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Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity

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The *Xenopus* vitellogenin (*vit*) gene B1 estrogen-inducible enhancer is formed by two closely adjacent 13 bp imperfect palindromic estrogen-responsive elements (EREs), i.e. ERE-2 and ERE-1, having one and two base substitutions respectively, when compared to the perfect palindromic consensus ERE (GGTCANNNTGACC). Gene transfer experiments indicate that these degenerated elements, on their own, have a low or no regulatory capacity at all, but *in vivo* act together synergistically to confer high receptor- and hormone-dependent transcription activation to the heterologous HSV thymidine kinase promoter. Thus, the DNA region upstream of the *vit*B1 gene comprising these two imperfect EREs separated by 7 bp, was called the *vit*B1 estrogen-responsive unit (*vit*B1 ERU). Using *in vitro* protein–DNA interaction techniques, we demonstrate that estrogen receptor dimers bind cooperatively to the imperfect EREs of the *vit*B1 ERU. Binding of a first receptor dimer to the more conserved ERE-2 increases ~4- to 8-fold the binding affinity of the receptor to the adjacent less conserved ERE-1. Thus, we suggest that the observed synergistic estrogen-dependent transcription activation conferred by the pair of hormone-responsive DNA elements of the *vit* B1 ERU is the result of cooperative binding of two estrogen receptor dimers to these two adjacent imperfect EREs.

Key words: cooperative DNA binding/estrogen receptor/estrogen-responsive elements/synergistic transcription activity/vitellogenin gene

Introduction

The direct activation of specific gene transcription by hormones of the 'steroid/thyroid group' requires the interaction of the hormone-receptor complexes to regulatory DNA sequences termed hormone-responsive elements (HREs). HREs for glucocorticoids and estrogens (Klock *et al.*, 1987; Martinez *et al.*, 1987) as well as for thyroid hormones (Glass *et al.*, 1988) are closely related but distinct palindromic DNA sequence motifs. In contrast, no specific HRE has yet been identified for progestins, androgens and mineralocorticoids, which all can regulate transcription via a glucocorticoid-responsive element (GRE; Cato *et al.*, 1988; Ham *et al.*, 1988; and references therein). HREs which are often found in multiple copies in the vicinity of the regulated promoters have been shown to behave as hormone-inducible DNA enhancers (for reviews see Green and Chambon, 1988; Beato, 1989; and references therein). In addition, several groups (Jantzen *et al.*, 1987; Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988a; Schüle *et al.*, 1988; Strähle *et al.*, 1988; Tsai *et al.*, 1989) have observed that HREs can act in synergy to confer high inducibility to the regulated target genes.

Interestingly, the strong estrogen-inducible enhancer of the *Xenopus* vitellogenin (*vit*) gene B1 is a modular unit composed of two, by themselves, inactive or very poorly active 13 bp imperfect palindromic estrogen-responsive elements (EREs) which act in synergy (Martinez *et al.*, 1987). These two imperfect palindromic EREs, ERE-1 and ERE-2, differ from the consensus ERE (5′-GGTCANNNT-GACC-3′; Walker *et al.*, 1984) only in their left arm by two and one nucleotides respectively. The synergistic estrogen-dependent regulatory activity of the two linked *vit*B1 imperfect EREs is similar to the activity of a single perfect palindromic ERE (5′-GGTCACTGTGACC-3′), such as the one found upstream of the *Xenopus vit*A2 gene (Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988a). Furthermore, it has also been demonstrated that the distance separating the two *vit*B1 imperfect EREs is important for synergism, since it only occurs when both EREs are closely adjacent (Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988a). Thus, both tandemly linked B1 imperfect EREs form a functional 'Estrogen-Responsive Unit' (ERU; see also Klein-Hitpass *et al.*, 1988a).

Here, we have further characterized the synergistic estrogen response conferred *in vivo* by the *vit*B1 imperfect EREs. Furthermore, we have analyzed the *in vitro* interaction of estrogen receptor either to each of the two imperfect EREs taken separately, or to both closely linked elements as they are naturally found in the *vit*B1 ERU. We demonstrate that two estrogen receptor dimers bind cooperatively to this pair of imperfect EREs. Thus, we propose that this cooperative receptor binding explains the synergistic activation of transcription conferred *in vivo* by these hormone regulatory DNA elements.

Results

Synergistic receptor- and hormone-dependent activation of transcription conferred by the two adjacent imperfect EREs of the vitB1 ERU

The vitellogenin gene B1 estrogen-responsive unit (*vit*B1 ERU, positions −302 to −334 from the *vit*B1 gene cap site) was shown previously to behave as a hormone-inducible enhancer in the human MCF-7 cells. It is formed by the two imperfect EREs (ERE-1 and ERE-2) acting in synergy (Martinez *et al.*, 1987). The primary structure of ERE-1 is 5′-AGTTATCATGACC-3′ and that of ERE-2 is 5′-AGTCACTGTGACC-3′. The nucleotides differing from the perfect palindromic consensus ERE (5′-GGTCANNNT-GACC-3′) are underlined. The fact that the 5 bp left arm of the imperfect *vit*B1 ERE-1 shows little similarity with that of the consensus ERE, prompted us to see whether this

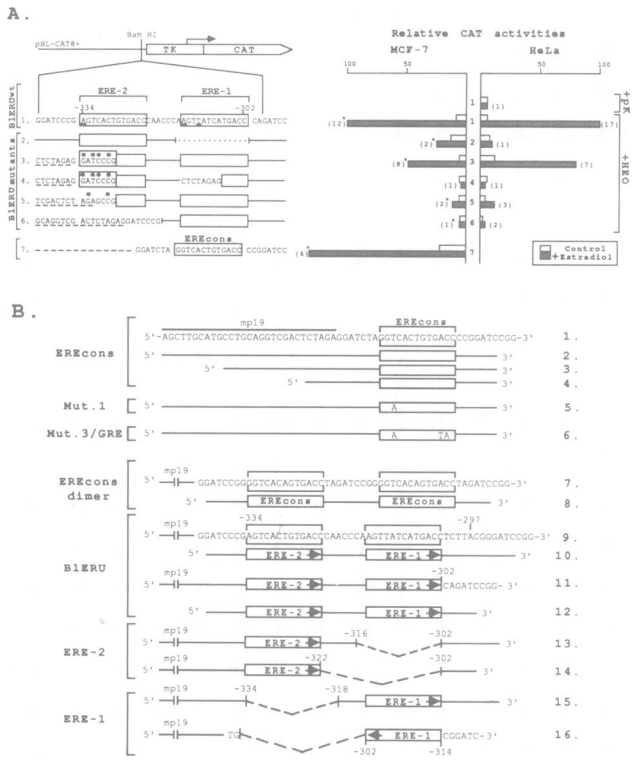


Fig. 1. (**A**) Hormone- and receptor-dependent synergistic activity of the two adjacent imperfect EREs of the *vit*B1 ERU. The *vit*B1 ERU (B1ERUwt, **lane 1**) containing both imperfect EREs (boxed sequences: ERE-1 and ERE-2) or mutants thereof (**lanes 2–6**) were inserted upstream of the tk-CAT gene. The nucleotides in the stems of ERE-1 and ERE-2 that differ from a perfect palindromic consensus ERE (EREcons, **lane 7**) are underlined in **lane 1**. A dotted line in **lane 2**, indicates deleted nucleotides. Thin lines and open boxes represent conserved flanking DNA and ERE sequences respectively (**lanes 2–6**). **Lanes 3–6** represent 5′ deletion mutants; the vector and linker sequences that replace *vit*B1 nucleotides are indicated. Vector sequences are underlined (dashed line). In **lanes 3, 4** and **5**, the black dots indicate nucleotides conserved with the left half of the EREcons. In **lane 4** the left arm of ERE-1 was replaced by new sequences which disrupt completely the palindromicity. At the right side are shown the relative CAT activities for each construct when transfected into MCF-7 cells and into HeLa cells, in the latter case either the control pKCR2 (pK) or the human estrogen receptor expression vector (HEO) were cotransfected with the receptor gene. The levels of CAT activity correlate with the amount of correctly initiated tk-CAT mRNAs (Martinez *et al.*, 1987 and data not shown). Open and hatched bars are CAT activities from unstimulated and estrogen-stimulated cells respectively. The induction factors are indicated in brackets. The asterisk (*) indicates values taken from Martinez *et al.* (1987). The expression vectors are: **lane 1**, pB1ERU(−334/−302)tk-CAT8+; **lane 2**, pB1ERU(−334/−316)tk-CAT8+; **lane 3**, pB1ERU(−327/−302)(a)tk-CAT8+; **lane 4**, pB1ERU(−327/−302) (a+LS)tk-CAT8+; **lane 5**, pB1ERU(−327/−302)(b)tk-CAT8+; **lane 6**, pB1ERU(−318/−302)tk-CAT8+; **lane 7**, pA2EREcons-tk-CAT8+. (**B**) DNA fragments used as probes in the bandshift and methylation interference experiments. Thin lines and open boxes represent conserved flanking DNA and responsive-elements respectively, compared with the probe above the one considered (otherwise changes are indicated). Numbers in B1 probes are the natural coordinates of the nucleotides. Arrow heads shown the orientation of the *vit*B1 EREs. The mp19 polylinker (mp19) in probes 7, 9, 11, 13, 14, 15 and 16 is not drawn to scale, but the sequence is the same as that in probe 1 (overlined nucleotides).

