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Ligand-dependent, transcriptionally productive association of the amino- and carboxyl-terminal regions of a steroid hormone nuclear receptor

(estrogen/antiestrogen/activation domain/transcriptional synergism)

W. LEE KRAUS*†‡, EILEEN M. MCINERNEY*‡, AND BENITA S. KATZENELLENBOGEN*§

*Department of Physiology and Biophysics, and §Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801

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ABSTRACT **The estrogen receptor (ER), a 66-kDa protein that mediates the actions of estrogens in estrogen-responsive tissues, is a member of a large superfamily of nuclear hormone receptors that function as ligand-activated transcription factors. ER shares a conserved structural and functional organization with other members of this superfamily, including two transcriptional activation functions (AFs), one located in its amino-terminal region (AF-1) and the second located in its carboxyl-terminal, ligand-binding region (AF-2). In most promoter contexts, synergism between AF-1 and AF-2 is required for full ER activity. In these studies, we demonstrate a functional interaction of the two AF-containing regions of ER, when expressed as separate polypeptides in mammalian cells, in response to 17β-estradiol (E_2) and antiestrogen binding. The interaction was transcriptionally productive only in response to E_2, and was eliminated by point or deletion mutations that destroy AF-1 or AF-2 activity or E_2 binding. Our results suggest a definitive mechanistic role for E_2 in the activity of ER—namely, to alter receptor conformation to promote an association of the amino- and carboxyl-terminal regions, leading to transcriptional synergism between AF-1 and AF-2. The productive reassembly of two portions of ER expressed in cells as separate polypeptides demonstrates the evolutionarily conserved modular structural and functional organization of the nuclear hormone receptors. The ligand-dependent interaction of the two AF-containing regions of ER allows for the assembly of a complete activation function from two distinct regions within the same protein, providing a mechanism for hormonally regulated transcription.**

The actions of estrogenic hormones are mediated through the estrogen receptor (ER), a member of a large superfamily of nuclear receptors that function as ligand-activated transcription factors (1). These receptors for steroid and thyroid hormones, vitamin D, and retinoic acid share a conserved structural and functional organization, which includes separable domains for ligand binding, DNA binding, and transcriptional activation (1–3). Like other members of the nuclear hormone receptor superfamily, ER has two transcriptional activation functions (AFs), one in its amino-terminal region (AF-1) and the second in its carboxyl-terminal, ligand-binding region (AF-2) (4–6). These two regions flank the DNA-binding domain of the receptor (see Fig. 1*A*). In most promoter contexts, synergism between AF-1 and AF-2 is required for full receptor activity (6, 7); however, the underlying mechanism of this synergism is not known. Although previous studies have demonstrated conformational changes in the ER ligand-binding domain in response to ligand binding (8, 9), the role of these ligand-induced changes in the biochemistry of ER activity has not been fully determined. It is generally thought that they free the receptor from inhibitory proteins or place it in a conformation suitable for productive interaction with the transcriptional machinery (8, 9). In the present study, we utilize an assay conceptually analogous to the two-hybrid assay originally described by Fields and coworkers (10, 11) to show that the binding of estrogen promotes a transcriptionally productive functional interaction between the amino- and carboxyl-terminal regions of ER. Our results suggest that this ligand-dependent interaction results from the association of the amino- and carboxyl-terminal regions of ER and is the underlying biochemical basis for the transcriptional synergism between AF-1 and AF-2.

