the overERE-CAT plasmid. In this case, also, the transcriptional activation of overERE was highly synergistic.

Thus, the synergistic activation of transcription elicited by the overERE sequence can be observed for either the natural hormone or the chemicals with estrogenic activity.

Effect of Antiestrogens. The basal activity of the ΔMTV-overERE-CAT plasmid was higher than that of both the ΔMTV-(ERE)2-CAT and the ΔMTV-ERE-CAT. These differences in basal activities could be due either to intrinsic properties of the various promoters or to an endogenous stimulation of the ER by a compound displaying estrogenic activity. To test the latter possibility, HepG2 cells that were transfected by one of the three plasmids cited above were incubated with 17-β-estradiol or the estrogen antagonist ICI164386 (10^{-5} M) or both. As expected, this compound decreased estrogen stimulation but it also dramatically inhibited the basal activity of these three constructs (Figure 7), suggesting that the increased basal activity of overERE is due to endogenous stimulation of the ER.

The A/B and E Domains are Responsible for the Cooperative Binding of the ER to the overERE unit. The cooperative binding of the ER to the overERE unit could be due either to an intrinsic property of the overERE sequence or to specific domains of the ER. To test the latter hypothesis, the effect of different truncated fragments of the ER were

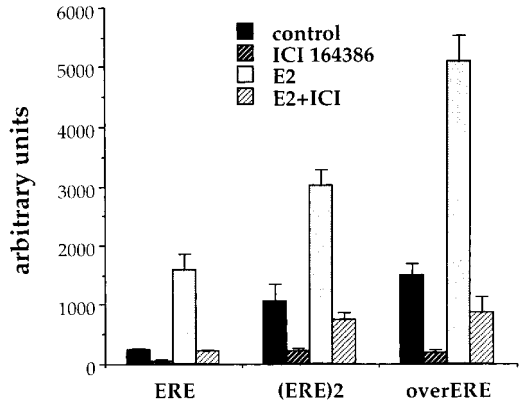


FIGURE 7: Effect of an estrogen antagonist on the basal activity. HepG2 cells were transiently transfected with either the ΔMTV-ERE-CAT, or the ΔMTV-(ERE)2-CAT, or the ΔMTV-overERE-CAT plasmid and 50 ng of the ER expression vector. Cells were incubated in the absence (control) or presence of either ICI164386 (10^{-5} M) or 17-β-estradiol (10^{-7} M) (E2) or both (E2 + ICI). Results are expressed in arbitrary units (ratio of the CAT activity over luciferase activity). The results are the mean ± SEM of 3 independent experiments.

evaluated (Figure 8A). Whole-cell extracts of CMT cells transfected by either truncated ER plasmids or wild-type ER were incubated with either radiolabeled ERE or overERE sequences. The migration of the complex containing dimerized ER was determined using the ERE probe. Using the overERE probe, the wild-type ER (HE0) formed the tetrameric complex even when small amounts of ER were added; it became predominant at intermediate and high concentrations of ER (Figure 8B). Using these extracts, the cooperativity ratio (KdII/KdIV) was close to 5, which indicates a cooperative binding of the ER to the overERE. An N-terminal truncated receptor, HE19, was also able to bind to the overERE as a tetramer (Figure 8D). Thus, the deletion of the A/B domain does not prevent tetramer formation. However, in this case, the cooperativity ratio is close to 1, suggesting that this domain contributes to cooperative binding. Several C-terminal truncated receptors were tested. HE13, which lacks the F domain, displayed properties similar to those of HE0 (KdII/KdIV = 4.9) (Figure 8F). In contrast, HE38, which also lacks the E domain, bound predominantly as a dimer (Figure 8G). A very faint band that could correspond to a tetramer was only observed at a very high receptor concentration. This was confirmed with the HE15 truncated receptor (Figure 8C). When both the C-terminal and N-terminal ends were truncated (HE70) (Figure 8E), no tetramer complex was observed even when high amounts of cell extracts were added and when the majority of the probe was complexed. In contrast, a tetramer was observed when the (ERE)2 probe was used with this ER fragment (Figure 8E). These results indicate that C-terminal and N-terminal domains of the ER are essential for the formation of a tetramer and for the cooperative binding of ER to the overERE unit and highlight the contribution of the E domain.