mutated half was essential for the synergistic activity observed. Reporter gene constructs containing the wild-type *vit*B1 ERU or mutants of it inserted upstream of the chimeric tk-CAT gene (tk is the HSV thymidine kinase promoter and CAT is the bacterial chloramphenicol acetyltransferase gene coding region) were transfected into the estrogen receptor-containing MCF-7 cells and into HeLa cells which do not contain functional estrogen receptors. However, in the latter, a human estrogen receptor cDNA expression vector (HEO; Green *et al.*, 1986) or the control vector without cDNA insert (pKCR2; Breathnach and Harris, 1983) was cotransfected with the reporter gene. The results reported in Figure 1A show that the transcription activity observed with the wild-type B1 ERU or with mutants of it in the MCF-7 cells is reproduced in the receptor-negative HeLa cells provided that the estrogen receptor expression vector is cotransfected with the reporter gene.

Briefly, no synergistic activity between the two linked imperfect B1 EREs is detected in HeLa cells in the absence of estrogen receptor (Figure 1A, lane 1, HeLa + pK) or in the absence of hormone (Figure 1A, lanes 1, open bars, HeLa + pK and +HEO). In contrast, in the presence of estrogen receptor the addition of hormone results in a 17-fold induction of transcription with this wild-type *vit*B1 ERU (Figure 1A, cf. lanes 1 HeLa + pK and HeLa + HEO). Deletion of any of the two imperfect EREs results in a loss of transcription activation (Figure 1A, lanes 2 and 6). Interestingly, the replacement of the left imperfect arm of ERE-2 with linker sequences that contain several nucleotides of a consensus half palindrome maintains synergism both in MCF-7 cells and in HeLa cells. In this case, a 7- to 8-fold hormone-dependent stimulation is still observed (Figure 1A, lane 3). This means that pairs of imperfect EREs different from that forming the *vit*B1 ERU are also able to act in synergy. In contrast, the replacement of the left imperfect arm of either of the two elements by nucleotide sequences that do not share significant homology with the left arm of a perfect palindromic consensus ERE completely abolishes synergism (Figure 1A, cf. lanes 3 and 4, and lanes 3 and 5). This result shows that even the less conserved left half palindrome of ERE-1 contributes to the synergistic transcription activation. In summary, this functional analysis demonstrates that the synergy first observed in MCF-7 cells between ERE-1 and ERE-2 of the *vit*B1 ERU can be reproduced in HeLa cells that artificially express the receptor and thus is not cell-specific (see also Klein-Hitpass *et al.*, 1988a; Seiler-Tuyns *et al.*, 1988). Also important is that the left half of ERE-1 and that of ERE-2 are both necessary for the hormone-dependent synergistic enhancer activity, despite the fact that they deviate from the consensus sequence.

Specific binding of estrogen receptor to the vitellogenin gene B1 ERU

Next, we analyzed the binding *in vitro* of the estrogen receptor to the *vit*B1 ERU using extracts from HeLa cells that overproduce the receptor protein. This strategy was previously used by Kumar and Chambon (1988) to demonstrate that the estrogen receptor binds to a single ERE as a ligand-induced homodimer. Thus, HeLa cells were transfected with the human estrogen receptor expression vector (HEO). Then, whole cell extracts were prepared from hormone-stimulated cells and from unstimulated cells and specific DNA binding activities were analyzed either by the gel retardation assay (Fried and Crothers, 1981) or by the DNase I protection assay (Galas and Schmitz, 1978).

Since whole cell extracts were used, it was necessary to first demonstrate that the high affinity ERE-binding activity

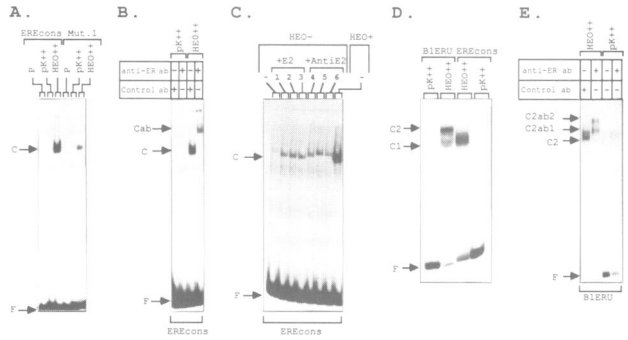


Fig. 2. Binding of the estrogen receptor to the EREcons and to the *vit*B1 ERU. (**A**) Specific binding of estrogen receptor to the ERE sequence. The perfect palindromic radiolabeled consensus ERE probe (EREcons, probe 2, Figure 1B) or a point mutant of it (Mut.1, probe 5, Figure 1B) were used in binding reactions with either the control (pK++) or the receptor-containing (HEO++) HeLa extract (20 μg of protein extract series A). The '++' in extracts 'pK++' and 'HEO++' indicates that estrogen was added *in vivo* to cells in culture and *in vitro* to the binding reaction. P indicates the purified probe. C is the specific protein−DNA complex. F is the position of the free probe. (**B**) Estrogen-receptor is present in the specific protein−DNA complex (C). The end-labeled EREcons probe (probe 4, Figure 1B) was incubated with the 'pK++' or the 'HEO++ ' extract (15 μg of protein extract series B) in the presence (+) or the absence (−) of either the rat monoclonal anti-human estrogen receptor H222 antibody (anti-ER ab) or the rat normal IgG control antibody (Control ab). Cab is the novel specific complex formed with the 'HEO++' extract only in the presence of anti-ER ab. (**C**) Ligand-dependent binding of the estrogen receptor to the ERE. The EREcons probe (probe 2, Figure 1B) was used in binding reactions with estrogen-receptor-containing extracts (10 μg of protein extract series A) from either unstimulated HeLa cells (HEO− extract) or *in vivo* estrogen-stimulated HeLa cells (HEO+ extract). Binding reactions *in vitro* were carried out either in the absence of hormone (lanes−) or in the presence of estradiol (+E2) at 10^{-9}, 10^{-8} and 10^{-7} M final concentrations (**lanes 1, 2** and **3** respectively), or in the presence of 10^{-6} M antiestrogens (+Anti E2): tamoxifen, hydroxytamoxifen and ICI 164,384 (**lanes 4, 5** and **6** respectively). (**D**) Comparison of receptor−DNA complexes formed with the *vit*B1 ERU and with the EREcons. The labeled B1 ERU probe (probe 10, Figure 1B) or the EREcons probe (probe 2, Figure 1B) was used in binding reactions with the 'pK++' or the 'HEO++' extract (8 μg of protein extract series C). C1 is a high mobility complex obtained with both probes. C2 is a low mobility specific complex obtained only with the B1 ERUprobe. (**E**) Estrogen receptor protein is present in the specific C2 complex. The end-labeled B1ERU probe (probe 11, Figure 1B) was incubated with either the 'pK++' or the 'HEO++' extract (7 μg of protein extract series C) in the presence (+) or the absence (−) of either the control (Control ab) or the H222 (anti-ER ab) antibody. C2ab1 and C2ab2 are the two specific complexes formed by the interaction of the H222 antibody with the receptor molecules bound to the B1 ERU probe.