MATERIALS AND METHODS

Plasmid Constructions. The ER expression vectors used in this study are derivatives of pCMV4-hER (12) and pCMV5-hER (13). The amino acids present in each of the ER derivatives expressed from the vectors are listed in Fig. 1*A*. pCMV-hER(ABCD) was constructed by release of the *Xba* I fragment from pCMV5-hER, followed by religation. pCMV-hER(CD) was constructed by release of the *Xba* I fragment from pCMV-hER(109-595), an expression vector for a mutant ER lacking residues 1–108 (14), followed by religation. pCMV-hER(EF) was made by release of the *Eag* I fragment from pCMV-hER(41–595), an expression vector for a mutant ER lacking residues 1–40, followed by religation. pCMV-hER(G521R) was made by site-directed mutagenesis of pCMV4-hER. Ligand binding and transfection assays were performed to confirm that the human ER G521R mutation is functionally homologous to the previously described mouse ER G525R mutation (15). pCMV-hER(EFG521R) and pCMV-hER(EFL540Q) were made by replacing the *Eag* I–*Bsm* I or *Eag* I–*Bam*HI fragment of pCMV-hER(EF) with the *Eag* I–*Bsm* I fragments from pCMV4-hER(G521R) or the *Eag* I–*Bam*HI fragment of pCMV5-hER(L540Q) (16), respectively. pCMV-hER(EF)-VP16, pCMV-hER(EFG521R)-VP16, and pCMV-hER(EFL540Q)-VP16 were made by replacing the *Bsm* I–*Bam*HI fragments of pCMV-hER(EF), pCMV-hER(EFG521R), and pCMV-hER(EFL540Q), respectively, with a PCR-generated fragment encoding the carboxyl-terminal 78 aa of herpes simplex virus protein VP16 (17) via *Bsm* I- and *Bam*HI-compatible ends.

The reporter vectors 3ERE-pS2-CAT and 4ERE-PR_{Dist}-CAT were made by cloning double-stranded ERE oligomers into the *Bam*HI sites of pS2-CAT (14) and PR_{Dist}-CAT (18),

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Abbreviations: AF, activation function; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; TOT, *trans*-hydroxytamoxifen.

†Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92093.
‡W.L.K. and E.M.M. contributed equally to this work.