We have tested the functionality of some of these truncated fragments of the ER. HepG2 cells were transfected with either the Tk-ERE-CAT or the Tk-overERE-CAT plasmid and increasing amounts of the ER truncated constructs. HE0 and HE13 elicited the same pattern of induction; that is, the transactivation displayed by the overERE unit was approximately 5 times that of ERE (Figure 9A,B). In the case of HE19, transcriptional activation elicited by overERE is 2−3 times higher than that of the ERE. HE19 has lost its ability to elicit a strong synergistic induction of overERE at least at a low concentration of the receptor (Figure 9C). Both HE15 and HE21 elicited a very weak transcriptional activation of either ERE- or overERE-containing promoters (data not shown). These results are in agreement with the gel retardation experiments, in which HE0 and HE13 showed a cooperative binding to overERE. In conclusion, the cooperative binding of the estrogen receptor to the overERE sequence in vitro correlates with its synergistic transcriptional activation in vivo.

DISCUSSION

In this report, we have evaluated the ability of the estrogen receptor to bind to two overlapping estrogen-responsive elements. We have studied the binding and functional properties of the overERE sequence which consists of two overlapping EREs (i.e., separated by 5 bp center-to-center, or approximately half of a helix turn). In addition to the dimeric ER complex, this sequence forms with the ER a specific low-mobility complex that is recognized by the anti-ER antibody. The structure of the overERE sequence (four half-sites) and the mobility of the complex suggest that it is an ER tetramer−overERE complex. This is supported by several lines of evidence. (i) It is unlikely that this additional low-migrating complex is due to a contamination by a protein that would shift the migration of the overERE−ER complex because identical patterns of migration were obtained using different ER preparations: ER prepared from baculovirus infected cells, commercially available purified ER, ER prepared in vitro by a transcription/translation system (not shown), and whole-cell extracts of CMT cells transfected with ER. (ii) The patterns of the migration of both (ERE)2 and overERE are similar. (iii) Methylation interference assays showed that the G residues located into the four ERE half-sites totally or partially interfered with binding (not shown).

The binding of ER to the overERE sequence was shown to be cooperative. The dissociation constant (Kd) of the tetrameric complex is 5−15-fold weaker than that of the dimeric complex, depending on the extract used. These results indicate that the binding of the first dimer facilitates the binding of the second one on the opposite side of the DNA double helix.

Two possible mechanisms could lead to a cooperative binding of the ER to the overERE. (i) A receptor−receptor interaction may stabilize the formation of the tetramer by increasing the affinity of the second dimer to the complex. (ii) The binding of the first dimer could modify the DNA structure, thus increasing its affinity for the second dimer. Although it is difficult to directly answer these questions, the use of receptor fragments gave us some insight into the mechanisms involved. Deletion of the A/B domain did not prevent the ER−tetramer−overERE complex formation, but it altered the kinetics of the binding. In contrast, deletion of the E domain gave dramatic results. In this case, the formation of a tetramer was severely restricted. When both domains were deleted, namely when the receptor consists mainly of the DBD, its binding to the overERE was