present in the extracts corresponds to the genuine estrogen receptor. To do this, a strongly active perfect palindromic ERE (called EREcons below: 5′-GGTCACTGTGACC-3′; see Figure 1) such as the one found upstream of the *Xenopus vit* gene A2 which matches perfectly the consensus ERE sequence (5′-GGTCANNNTGACC-3′) and a point-mutant of it which is an inactive ERE *in vivo* (Mut.1: 5′-GGACACTGTGACC-3′, mutated nucleotide underlined; Martinez *et al.*, 1987) were radioactively labeled. These radiolabeled EREcons and Mut.1 probes were incubated with extracts from estrogen-stimulated HeLa cells transfected either with the human estrogen receptor expression vector (HEO) or with the control vector (pKCR2). The binding reactions were then analyzed by gel retardation assay. As shown (Figure 2A) a specific protein−DNA complex (C) is formed with the consensus ERE probe (EREcons) incubated with the receptor-containing extract (HEO++, see description in the legend of Figure 2A) and no complex is formed with the control extract (pK++) that does not contain the receptor. In addition one mutation in the left arm of the perfect ERE generating the Mut.1 probe reduces specific binding ~10-fold. That estrogen receptor is present in the protein−EREcons complex (C) was further demonstrated by the addition to the binding reaction of the specific rat monoclonal H222 antibody directed against the hormone-binding domain of the human estrogen receptor protein (Greene *et al.*, 1984). Through binding to the receptor, this specific antibody (Figure 2B, anti-ER ab) increases the size of the resulting protein−DNA complex, which now presents a lower mobility (Figure 2B, cf. complexes C and Cab). More important is the observation that no significant binding to the ERE consensus probe occurs in the absence of hormone with a receptor-containing extract from cells that were not stimulated with estrogen (Figure 2C, HEO−/lane−). The *in vitro* addition of estrogen to this extract induces specific binding to the consensus ERE probe (Figure 2C, HEO−/+E2 lanes 1−3). The reason why receptor-containing extracts from estrogen-stimulated cells (HEO+, Figure 2C) show higher specific binding activities in the absence of *in vitro* added hormone than extracts from unstimulated cells (HEO−, Figure 2C) complemented with estrogen *in vitro* (Figure 2C, cf. HEO+/lane− with HEO−/lanes +E2) is not known, but may be the result of a higher stability of the hormone-activated receptor during extract preparation. Interestingly, the anti-estrogens tamoxifen, hydroxytamoxifen and ICI 164,384 (Wakeling and Bowler, 1988) also induce binding to the ERE probe (Figure 2C, HEO−/+AntiE2 lanes 4−6), but the complexes thus formed have a slightly lower mobility in the native polyacrylamide gel, suggesting that these ligands induce different receptor conformations by their interaction with the hormone-binding domain. Altogether, our binding results which are in agreement with those described by Kumar and Chambon (1988), demonstrate that the ERE-binding activity present in our extract preparations is the authentic estrogen receptor.

Then, we analyzed the binding of estrogen receptor to the *vit*B1 ERU. As shown in Figure 2D, using the receptor-containing extract (HEO++), two specific receptor−DNA complexes (Figure 2D, C1 and C2) are formed with the B1 ERU probe which contains both imperfect EREs (ERE-1 and ERE-2). The complex C1, which is scarce under these conditions, has the same mobility in the polyacrylmide gel as the specific complex formed with the perfect palindromic consensus ERE probe (EREcons). However, the predominant complex C2 has a reduced mobility suggesting that the number of receptor molecules that bind to the *vit*B1 ERU is higher than that bound to a single perfect palindromic ERE. Since it has been previously shown that a receptor homodimer binds to a perfect ERE (Kumar and Chambon, 1988), we suggest from this experiment that more than two receptor molecules bind to the *vit*B1 ERU. The presence of estrogen receptor in the C2 complex formed with the B1 ERU probe was confirmed by the addition of the specific H222 monoclonal antibody (Figure 2E, anti-ER ab), which resulted in the replacement of the C2 complex by two slower migrating complexes (Figure 2E, C2ab1 and C2ab2). The

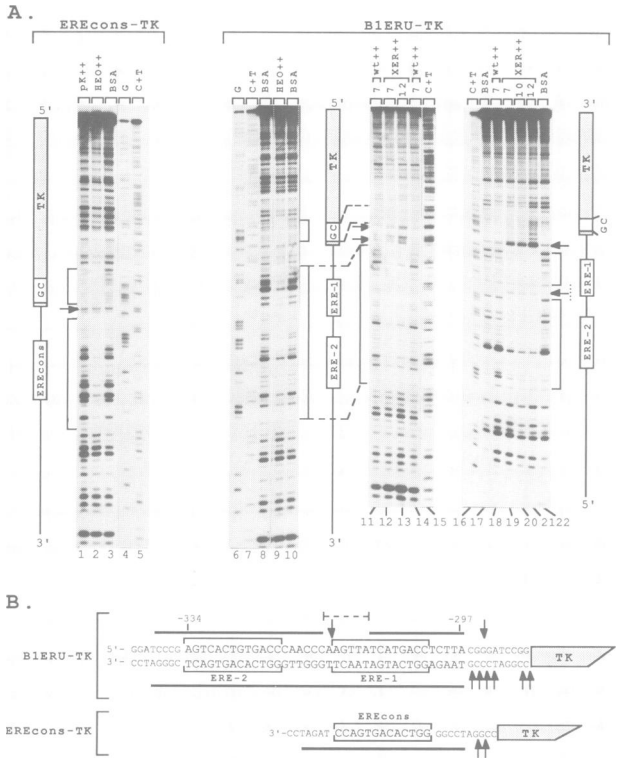


Fig. 3. DNase I footprinting of estrogen receptor binding sites. (**A**) The EREcons-TK fragment radiolabeled at one 3′ end (**lanes 1–5**) was incubated in binding reactions with 10 μg of protein (extract series C) from either the control (pK++, **lane 1**) or the human estrogen receptor-containing extract (HEO++, **lane 2**) from transfected HeLa cells. Alternatively, this same labeled fragment was also incubated with 10 μg of BSA (**lane 3**) as a control. The DNase I protected regions are shown in brackets. DNase I hypersensitive sites are indicated by arrows. The positions of the ERE consensus palindrome (EREcons) and of the distal GC-box (GC) of the tk promoter (TK) are shown in the scheme. The B1 ERU-TK fragment asymmetrically 3′ labeled (**lanes 6–10** and **11–15**) was either processed exactly as above (**lanes 6–10**) or was used in footprinting reactions with extracts from estrogen-induced HeLa cells infected with either the wild-type vaccinia virus (wt++) as a control or a recombinant virus expressing the *Xenopus* estrogen receptor (XER++, **lanes 11–15**). The meaning of '++' in the names of these extracts is as in the legend to Figure 2A. 7 μg of protein from the 'wt++' extract (**lanes 11** and **14**) or 7 and 12 μg of protein from the 'XER++' extract (**lanes 12** and **13** respectively) were used. The positions of the B1 ERE-1 and ERE-2 as well as the GC-box relative to the footprints are also indicated. The B1 ERU-TK fragment was also labeled at one 5′ end and receptor binding to the upper strand (**lanes 16–22**), was analyzed with either 7 μg of the protein extract 'wt++' (**lane 18**) or 7, 10 and 12 μg of the protein extract 'XER++' (**lanes 19, 20** and **21** respectively). As a control, 10 μg of BSA was used (**lanes 17** and **22**). A vertical dotted line between the two main protected regions indicates that this part is protected only at high protein concentration (lane 21). The position of ERE-1, ERE-2 and of the GC-box relative to the footprints is indicated in the scheme. G (**lanes 4** and **6**) and C+T (**lanes 5, 7, 15** and **16**) are chemical sequencing reactions (Maxam and Gilbert, 1980). (**B**) Summary of the footprinting results presented in (A). Solid bars indicate the receptor-dependent protected regions. The ERE sequences (ERE-1 and ERE-2 of the *vit*B1 ERU and EREcons) are boxed. Arrows indicate the DNase I hypersensitive sites. Numbers indicate the natural coordinates of the B1 ERU nucleotides with respect to the *vit*B1 cap site. The dashed line above the upper strand of the B1 ERU indicates the region that is protected only at high protein concentration (see above).

stoichiometry of the two resulting antibody–receptor–DNA complexes was not further investigated.