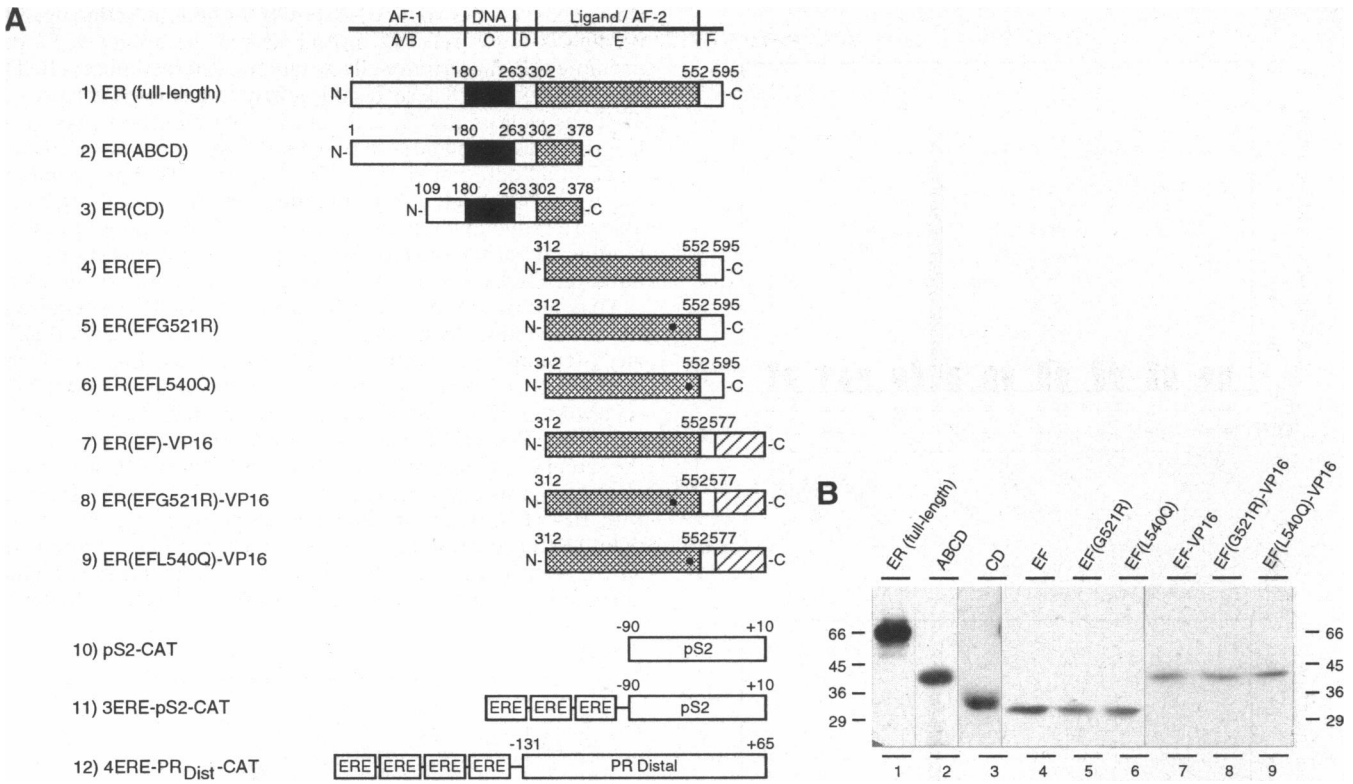

FIG. 1. Structure and expression of ER derivatives used in this study. (*A*) The structural domains of ER (A/B, C, D, E, and F), as well as the AF-1, AF-2, DNA-binding (solid boxes), and ligand-binding (cross-hatched boxes) functional domains, are shown above the schematics for the receptors. Hatched boxes represent the VP16 activation domain (residues 413–490). Schematics of the pS2 and the PR_{Dist} promoter–chloramphenicol acetyltransferase (CAT) reporter genes are also shown. ERE, estrogen response element. (*B*) The appropriate expression of the ER derivatives from cytomegalovirus (CMV) promoter-based expression vectors was tested by transient transfection into COS-1 cells, followed by immunoblotting with the anti-ER monoclonal antibodies H226 (lanes 1–3) and H222 (lanes 4–9). The sizes of the molecular weight markers in kilodaltons are indicated.

respectively, as described (18). The β-galactosidase expression plasmid pCMVβ, used as an internal control to normalize for transfection efficiency, was from Clontech. Plasmid pTZ19R, used as carrier DNA, was from Pharmacia.

Cell Culture, Transfections, and CAT Assays. ER-negative human breast cancer MDA-MB-231 cells (19) and ER-negative 3T3 mouse fibroblasts were maintained and transfected as described (13, 20). Each 100-mm-diameter dish of cells received calcium phosphate/DNA crystals containing 1.5 μg of each ER expression vector, 10 μg of reporter vector, 800 ng of pCMVβ, and pTZ19R to give 15 μg of total DNA. After transfection, the cells were then treated with vehicle, 17β-estradiol (E_2, 10 nM), and/or the antiestrogen *trans*-hydroxytamoxifen (TOT; 1 μM) as indicated for 24 hr. CAT activity, normalized for β-galactosidase expression from the internal control plasmid, was determined as described (21).

Immunoblot Assays. The appropriate expression of the ER derivatives from the CMV promoter-based expression vectors was tested by transient transfection into COS-1 and MDA-MB-231 cells (12, 20). Extracts from the cells were then analyzed by immunoblotting with the anti-ER monoclonal antibodies H226 or H222 (22).

RESULTS

Although many structure/function analyses of ER have been described, a clear picture of the biochemical basis for the ligand-dependent events leading to receptor activation, including transcriptional synergism between AF-1 and AF-2, has not emerged. Since many functional interactions between proteins involve a physical association of those proteins, we hypothesized that protein–protein interactions between the amino-terminal (AF-1-containing) and carboxyl-terminal (AF-2-containing) regions of ER might underlie the transcriptional synergism between AF-1 and AF-2. To test this, we expressed truncated human ERs containing either the amino-terminal A/B and DNA-binding domains (ABCD) or the carboxyl-terminal, ligand-binding domain (EF; see Fig. 1) individually or together in MDA-MB-231 ER-negative breast cancer cells (MDA-231 cells). We then assayed for ligand-induced activity from a reporter construct containing three EREs linked upstream of the promoter from the estrogen-responsive human pS2 gene and the CAT reporter gene (3ERE-pS2-CAT; see Fig. 1*A*). Conceptually, this assay is similar to the two-hybrid system described by Fields and coworkers (10, 11), in which the noncovalent physical association of a pair of interacting proteins is used to tether an activation function to a DNA binding site adjacent to an inducible promoter in a reporter gene.