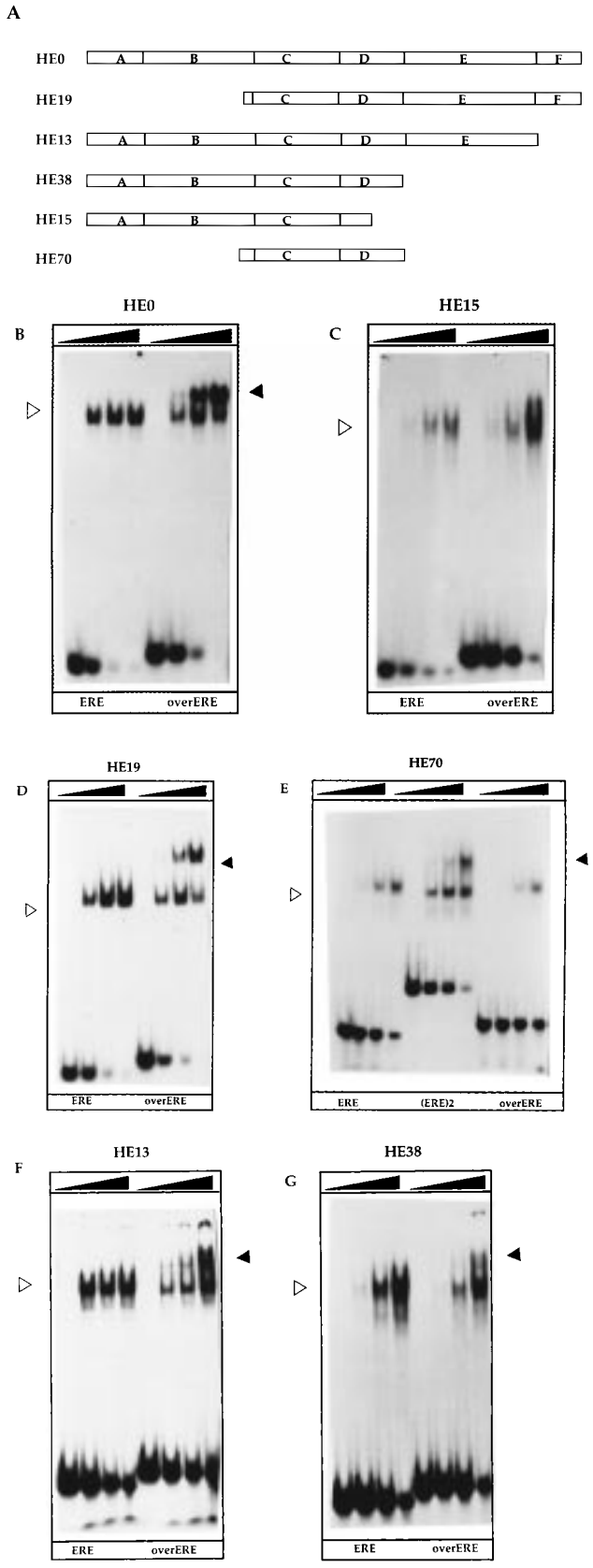


FIGURE 8: EMSA performed with ER truncated fragments. The structure of wild-type ER and truncated receptors is shown in A. Either radiolabeled ERE or overERE or (ERE)2 oligonucleotides were incubated with inceasing amounts of CMT whole-cell extracts transfected with HE0 (B), HE15 (C), HE19 (D), HE70 (E), HE13 (F), or HE38 (G) expression vectors. This experiment was repeated 4 times. The empty arrow indicates the position of the dimeric complex. The full arrow indicates the position of the tetrameric complex.

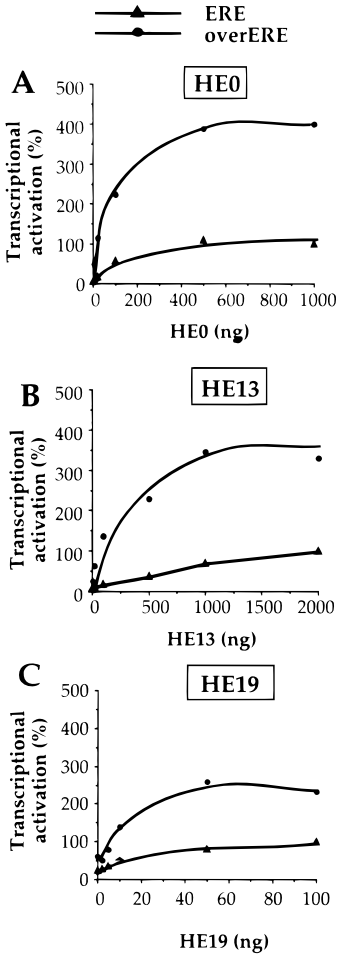


FIGURE 9: Functionality of the ER truncated fragments. HepG2 cells were transiently transfected with either the Tk-ERE-CAT (▲), or the Tk-overERE-CAT (●) plasmids and increasing amounts of either the HE0 (A), HE13 (B), or HE19 (C) expression vector; 100% corresponds to the maximal activity of the ERE construct. These results are the mean of 2 independent experiments.

indistinguishable from its binding to an ERE even at high concentrations. In contrast to the wild-type receptor, the binding of an ER DBD dimer actually prevented the binding of an additional ER DBD dimer. In order to recover such a possibility, the E domain appears to be critical. This constitutes an additional function of this domain, which consists of providing the receptor−DNA complex with a conformation allowing the binding of an additional receptor dimer on an overlapping site.