To delimitate the DNA sequences covered by the estrogen receptor after binding either to a single perfect palindromic ERE (EREcons) or to the *vit*B1 ERU, DNase I protection

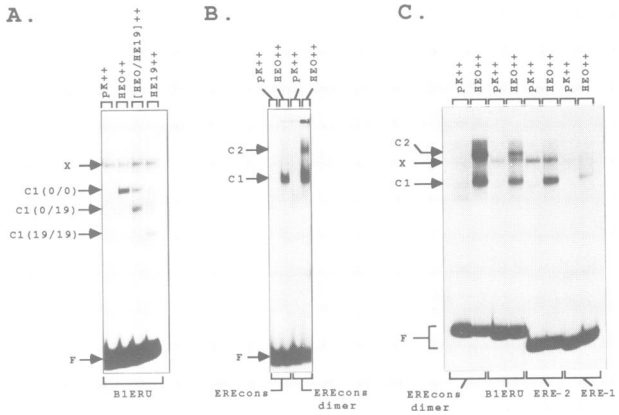


Fig. 4. Two receptor dimers interact with the *vit*B1 ERU. (**A**) One receptor dimer is bound to the *vit*B1 ERU in the specific C1 complex. The labeled B1 ERU probe (probe 11, Figure 1B) was incubated with extracts (10 μg of protein extract series B) from stimulated HeLa cells transfected with either the control pKCR2 (pK++ extract), the receptor expression vector HEO (HEO++ extract), the N-terminal truncated receptor expression vector HE19 (HE19++ extract) or with a mixture of both HEO and HE19 [(HEO/HE19)++ extract]. The specific C1 complexes formed on the bandshift gel are C1(0/0) with the 'HEO++' extract, C1(19/19) with the 'HE19++' extract and C1(0/19) with the '(HEO/HE19)++' extract. The complex X is not estrogen-receptor dependent since it appears with the control 'pK++' extract and its intensity depends on the quality of the extract and on the probe used (see also part C). F is the position of the free probe. (**B**) Gel-shift analysis of the specific complexes C1 and/or C2 formed with the radiolabeled EREcons probe (probe 3, Figure 1B) and the probe formed by two linked copies of the consensus ERE (EREcons dimer, probe 8, Figure 1B) when incubated with either the 'pK++' or the 'HEO++' extract (10 μg of protein extract series B). (**C**) Gel retardation assay with the radiolabeled probes: EREcons dimer (probe 7, Figure 1B), B1ERU (probe 11, Figure 1B), ERE-2 (probe 13, Figure 1B) and ERE-1 (probe 15, Figure 1B), and either the 'pK++' or the 'HEO++' extract (4 μg of protein extract series C). Receptor-dependent specific complexes are C1 and C2; X is the non-specific complex.

experiments were carried out (Figure 3). These footprint analyses were made with fragments containing ERE sequences fused upstream of the tk promoter (positions −105 to +51). Analysis of protein binding to the EREcons-TK fragment using the control HeLa extract (pK++) that does not contain estrogen receptor, reveals only one main protected region in the tk promoter (Figure 3A, lane 1) which corresponds to the distal GC-box that is most likely bound by the transcription factor Sp1 present in HeLa cells (Dynan and Tjian, 1983). In addition, no protection is observed over the consensus ERE sequence with this control extract (Figure 3A, cf. lane 1 with lane 3). In contrast, when using the receptor-containing extract (HEO++), the consensus ERE sequence is protected from DNase I digestion in addition to the GC-box (Figure 3A, lane 2).

Protein binding to the B1ERU-TK fragment (Figure 3) was tested in the same way, but two different types of receptor-containing extracts were compared, i.e. the extract from hormone-stimulated HeLa cells transfected with the human estrogen receptor expression vector (extract HEO++) and an extract from stimulated HeLa cells infected with a recombinant vaccinia virus expressing the *Xenopus* estrogen receptor protein (extract XER++). Analysis of the protected DNA sequences in the bottom strand of the B1ERU-TK fragment (Figure 3A, lanes 6–15) reveals two footprints with the extract 'HEO++': one on the distal GC-box of the tk promoter and another that covers both

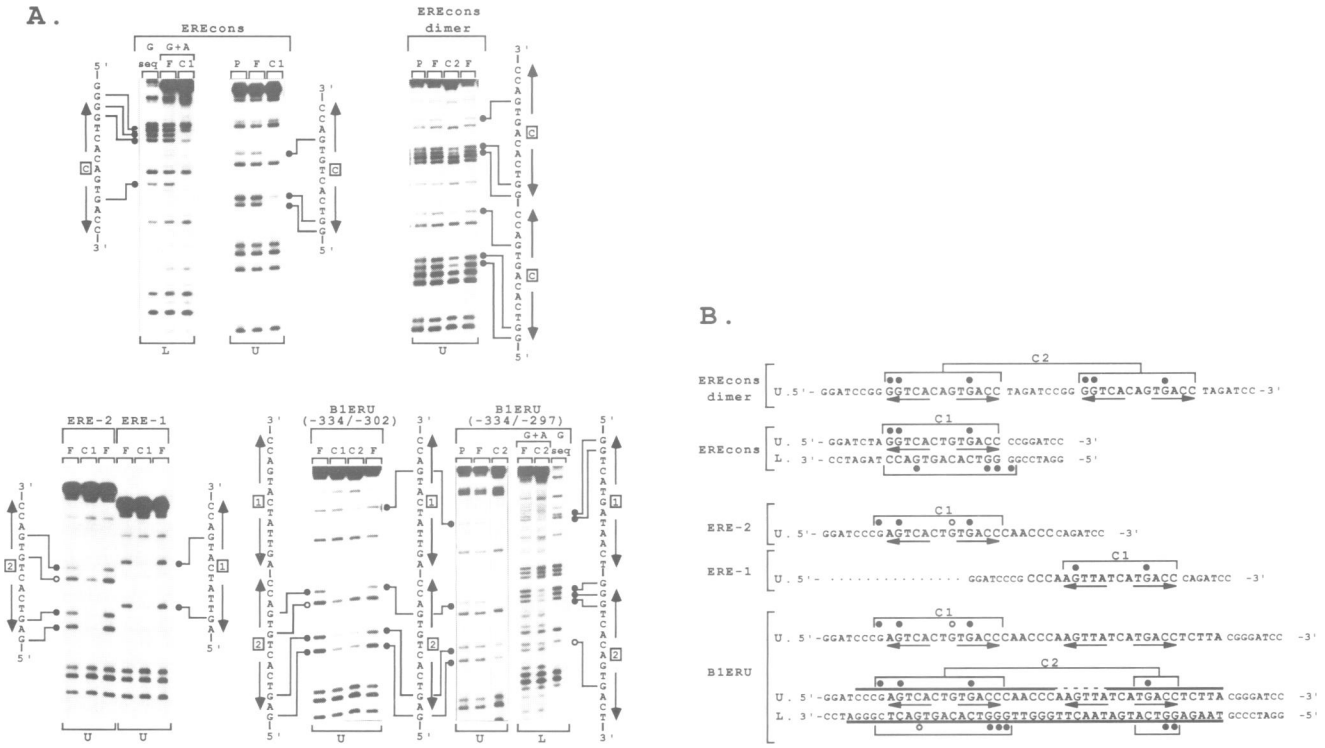


Fig. 5. Methylation interference analysis of the estrogen receptor interaction with the different target EREs. (**A**) L and U indicate the lower and upper strands of the ERE probes. EREcons is probe 1, EREcons dimer is probe 7, ERE-2 is probe 13, ERE-1 is probe 15, B1 ERU (−334/−302) is probe 11, B1 ERU (−334/−297) is probe 9 (see Figure 1B for probe code number). P is the purified methylated probe. F is the protein-free probe isolated from the F position in the bandshift gel. C1 and C2 represent probes bound in the C1 and C2 complexes respectively (see Figure 4). G seq, indicates a chemical G sequencing reaction. G+A, indicates cleavage at N-7 methylated guanines and N-3 methylated adenines. Only the nucleotide sequences of the probes in the region of the EREs are shown. Boxed C indicates a perfect palindromic consensus ERE sequence. Boxed 1 and 2 represent B1 ERE-1 and ERE-2 respectively. The G positions that interfere strongly are indicated by closed circles and open circles represent weak interfering positions. The N-3 methylated adenines do not interfere with receptor binding to the EREs tested. (**B**) Summary of the methylation interference experiments shown in (A). Only the nucleotide sequences of the probes in the region of the EREs are shown. Methylated G residues that strongly interfere with receptor binding are indicated by closed circles, and weak interfering G residues are indicated by open circles. C1 and C2 indicate the DNA region contacted by the receptor protein in the C1 and C2 receptor−DNA complexes respectively. Inverted arrows indicate the palindromic EREs. The solid bars and the dashed line in the *vit*B1 ERU (C2 complex) are regions protected by the receptor from DNase I digestion (from the results in Figure 3).

imperfect EREs of the *vit*B1 ERU (Figure 3A, cf. lane 9 with control lanes 8 and 10). With the 'XER++' extract the same region containing the two imperfect EREs of the *vit*B1 ERU is protected from DNase I digestion (Figure 3A, cf. lanes 12 and 13 with the control lanes 11 and 14). In contrast, the footprint pattern with this 'XER++' extract on the top strand of the B1ERU-TK fragment (Figure 3A, lanes 16−22) shows that at low protein concentrations only ERE-2 and the right half of ERE-1 are protected by the receptor but not the left less conserved half of ERE-1 (Figure 3A, cf. lanes 19 and 20 with control lanes 17, 18 and 22). However, increasing the protein concentration also leads to protection of the left half of the imperfect ERE-1 (Figure 3A, lane 21; see the region indicated by a dotted vertical line). No protection is observed with this extract over the GC-box of the tk promoter, which is not surprising since infection by vaccinia virus is known to strongly inhibit cellular synthesis and disturb cell organization (Person *et al.*, 1980; Rice and Roberts, 1983; and references therein). These footprinting results (see summary in Figure 3B) show that the DNA region protected by the receptor on the *vit*B1 ERU comprises both imperfect B1 EREs (ERE-1 and ERE-2). However, on the top strand the left imperfect arm of the *vit*B1 ERE-1 is protected only at the highest receptor concentration used. Taken together, the bandshift and footprint experiments strongly suggest that estrogen receptor binds specifically to both imperfect EREs of the *vit*B1 ERU and that the complex thus formed comprises more than two receptor molecules since it is larger than the one formed on a single perfect ERE.