When expressed individually, neither ABCD, which has intact DNA-binding and AF-1 activities but no ligand-binding activity, nor EF, which has intact ligand-binding and AF-2 activities but no DNA-binding activity, could activate transcription from the reporter in response to treatment with E_2 (Fig. 2*A*). However, when ABCD and EF were coexpressed, the two separate polypeptides were capable of interacting in an E_2-dependent manner to reconstitute the intrinsic transcriptional activity of ER, causing an 11-fold induction of CAT activity (Fig. 2*A*). To test the generality of these observations, we also examined the transcriptional activity of these ER fragments in a different cell line and with a different promoter—namely, 3T3 mouse fibroblast cells and a CAT reporter construct containing EREs linked upstream of the rat progesterone receptor gene distal promoter. Very similar results

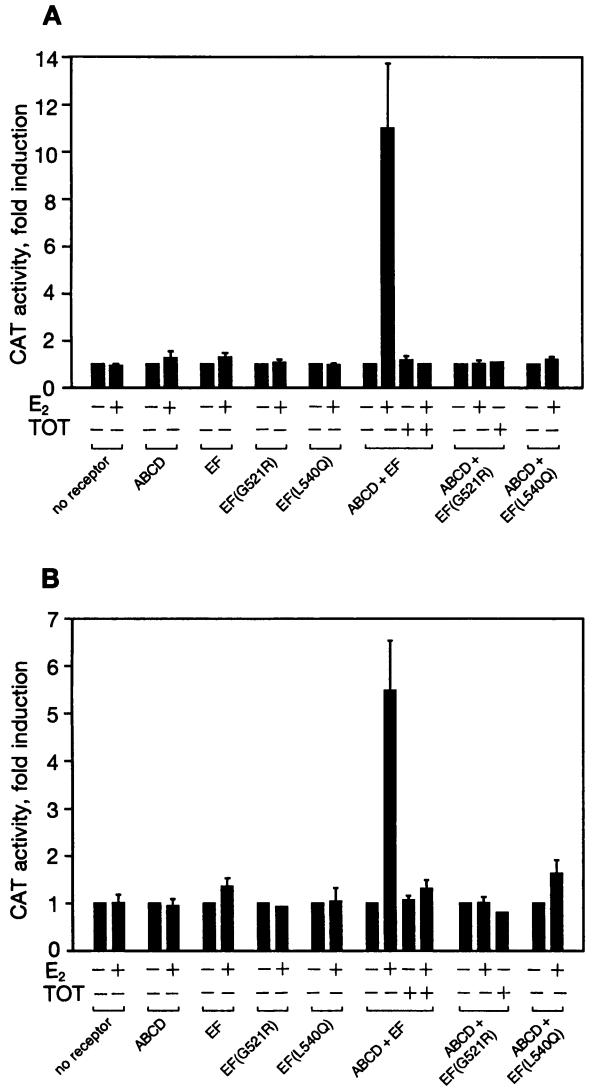

FIG. 2. Ligand-dependent, transcriptionally productive interaction of the amino- and carboxyl-terminal regions of ER. ER-negative MDA-231 (*A*) and ER-negative 3T3 (*B*) cells were transfected with expression vectors for the ER derivatives listed and 3ERE-pS2-CAT (*A*) or 4ERE-PR_{Dist}-CAT (*B*). They were then treated with vehicle, E_2 (10 nM), and/or TOT (1 μM) as indicated for 24 hr. CAT activity, normalized for β-galactosidase expression from an internal control plasmid, was determined as described. Each bar represents the mean ± SEM for three or more determinations. Some error bars are too small to be visible.

were obtained (Fig. 2*B*). In both cell types, cotransfection of ABCD and EF with subsequent E_2 treatment resulted in 10–20% of the transcriptional activity of full-length ER assayed under the same conditions. However, in these experiments ABCD and EF were expressed to only approximately 40% and 10% the levels of full-length ER, respectively (Fig. 1*B* and data not shown). From other studies in which we have transfected different amounts of expression plasmids encoding full-length ER or ABCD plus EF into MDA-231 cells, we find that expression of ABCD plus EF at levels comparable to that of intact ER gives transcriptional activity approximately one-third to one-half that achieved with intact ER.