The bending of DNA upon receptor interaction could possibly account for the above observation. Indeed, the binding of an ER DBD dimer is sufficient to bend DNA (*40, 41*). This could prevent the binding of an additional DBD dimer on an overlapping site. Interestingly, this did not prevent the binding of the DBD to an adjacent site (Figure 8E). The addition of an E domain apparently overcomes this inhibition and a full-length receptor displays binding cooperativity. Because the E domain is known to interact with several proteins, it is likely that cooperative binding in our case could be accounted for by receptor−receptor interaction. However, we cannot exclude that this domain, by weakly interacting with the DNA phosphate backbone, could favor the binding of a second receptor dimer.

The ability to form a tetramer is not a unique property of the ER. Another member of the nuclear receptor superfam-

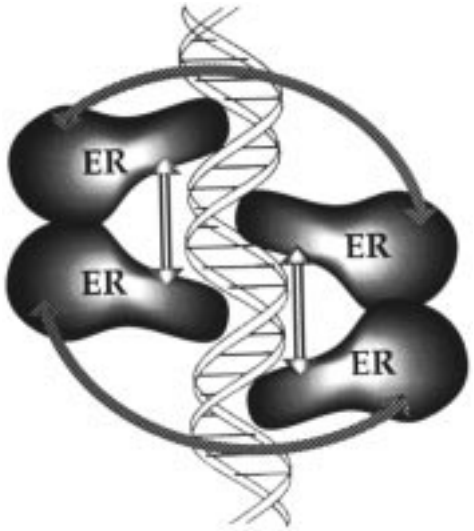


FIGURE 10: Schematic model of the interaction of four molecules of ER with the overERE sequence. White arrows represent receptor−receptor interactions implicated in the formation of a dimeric complex. Hatched arrows indicate hypothesized dimer−dimer interactions elicited by the A/B and E domains implicated in the formation of a tetrameric complex.

ily, the GR, is able to form a tetramer when interacting with overlapping GREs located in the aspartate aminotransferase gene promoter (*29*). The binding of the GR tetramer to overlapping response elements appears to be more cooperative than in the case of the ER. We hypothesize that this may be due to different effects of the GR and the ER on DNA bending. Indeed, while several studies show that the ER bends DNA by approximately 56° (*40, 42*), initial studies of the GR failed to show bending (*43*), while recent studies have suggested a moderate effect (28°) (*44*). Another nuclear receptor, RXRα, has also been shown to form tetramers upon binding to adjacent response elements (*45*). In addition, this receptor has been shown to form tetramers in solution particularly at high protein concentration (*46*). Other DNA binding proteins also form tetramers; the tumor suppressor p53 forms antiparallel tetramers in solution through a specific C-terminal sequence (*47*−*49*). Another example of tetramer formation is the λ phage repressor. Two dimers bind cooperatively to adjacent sites of the DNA. In this case, the C-terminal domain is responsible for contacts holding each dimer together and also for the cooperative interaction between the dimers (*50*). The lac repressor also binds DNA. In this case, too, the C-terminal domain is crucial for tetramerization (*51*−*53*).

The two overlapping EREs of the overERE sequence displayed a high synergistic transcriptional activation. The binding assays and the functional transfection experiments, performed on wild-type and deleted ER, suggest that the cooperative binding of the estrogen receptor to overERE may explain the functional synergism observed ex vivo (Ptashne, 1986). We do not exclude that, in addition to the cooperative binding described here, other mechanisms leading to synergistic activation of gene expression also contribute to the ex vivo estrogen-dependent functional synergism. In the case of adjacent EREs, cooperative binding was found to correlate with synergism is some studies (*23*, *24*, *54*, *55*) but not in others (*22*). The differences between the various studies on adjacent EREs, including ours, could be due to differences in the absolute position of the receptor binding sites relative to the basal transcription complex.

In conclusion, we have shown that overlapping estrogen responsive elements can cooperatively bind receptor tetramers and are very efficient in mediating hormonal gene regulation (Figure 10). The formation of such receptor−receptor interaction upon binding to overlapping responsive elements reveals a high flexibility of the receptor molecule. It is possible that such interactions could also occur between receptors bound to widely spaced imperfect responsive elements or even half EREs and could stabilize such DNA complexes (*54*). This could account for the efficiency of such sequences in mediating hormonal regulation.

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