Two estrogen receptor dimers bind to the vitellogenin gene B1 ERU

To determine the number of receptor molecules bound to the *vit*B1 ERU in the C1 complex (see Figure 2D and below), an extract from stimulated HeLa cells transfected with the wild-type estrogen receptor expression vector (HEO) together with a mutant estrogen receptor expression vector (HE19) which has the N-terminal 178 amino acids forming the A/B domains deleted (Kumar *et al.*, 1987) was incubated with the B1 ERU probe in conditions where only C1 complex can form and the binding reaction was analyzed by band-shift assay. As shown in Figure 4A, the use of the cell extract containing both, the wild-type and the mutant receptors [extract (HEO/HE19)++], gives rise to an additional specific complex C1(0/19) of intermediate mobility compared with the complexes C1(0/0) and C1(19/19) formed with extracts containing the wild-type receptor alone (HEO++) or the mutant receptor alone (HE19++) respectively (complex X which is also formed with the

control extract is non-specific; see also legend of Figure 4). From this result and from the observation that the C1 complex formed on the B1 ERU probe has the same mobility on a native polyacrylamide gel as the complex formed on a single perfect palindromic ERE (Figure 2D, see also below and Figure 4B and C) which is known to be bound by a single receptor homodimer (Kumar and Chambon, 1988), we conclude that the C1 complex formed with the *vit*B1 ERU comprises one receptor dimer. To estimate the number of receptor molecules bound to the *vit*B1 ERU in the C2 complex, we compared the gel mobilities of the specific receptor–DNA complexes formed with a single-copy consensus ERE probe (EREcons), with two linked consensus EREs (EREcons dimer) and with the B1 ERU probe. The results show that two specific complexes, C1 and C2, are also formed with the probe having two copies of a perfect ERE (Figure 4B, EREcons dimer) and that the C1 complex has the same mobility as the complex formed with a single copy of the perfect ERE (Figure 4B, EREcons) thus corresponding to one receptor dimer bound to one of the two linked perfect EREs. The C2 complex therefore would represent two receptor dimers bound, one on each perfect ERE of the EREcons dimer probe (Figure 4B). Interestingly the C2 complex formed on the probe containing two copies of a perfect consensus ERE (EREcons dimer) has the same gel mobility as the C2 complex formed on the B1 ERU probe containing the two linked imperfect B1 EREs (Figure 4C, cf. C2 complexes with EREcons dimer and with B1 ERU). Taken together these results strongly suggest that the specific C2 complex formed with the B1 ERU probe results from the binding of two estrogen receptor dimers. That each imperfect B1 ERU taken separtely is bound by a receptor dimer was demonstrated in the experiment of Figure 4C. It shows that the specific complexes formed with the imperfect ERE-1 and ERE-2 probe migrate in the gel at the same C1 position corresponding to probes bound by a single receptor dimer (see above). Interestingly, the B1 imperfect ERE-1 (2 bp deviating from the consensus) has a lower affinity for the receptor than ERE-2 (1 bp deviating from the consensus) as indicated by the amount of the specific retarded complex obtained with each probe (Figure 4C, cf. C1 complexes with ERE-1 and ERE-2).

In order to identify the G residues of the different ERE probes described above interacting with the estrogen receptor in the specific complexes C1 and/or C2, we performed methylation interference experiments. This method also allows an indirect determination of the number of receptor molecules bound to the probe in a specific complex, since it has been previously demonstrated that palindromic HREs interact directly with receptor dimers and that each monomer interacts with one arm of the palindromic responsive element (Kumar and Chambon, 1988; Tsai *et al.*, 1988). First, we confirmed our preceding results based on bandshift comparisons, by showing that the B1 imperfect ERE-1 and ERE-2 taken separately as well as a perfect palindromic ERE (EREcons) are bound by a receptor dimer since methylated G residues on each half of the palindrome interfere with specific binding (Figure 5A and B, ERE-1/C1, ERE-2/C1 and EREcons/C1). Second, based on the same reasoning, we conclude that two receptor dimers interact with the probe formed by two linked perfect palindromic EREs (EREcons dimer) in the specific C2 complex, because methylated G residues present on the four arms of the two linked palindromes interfere with C2 complex formation (Figure 5A and B, EREcons dimer/C2). Taking these results into account, we then analyzed in the same way the contact points of the estrogen receptor with the B1 ERU probe in the specific C1 and C2 complexes. The results presented in Figure 5A and summarized in Figure 5B show that in the C1 complex only the ERE-2 interferes but not the ERE-1 (Figure 5A and B, B1 ERU/C1), indicating that a receptor dimer is bound only to the imperfect ERE-2. In contrast, in the C2 complex both imperfect EREs make contacts with the receptor protein (Figure 5A and B, B1 ERU/C2). In addition, only the right perfect but not the left less conserved arm of the ERE-1 interferes with specific C2 complex formation. This would suggest at first sight that only one receptor molecule binds to the ERE-1, namely on its right perfect arm and that a receptor dimer binds to the ERE-2. However, this C2 complex has exactly the same mobility on a native polyacrylamide gel as the C2 complex obtained with the EREcons dimer probe (Figure 4C). Furthermore, we have shown that two receptor dimers bind to the EREcons dimer probe in the C2 complex (see above and Figure 5). In addition, we know from the functional studies described in Figure 1A that the integrity of the left imperfect half palindrome of ERE-1 is required for synergistic enhancer activity (Figure 1A, cf. lanes 3 and 4). Moreover, it is known (Kumar and Chambon, 1988) that the hormone-activated estrogen receptor exists as stable homodimers in solution. Thus, these results strongly suggest that two receptor dimers are bound to the B1 ERU probe in the C2 complex, one receptor dimer on each imperfect ERE. However, the interaction of one receptor molecule with the left arm of ERE-1 is either transient and/or too weak to be revealed by the methylation interference technique used. This interpretation is consistent with the footprinting data described in Figure 4 showing that the left half of the imperfect ERE-1 is protected from DNase I digestion, although weakly on the top strand, at high concentration of receptor-containing extract (see also Figure 5B, B1ERU/C2). Interestingly, however, methylation of the G residue on the left less conserved half of ERE-1 interferes strongly with specific binding when this element is tested independently (Figure 5A and B, ERE-1/C1). This apparent discrepancy with the above observation may indicate that the stability of the C1 complex obtained with an isolated ERE-1 probe is mainly achieved by protein–DNA interactions, whereas the stability of the C2 complex obtained with the B1 ERU probe may not only be achieved by protein–DNA contacts but also by protein–protein interactions. This would explain why methylation of the left arm of ERE-1 does not affect the stability of the C2 complex to a great extent. Similarly, methylation of the G residue in the loop of ERE-2 interferes weakly with C1 complex formation with the probe ERE-2 (Figure 5A and B, ERE-2/C1) and with the B1 ERU probe (Figure 5A and B, B1ERU/C1). In contrast, this same G residue does not interfere with C2 complex formation with the B1 ERU probe (Figure 5A and B, B1ERU/C2). This also supports the idea that protein–protein interactions contribute to the stability of the C2 complex. Taken together the results described above demonstrate that an estrogen receptor dimer interacts preferentially with the imperfect ERE-2 of the *vit*B1 ERU, which has a single point mutation with respect to the consensus ERE, and that a second receptor dimer binds to the imperfect ERE-1, which has two