The functional interaction of ABCD and EF leading to the reconstitution of transcriptional activity was not promoted by the antiestrogen TOT (Fig. 2). Furthermore, TOT blocked the stimulatory effect of E_2 (Fig. 2). A point mutation in EF that selectively destroys E_2 binding without affecting TOT binding or AF-2 activity (G521R; ref. 15), as well as a point mutation that selectively destroys AF-2 activity without affecting ligand binding (L540Q; ref. 16), also abolished the ability of E_2 to promote the transcriptionally productive interaction of ABCD and EF (Fig. 2). These results mirror the known effects of TOT, as well as the G521R and L540Q mutations, on full-length ER assayed under similar conditions (refs. 15, 16, 20, 22, and 23 and data not shown). The failure of TOT to promote the transcriptionally productive interaction was not attributable to a reduction in the expression of EF in response to TOT binding (data not shown). Similarly, the point mutations did not alter the expression of EF (Fig. 1*B* and data not shown).

To demonstrate that the A/B region of ABCD was required for transcriptionally productive interaction with liganded EF, an N-terminal truncation of ABCD (CD; see Fig. 1) which lacks the first 108 residues was used in the assay in MDA-231 cells with an EF-VP16 activation domain fusion protein (EF-VP16; see Fig. 1). EF-VP16 was used in place of EF to make the screening system more sensitive, since the VP16 activation domain is transcriptionally potent (24). ABCD cotransfected with EF-VP16 was more active in response to E_2 treatment than ABCD cotransfected with EF (compare Fig. 2*A* and Fig. 3*A*). EF-VP16 was incapable, however, of interacting with CD in response to E_2 treatment, as shown by the lack of activity in

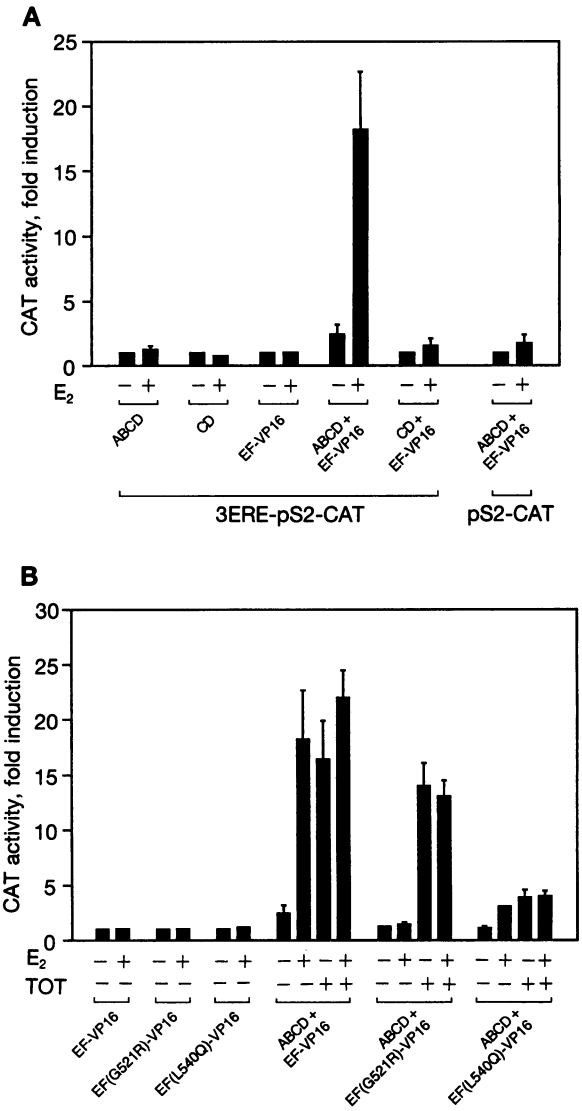

FIG. 3. Determinants for the transcriptionally productive interaction of the amino- and carboxyl-terminal regions of ER. MDA-231 cells were transfected and treated and CAT activity was analyzed as described in the legend to Fig. 2.

the assay (Fig. 3*A*). These results demonstrate that the ability of ABCD and EF to productively interact requires the N-terminal (AF-1-containing) region of ABCD. No signal was observed in response to E_2 treatment when ABCD and EF-VP16 were tested with pS2-CAT, a reporter lacking EREs, demonstrating the need for a specific ER binding site in the reporter (Fig. 3*A*).

The results from the original assay described above (Fig. 2) showed that the ligand-dependent, transcriptionally productive interaction of ABCD and EF was abolished by TOT treatment, as well as by point mutations that destroy E_2-binding (G521R) or AF-2 activity (L540Q). However, these results did not address whether transcriptionally unproductive interactions between ABCD and EF could occur in the presence of the antiestrogen or with the point mutations. Again, we used EF-VP16, since the presence of the VP16 activation domain, which is constitutively active (24), eliminated the need for ligand-dependent transcriptional synergism between AF-1 and AF-2 for a signal to be observed in the assay. Thus, by comparing the results obtained for ABCD plus EF (Fig. 2) with those obtained for ABCD plus EF-VP16 (Fig. 3*B*), we could determine under what conditions interactions between ABCD and EF occurred, even if the interaction of AF-1 and AF-2 was itself transcriptionally unproductive.

By using this approach, we showed that ABCD and EF could interact in the presence of TOT, but the interaction was not intrinsically transcriptionally productive (Figs. 2 and 3*B*). Similar results were observed in response to TOT with the G521R mutation, which destroys E_2 binding but not TOT binding; however, no interaction was observed in response to E_2 with this mutant (Figs. 2 and 3*B*). This result highlights the ligand dependence of the interaction between ABCD and EF, and suggests that although antiestrogens such as TOT can place the receptor in a conformation that promotes the interaction of these two regions, the interaction is transcriptionally unproductive. With the L540Q mutation, which destroys the AF-2 activity of ER, little or no ligand-dependent interaction (transcriptionally productive or unproductive) was observed (Figs. 2 and 3*B*). Thus, an intact AF-2 is required not only for transcriptional activity, but also for the efficient association of ABCD and EF.

DISCUSSION

Our results suggest a definitive role for E_2 binding and the conformational changes that follow in the activity of ER—namely, to promote the transcriptionally productive association of the amino- and carboxyl-terminal regions of the receptor. Furthermore, our results suggest that this ligand-dependent association is the underlying biochemical basis for synergism between the different AFs contained within these two regions of ER. Our findings are consistent with an association, either direct or indirect, of the amino- and carboxyl-terminal regions of ER. This interpretation is supported by the fact that EF lacks most of the sequences necessary for efficient nuclear localization but does contain a sequence that can cooperate with nuclear localization sequences present in ABCD (25). Thus, in our assay, a physical association of EF with ABCD may promote nuclear localization, as well as tether the non-DNA-binding EF near the promoter. We cannot, however, completely rule out independent interaction of ABCD and EF with the basal transcriptional machinery or ligand-induced squelching of an ABCD-masking factor by EF, although they seem less likely possibilities. Nonetheless, our results show an important functional interaction between the amino- and carboxyl-terminal regions of ER. This assay does not distinguish between a direct interaction of ABCD and EF and one that involves additional intermediary proteins. Since ligand-bound ER has been shown to interact with other proteins that are potential modulators or mediators of its activity (26–28), it is likely that an interaction between the amino- and carboxyl-terminal regions would involve additional factors (see model, Fig. 4).