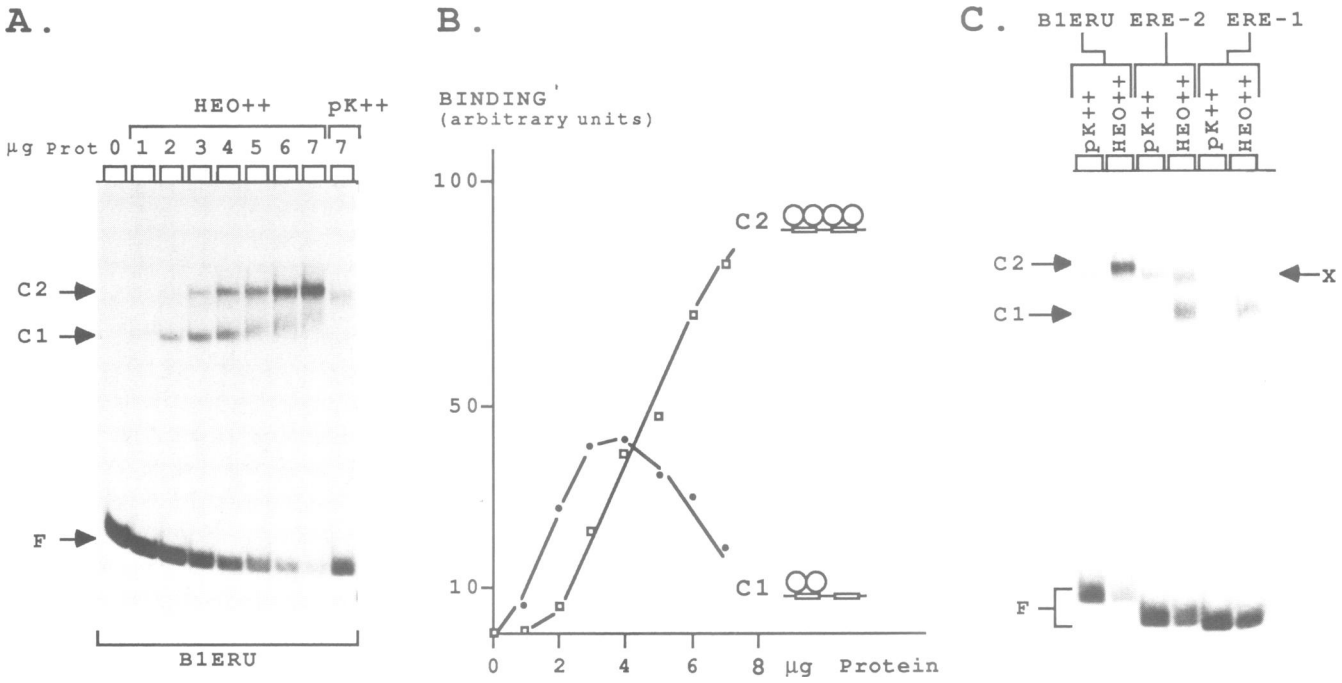


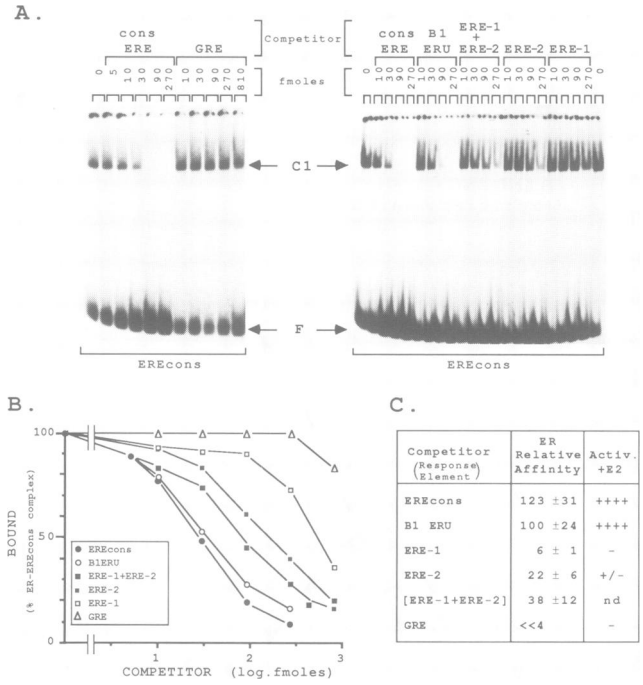
Fig. 6. Cooperative binding of estrogen receptor to the *vit*B1 ERU. (**A**) Gel retardation assay using the radiolabeled B1 ERU probe (probe 11, Figure 1B) incubated with increasing amounts of the receptor-containing extract HEO++ (extract series C) from 0 to 7 µg of protein (µg prot). A control binding was carried out with 7 µg of protein from the receptor-negative 'pK++' extract. F indicates the position of the free probe. The reason for the slightly reduced mobility of the C1 complex in lanes with 5–7 µg prot (HEO++) is unclear. This effect is not observed under the same conditions when no C2 complex can form with the ERE-1 or ERE-2 probes taken separately (see part C). (**B**) Graph showing the amount of bound B1 ERU probe in the specific C1 and C2 complexes determined by quantitative densitomery and plotted (in arbitrary binding units) as a function of the amount of protein extract 'HEO++' (µg protein). Fifty binding units correspond to 34% of the probe bound in a given complex. A scheme for bound receptor dimers (OO) to the B1 ERU in each complex C1 and C2 is shown. (**C**) The probes B1 ERU (probe 11, Figure 1B), ERE-2 (probe 13, Figure 1B) and ERE-1 (probe 15, Figure 1B) were ^{32}P end labeled to similar specific activities and incubated in binding reactions containing either the 'pK++' or the 'HEO++' extract (7 µg of protein extract series C). The complexes were then separated by gel retardation. C1 and C2 are the specific receptor–DNA complexes, X is the non-specific receptor-independent complex.

nucleotide positions deviating from the consensus ERE sequence.

Cooperative binding of two estrogen receptor dimers to the two adjacent imperfect EREs of the vitB1 ERU

To characterize the assembly of estrogen receptor dimers on the *vit*B1 ERU, various protein concentrations of receptor-containing extract (HEO++) were used in binding reactions that were analyzed by gel retardation assay. As shown in Figure 6A and B the high mobility complex C1, which represents a receptor dimer bound to the ERE-2 (see above), appears first at low protein concentrations. This specific C1 complex is then replaced by the lower mobility specific complex C2, which becomes predominant as the protein concentration is increased, indicating that a second receptor dimer has bound to the adjacent ERE-1 (see above). The bound B1 ERU in the C1 complex never exceeds 30% of the total probe used (see legend to Figure 6B). Although this result suggests a cooperative binding of the receptor to the *vit*B1 ERU, additional experiments were performed to demonstrate it.

Thus, in order to see whether the binding of a first estrogen receptor dimer to the ERE-2 increases the affinity of the receptor to the neighboring ERE-1, we tested in a gel retardation assay the B1 ERU probe containing both imperfect B1 EREs linked, and each imperfect B1 ERE, i.e. ERE-1 probe and ERE-2 probe, taken separately. These three probes were radioactively labeled to similar specific activities and used in binding reactions with control (pK++) or receptor-containing (HEO++) extracts from estrogen-stimulated HeLa cells. As shown in Figure 6C, at high protein concentration almost all the B1 ERU probe is retarded into the specific C2 complex corresponding to both adjacent imperfect EREs bound by the receptor. In contrast, only a small fraction of the probes ERE-1 and ERE-2, taken separately, is bound by the receptor protein under the same conditions (Figure 6C, cf. C2 complex with B1ERU and C1 complexes with ERE-2 and ERE-1; X is a non-specific complex, see legend to Figure 4). A similar observation was also made at low protein concentration, in conditions where there is a large excess of free probe (Figure 4C, see the lower amount of C1 complex with ERE-1 compared with C2 complex with B1ERU). These results indicate a cooperative binding of estrogen receptor to the imperfect EREs of the *vit*B1 ERU because the affinity of estrogen receptor for ERE-1 is increased when ERE-2 is bound by a receptor dimer, and are consistent with the above observation (Figure 6A and B). To further confirm the cooperative binding of



Competitor (Response Element)	ER Relative Affinity	Activ. +E2
EREcons	123 ±31	++++
B1 ERU	100 ±24	++++
ERE-1	6 ± 1	-
ERE-2	22 ± 6	+/-
[ERE-1+ERE-2]	38 ±12	nd
GRE	<<4	-