Our observations for ER may be broadly applicable to a range of transcriptional activators that contain more than one AF, such as other members of the nuclear hormone receptor superfamily (1–3). In Fig. 4, we have modeled our results in the context of the full-length receptor. Unliganded ER (shown) and mutant ERs incapable of binding ligand exhibit no association of the amino- and carboxyl-terminal regions and are transcriptionally inactive (Fig. 4*A*). Estrogen-occupied ER exhibits ligand-induced conformational changes in the ligand-binding domain (8, 9), leading to the association of the amino- and carboxyl-terminal regions and subsequent synergism between AF-1 and AF-2 (Fig. 4*B*). The interaction may involve additional intermediary proteins, and this possibility is shown (Fig. 4*B Right*). Antiestrogen-occupied ER exhibits ligand-induced conformational changes in the ligand-binding domain that are distinct from those induced by estrogen (8, 9) and that lead to the association of the amino- and carboxyl-terminal regions but do not promote synergism between AF-1 and AF-2 (Fig. 4*C*). Again, the interaction may involve additional intermediary proteins (Fig. 4*C Right*). ER AF-2 mutants (Fig. 4*D*) and AF-1 mutants (Fig. 4*E*) exhibit ligand-induced conformational changes in the ligand-binding domain but show no association of the amino- and carboxyl-terminal regions and are transcriptionally inactive.

The productive reassembly of two portions of ER expressed in cells as separate polypeptides resembles the phenomenon of α-complementation of β-galactosidase (29), in which enzymatic activity can be reconstituted from two fragments of this

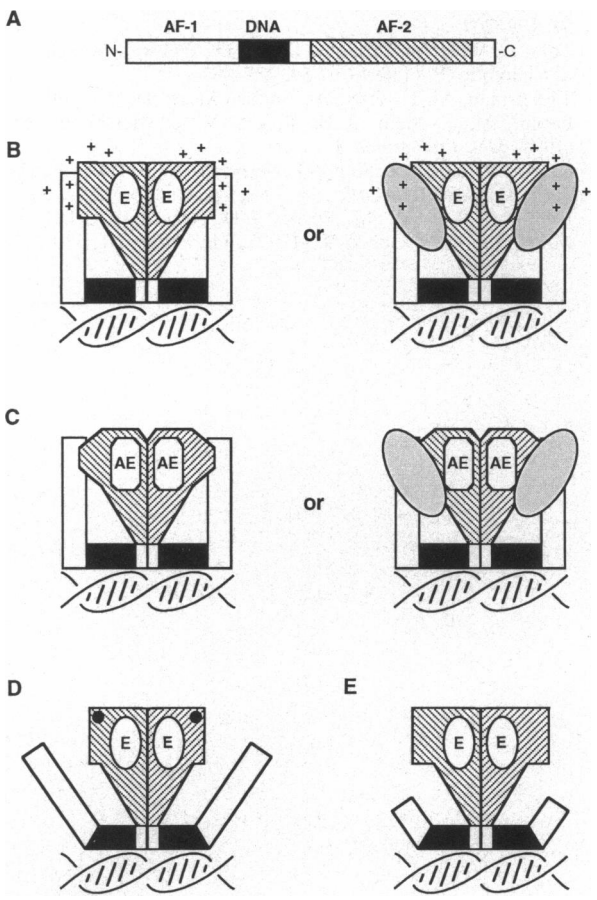

FIG. 4. Model for the ligand-dependent, transcriptionally productive interaction of the amino- and carboxyl-terminal regions of ER. A description of the model is provided in *Discussion*.

protein. Our reconstitution of ER transcriptional activity by coexpression of the amino- and carboxyl-terminal portions of this protein highlights the evolutionarily conserved modular structural and functional organization of nuclear hormone receptors (1–3). Perhaps the ligand-binding and regulatory functions of the carboxyl-terminal region were distinct from the activational function of the amino-terminal region at some point in the evolution of these receptors. These physically separate functional domains may have been analogous to other activational/catalytic and regulatory pairings such as NF-κB and IκB, an activation system in which the DNA-binding/activation functions and the regulatory/inhibitory functions are in two separate but interacting proteins (30), or the catalytic and regulatory subunits of enzymes such as protein kinase A (31, 32). The linking of these two functional domains as in the nuclear receptors provides a mechanism for the ligand-dependent assembly of a complete activation function from two distinct regions within the same protein, yielding yet another level of control in the complex process of regulated transcription.

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1. Evans, R. M. (1988) *Science* **240,** 889–895.
2. Gronemeyer, H. (1991) *Annu. Rev. Genet.* **25,** 89–123.
3. Tsai, M.-J. & O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486.
4. Webster, N. J. G., Green, S., Jin, J. R. & Chambon, P. (1988) *Cell* **54,** 199–207.
5. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E. & Chambon, P. (1989) *Cell* **59,** 477–487.
6. Tzukerman, M. T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M. G., Stein, R. B., Pike, J. W. & McDonnell, D. P. (1994) *Mol. Endocrinol.* **8,** 21–30.
7. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R. & Chambon, P. (1987) *Cell* **51,** 941–951.
8. Fritsch, M., Leary, C. M., Furlow, J. D., Ahrens, H., Schuh, T. J., Mueller, G. C. & Gorski, J. (1992) *Biochemistry* **31,** 5303–5311.
9. Beekman, J. M., Allan, G. F., Tsai, S. Y., Tsai, M.-J. & O'Malley, B. W. (1993) *Mol. Endocrinol.* **7,** 1266–1274.
10. Fields, S. & Song, O.-K. (1989) *Nature (London)* **340,** 245–246.
11. Chien, C. T., Bartel, P. L., Sternglanz, R. & Fields, S. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9578–9582.
12. Reese, J. C. & Katzenellenbogen, B. S. (1991) *Nucleic Acids Res.* **19,** 6595–6602.
13. Kraus, W. L., Montano, M. M. & Katzenellenbogen, B. S. (1993) *Mol. Endocrinol.* **8,** 952–969.
14. Kraus, W. L., Weis, K. E. & Katzenellenbogen, B. S. (1995) *Mol. Cell. Biol.* **15,** 1847–1857.
15. Danielian, P. S., White, R., Hoare, S. A., Fawell, S. E. & Parker, M. G. (1993) *Mol. Endocrinol.* **7,** 232–240.
16. Wrenn, C. K. & Katzenellenbogen, B. S. (1993) *J. Biol. Chem.* **268,** 24089–24098.
17. Triezenberg, S. J., LaMarco, K. L. & McKnight, S. L. (1988) *Genes Dev.* **2,** 730–742.
18. Kraus, W. L., Montano, M. M. & Katzenellenbogen, B. S. (1993) *Mol. Endocrinol.* **7,** 1603–1616.
19. Horwitz, K. B., Zava, D. T., Jensen, E. M. & McGuire, W. L. (1978) *Cancer Res.* **38,** 2434–2437.
20. Montano, M. M., Müller, V., Trobaugh, A. & Katzenellenbogen, B. S. (1995) *Mol. Endocrinol.* **9,** 814–825.
21. Reese, J. C. & Katzenellenbogen, B. S (1991) *J. Biol. Chem.* **266,** 10880–10887.
22. Wrenn, C. K. & Katzenellenbogen, B. S. (1990) *Mol. Endocrinol.* **4,** 1647–1654.
23. Jordan, V. C. & Murphy, C. S. (1990) *Endocr. Rev.* **11,** 578–610.
24. Sadowski, I., Ma, J., Triezenberg, S. & Ptashne, M. (1988) *Nature (London)* **335,** 563–564.
25. Ylikomi, T., Bocquel, M. T., Berry, M., Gronemeyer, H. & Chambon, P. (1992) *EMBO J.* **11,** 3681–3694.
26. Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C. & Brown, M. (1994) *Science* **264,** 1455–1458.
27. Cavailles, V., Dauvois, S., Danielian, P. S. & Parker, M. G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10009–10013.
28. Le Douarin, B., Zechel, C., Garnier, J., Lutz, Y., Tora, L., Pierrat, B., Heery, D., Gronemeyer, H., Chambon, P. & Losson, R. (1995) *EMBO J.* **14,** 2020–2033.
29. Ullman, A. & Perrin, D. (1970) in *The Lactose Operon*, eds. Beckwith, J. & Zipser, D. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 403–420.
30. Beg, A. A. & Baldwin, A. S., Jr. (1993) *Genes Dev.* **7,** 2064–2070.
31. Taylor, S. S. (1987) *Bioessays* **7,** 24–29.
32. Hardie, G. (1988) *Nature (London)* **335,** 592–593.