Fig. 7. Determination of the relative binding affinities of the estrogen receptor for the different EREs by bandshift competition assay. (**A**) 5 fmol of the radiolabeled consensus ERE probe (EREcons, probe 2, Figure 1B) was used in binding reactions with the receptor-containing extract (HEO++, 16 μg of protein extract series B) in the absence of competitor (lane 0 fmoles competitor) or in the presence of increasing amounts (5, 10, 30, 90, 270 and 810 fmol) of the non-labeled competitor DNAs: EREcons (probe 2, Figure 1B), GRE (probe 6, Figure 1B), B1 ERU (probe 10, Figure 1B), ERE-1 (probe 16, Figure 1B), ERE-2 (probe 14, Figure 1B), and the mixture ERE-1 + ERE-2 (probe 16 + probe 14) where the amount of each competitor probe in the mixture is either 10, 30, 90 or 270 fmol as indicated. C1 indicates the specific 'receptor-EREcons' complex. F shows the position of the free probe. (**B**) The amount of specific C1 complex formed in each competition reaction (determined by quantitative densitometry) was normalized to the amount of C1 complex formed in the absence of competitor (lanes 0 fmoles) which was taken as 100. Mean values from two to six different series of bandshift competitions, with two different competitor probe preparations, were plotted as a function of the amount of competitor used (log. fmoles). The competitor probes used are boxed (see also part A). Two B1 ERU competitors: probe 10 and probe 12 (Figure 1B), give identical competition results (not shown). (**C**) The relative affinity of the estrogen receptor (ER Relative Affinity) for each competitor DNA (Competitor, Response Element) was determined from the inverse of the amount of competitor required to titrate 50% of the specific 'ER-EREcons' complex (see part B) and normalized to the affinity of the B1 ERU which was arbitrary taken as 100. The relative affinity of the estrogen receptor for the GRE (<<4) is a large overestimation determined from the last point in the GRE curve in part (B). The level of estrogen-dependent transcription activity (Activ. + E2) conferred by each regulatory element is shown in parallel by the '+' (active) and '−' (inactive) signs (from the results in Figure 1A). nd indicates not determined.

estrogen receptor to the adjacent imperfect EREs of the *vit*B1 ERU, we tested by bandshift competition experiments whether the estrogen receptor has a higher affinity for the *vit*B1 ERU fragment containing both linked imperfect EREs compared with each imperfect ERE taken separtely. Specific receptor binding to the radioactively labeled perfect palindromic ERE (EREcons probe) resulting from the incubation with the receptor-containing extract (HEO++) from stimulated HeLa cells, was competed with increasing amounts of non-labeled competitor DNAs, and analyzed by gel retardation assay. The results reported in Figure 7 show that a strong GRE (Mut.3/GRE probe in Figure 1B; Martinez *et al.*, 1987) does not significantly compete for estrogen receptor binding and that the perfect palindromic ERE (EREcons) and the B1 ERU are almost equivalent competitors, which is in agreement with their similar hormone-dependent enhancer activities *in vivo* (see Figure 1A). In contrast, the B1 ERU is ~5 and 17 times more efficient in reducing the formation of the specific complex, than ERE-2 and ERE-1 respectively, and ~3 times more efficient than a mixture of ERE-1 and ERE-2 on separate DNA fragments (Figure 7B and C). These results are consistent with the lack of activity or the very low activity *in vivo* of the imperfect B1 EREs (ERE-1 and ERE-2; see Figure 1A). The relative competition efficiencies given above were estimated from the amount of competing DNA required to titrate 50% of the labeled specific 'receptor-EREcons' complex (Figure 7B and C). Thus, they reflect different affinities of the estrogen receptor for these EREs. These competition analyses thus also show a cooperative interaction of the estrogen receptor with the two low affinity binding sites of the *vit*B1 ERU. In summary, these results demonstrate that the estrogen receptor has an ~4-fold higher affinity for the imperfect ERE-2 than for the less conserved ERE-1 of the *vit*B1 ERU (Figure 7C). The binding of a receptor dimer first to the ERE-2 increases ~4- to 8-fold the binding affinity of the estrogen receptor to the neighboring imperfect ERE-1, as determined from the curves of C1- and C2-specific complex formation with the *vit*B1 ERU as a function of the protein concentration (Figure 6B) and from the relative affinities of estrogen receptor for ERE-1 and ERE-2 (Figure 7C and see Materials and methods for the calculations). Finally, we show that the cooperative binding of estrogen receptor to the imperfect EREs of the

*vit*B1 ERU correlates with their synergistic hormone-dependent enhancer activity *in vivo* (Figure 7C, cf. 'ER Relative Affinity' with 'Activ. + E2').

Discussion

The results presented here demonstrate that the synergistic hormone-dependent enhancer activity of the two adjacent imperfect EREs (ERE-1 and ERE-2) of the *Xenopus* vitellogenin gene B1 estrogen-responsive unit (*vit*B1 ERU) is strictly dependent on the presence of estrogen receptor and the hormone *in vivo*. Both imperfect ERE-1 and ERE-2 have their 5 bp right arm conserved when compared with that of the consensus ERE; in contrast, their left arm contains either two (ERE-1) or one (ERE-2) nucleotide positions differing from the left arm of the perfect palindromic consensus ERE. Interestingly, we show that each left mutated half of ERE-1 and ERE-2 is required for this synergistic functional activity, indicating that these imperfect sequences must interact with the estrogen receptor in spite of the mutations. Furthermore, we have demonstrated a cooperative binding *in vitro* of estrogen receptors to the two imperfect EREs of the *vit*B1 ERU. Our results indicate that in the presence of hormone the estrogen receptor dimer binds weakly to the separate imperfect B1 EREs, since the ERE-1 and the ERE-2 show ~20- and 5-fold lower affinity for the receptor respectively, than a perfect ERE or than the *vit*B1 ERU formed by the two linked imperfect EREs. We calculated that the preferential binding of a first receptor dimer to the B1 ERE-2 increases the binding affinity of a second receptor dimer for the adjacent less conserved ERE-1 ~4- to 8-fold. Thus, this cooperative binding of estrogen receptor dimers to the imperfect *vit*B1 EREs most likely explains the functional synergism observed *in vivo* with these regulatory DNA elements. At the moment, however, we cannot discriminate between cooperative binding resulting from protein−protein interactions or alternatively from a change in DNA conformation induced by receptor binding. Furthermore, we do not exclude that in addition to the cooperative binding described here other mechanisms leading to synergistic regulation of gene expression (Ptashne, 1988) also contribute to the *in vivo* estrogen-dependent functional synergism. Similar to this report, it has been shown recently (Tsai *et al.* 1989; Schmid *et al.*, 1989) that a pair of glucocorticoid/progesterone-responsive elements (G/PREs) that can act in synergy *in vivo*, are bound by progesterone and glucocorticoid receptors in a cooperative manner. In addition, synergistic regulations have also been shown to occur between an inactive or weak hormone-responsive element (HRE) and a DNA-binding site for either an ubiquitous basal transcription factor such as CTF/NF-I, Sp1, CACCC-box binding protein and OTF (Strähle *et al.*, 1988; Schüle *et al.*, 1988) or a cell-specific transcription factor (Tora *et al.*, 1988). Whether cooperative binding contributes to these functional synergisms is still unknown. Thus, it appears that the formation of a stable receptor−DNA complex is at least in a first step, a prerequisite for transcription activation. This strong interaction which depends on the presence of hormone as it is demonstrated for the estrogen receptor−ERE interaction (Kumar and Chambon, 1988; and our results presented here), can be achieved by a single HRE matching perfectly the corresponding consensus sequence as for instance the 13 bp perfect palindromic ERE: 5′-GGTCACTGTGACC-3′ (Kumar and Chambon, 1988; Klein-Hitpass *et al.*, 1989; and our results presented here) or the 15 bp GRE II of the tyrosine aminotransferase (TAT) gene: 5′-TGTACAGGATGTTCT-3′ (Jantzen *et al.*, 1987; Strähle *et al.*, 1987). The latter regulatory element has a single base (underlined) deviating from the consensus GRE sequence: 5′-GGT(A/T)CANNNTGTYCT-3′ (Scheiderheit *et al.*, 1986). Alternatively, weakly active or inactive imperfect HREs having a low intrinsic affinity for the receptor, when closely adjacent to each other form highly active hormone-responsive units (HRUs). These HRUs can achieve strong interaction with the receptor through cooperative binding, as we have reported in the present paper for the imperfect *vit*B1 EREs, and as it has been recently shown for two weak G/PREs (Tsai *et al.*, 1989). Interestingly, however, only HRUs formed by either several HREs or by an HRE and a regulatory transcription factor binding site, have been unambiguously demonstrated to activate transcription over long distances (Jantzen *et al.*, 1987; Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988b; Strähle *et al.*, 1988). Moreover, it has been recently demonstrated that the 15 bp GRE II of the TAT gene when close to the HSV tk promoter (at position −105 bp from the cap site) or immediately upstream of its TATA-box (at position −37 bp from the tk cap site), is able to confer strong regulation, but is inactive when present at −351 bp from the TAT gene cap site. However, at that same distant position, two closely linked copies or one copy plus one adjacent transcription factor binding site can restore high inducibility (Strähle *et al.*, 1987, 1988). This could mean that at a long distance strong binding to one single responsive DNA element may not be sufficient for activation and that a second mechanism is involved which would depend to a certain extent on the number of transcription factors bound on the enhancer. By taking our results and these observations into account, we propose as a model that the hormone-dependent enhancer activity of the *vit*B1 ERU could mainly be achieved in three steps. First, the hormone through its interaction with the receptor induces the formation of stable receptor dimers in solution (Kumar and Chambon, 1988). Second, the activated receptor dimers interact cooperatively with the imperfect EREs of the *vit*B1 ERU to achieve stable occupation of the enhancer with a total of two receptor dimers bound (from the results presented here). Finally, the bound receptors may promote the formation at a distance of active transcription initiation complexes, either by recruiting transcription factors or/and by stabilizing the interaction of the transcription factors with the promoter through protein−protein interactions and looping out of the intervening DNA (Ptashne, 1988; and references therein). Interestingly, we have previously demonstrated that duplication of the *vit*B1 ERU ~2 kbs downstream of the transcription initiation site of the tk-CAT chimeric gene resulted only in additive hormone-dependent enhancer activity (Martinez *et al.*, 1987). This could mean that two receptor dimers bond to the *vit*B1 ERU already saturate the interaction with the transcription factors at the promoter; similar observations were also reported for GREs (Strähle *et al.*, 1988). Clearly this last activation step is the less well understood. Further studies using *in vivo* and *in vitro* reconstituted transcription systems employing wild-type or mutant transcription factors

should allow a better understanding of this final transcription activation process.

In conclusion, the cooperative binding at low affinity binding sites, of ligand-dependent transcription factors as well as tissue-specific transcription factors, may be a general biological mechanism to achieve fine regulation of gene expression in a cell-, tissue-, sex- and developmental stage-specific manner.

Materials and methods

Recombinant plasmid DNAs

The chimeric expression vectors used in this study have been constructed and prepared as described previously (Martinez *et al.*, 1987; Martinez, 1989). The human estrogen receptor cDNA expression vector HEO (Green *et al.*, 1986) and the control parental vector pKCR2 (Breathnach and Harris, 1983) were a kind gift of P.Chambon.

Cell transfection and CAT assay

Transfection of MCF-7 and HeLa cells, and the CAT assays have been carried out as described (Matinez *et al.*, 1987; Martinez, 1989).

Cell extract preparation for receptor binding analysis

HeLa cells were transfected (Martinez, 1989) with the receptor expression vector HEO or the control vector pKCR2 by either the calcium phosphate coprecipitation technique (Wigler *et al.*, 1979) or the lipofection technique (Felgner *et al.*, 1987). The extracts obtained from lipofected cells are called the 'extract series A'. The extracts from calcium phosphate transfected cells are called 'extract series B' and 'C'. Alternatively, HeLa cells have been infected with a recombinant vaccinia virus (C.Bertholet and W.Wahli, unpublished data) expressing the *Xenopus* estrogen receptor (Weiler *et al.*, 1987).

Whole cell extracts (WCE) from estrogen stimulated or unstimulated, transfected or infected HeLa cells were prepared as described (Kumar and Chambon, 1988).

Gel retardation and DNase I protection analyses

The probes used for bandshift experiments are listed in Figure 1B. These are double-stranded DNA fragments that were excised from plasmids by restriction enzyme digestion. The ends of the probes were flushed by nuclease S1 treatment or by filling with Klenow enzyme in the presence of an [α-^{32}P]dNTP to label the ends. For the competitor DNA fragments, the Klenow filling reaction was done in the absence of radiolabeled nucleotides. For gel retardation, WCE from uninduced or 17β-estradiol-induced cells, containing 10–20 μg protein for extract series A and B or 1–8 μg protein for extract series C, was incubated at 0°C for 15 min with 2 μg or 0.2 μg poly(dI.dC)·poly(dI.dC) respectively. The binding reaction (Kumar and Chambon, 1988) was started by adding the end-labeled DNA probe (~5 fmol, ~5 × 10^4 c.p.m.) and was then incubated at 20°C for 15 min. The complexes were analyzed on 5% native polyacrylamide gels. For the antibody-containing binding reactions, 0.4 ng of either the rat monoclonal anti-estrogen receptor H222 antibody or the control normal rat IgG (Abbott ER-ICA monoclonal kit, Abbott Laboratories) were included in the preincubation mixture at 0°C for 15 min. For the gel retardation competition experiments (see Figure 7) different concentrations of unlabeled competitor DNA were added after the first 15 min precincubation step at 0°C, and the mixture was further incubated for 15 min at 0°C, then 5 fmol of labeled DNA probe was added.

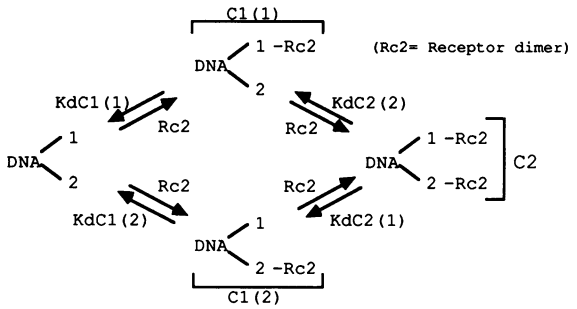
The probes used in the footprinting analyses are displayed in Figure 3A and correspond to the *Hin*dIII–*Bgl*II fragments (~200 bp) from either pB1ERU (–334/–297)tk–CAT8+ or pA2EREcons-tk-CAT8+, containing the EREs fused upstream of the HSV tk promoter (positions –105 to +51). The probes were labeled at the *Hin*dIII site. The binding and footprinting reactions were carried out as described (Martinez, 1989).

Methylation interference assay

Probes 1, 7, 9, 11, 13 and 15 (Figure 1B) were ^{32}P-labeled only at the *Hin*dIII site, either at the 5′-end or at the 3′-end. The labeled probes were partially methylated at purines with dimethylsulfate (Maxam and Gilbert, 1980). Each methylated probe (100 000 c.p.m.) was used in binding reactions with receptor containing extract (10 μg protein, HEO++ extract series C) and either 2 μg poly(dI.dC)·poly(dI.dC) (probes 7, 11, 13) or 0.5 μg poly(dI.dC)·poly(dI.dC) (probes 1, 9 and 15). The protein–DNA complexes were analyzed by gel retardation assay. After 2 h autoradiography of the wet gel at 4°C, the receptor-bound and free DNA probes were cut out from the gel. The DNA was eluted and cleaved at G residues with piperidine (Maxam and Gilbert, 1980) or at G+A residues (Raymondjean *et al.*, 1988). The cleavage products were then analyzed on a 8% polyacrylamide–7.5 M urea gel.

Equations to calculate the cooperativity factor

The equations reported by Tsai *et al.* (1989) have been adapted for the two imperfect B1 EREs, i.e. ERE-1 and ERE-2, which have different affinities for the estrogen receptor:



$$C1 = C1(1) + C1(2)$$

Since C1 is formed predominantly by receptor bound on ERE-2 (C1(2)) and ERE-2 has ~4-fold higher affinity for the receptor than ERE-1 (Figure 7), we can simplify the above equation by approximating that C1(1) is negligible, thus: C1 = C1 (2). The measure of the cooperativity (cooperativity factor = CF) is given by the ratio between the dissociation constant at ERE-1 in the absence of receptor bound to the ERE-2 [KdC1(1)] and that in the presence of a bound ERE-2 [KdC2(1)]:

$$CF = \frac{KdC1\ (1)}{KdC2\ (1)} \qquad (a)$$

Using the approximation described above applied to the equations described previously (Tsai *et al.*, 1989), we find that the maximum fraction of C1 complex formed ($f_{max}C1$) is:

$$f_{max}C1 = \frac{\sqrt{KdC1\ KdC2}}{2KdC1 + \sqrt{KdC1\ KdC2}} \qquad (b)$$

$$\frac{KdC1\ (2)}{KdC2\ (1)} = \frac{KdC1}{KdC2} = \frac{1}{4}\left(\frac{1}{f_{max}C1} - 1\right)^2 \qquad (c)$$

From the relative affinities determined in Figure 7, we can calculate the following ratio (x):

$$x = \frac{KkC1\ (2)}{KdC1\ (1)} \qquad (d)$$

$$KdC1\ (2) = x\ KdC1\ (1) \qquad (e)$$

Substitute (e) into equation (c):

$$CF = \frac{KdC1\ (1)}{KdC2\ (1)} = \frac{\frac{1}{4}\left(\frac{1}{f_{max}C1} - 1\right)^2}{x} \qquad (f)$$

$f_{max}C1$ = 0.30 (Figure 6B); x = 0.27 ± 0.11 (Figure 7C).

By replacing $f_{max}C1$ by the value determined from the experiment in Figure 6B (maximum 30% of bound probe in the C1 complex) and replacing x by the ratio of the relative affinities determined in Figure 7C (6 ± 1/22 ± 6), we find a cooperativity factor (CF) of 4- to 8-fold.

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