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PRINCIPAL INVESTIGATOR: Aliccia Bollig
Richard J. Miksicek, Ph.D.

CONTRACTING ORGANIZATION: Michigan State University
East Lansing, Michigan 48824-1046

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Introduction

Analysis of messenger RNA (mRNA) prepared from a variety of estrogen responsive cells and tissues has established that estrogen receptor- α (ER α) mRNA is typically expressed as a mixture of transcripts (1-4). This heterogeneity results largely from a pattern of alternative mRNA splicing that gives rise to a family of correctly processed and exon-skipped ER α mRNAs. Although there is no consistent ratio of relative expression, wild-type and variant ER transcripts are always coexpressed in ER-positive tumor cell lines and normal and tumorous breast tissue. Quantitation of individual variants shows that they generally represent a minority of ER mRNA; however, as a population, splicing variants typically constitute as much as 50% of the total ER mRNA in the tissues and cell lines examined (1,2,4). While there has been extensive analysis at the RNA level of the pattern of expression and abundance of ER α splicing variants, limited information is available on their functional activity. In a recent report (*Molecular Endocrinology* 14: 634-649, May 2000) I describe results from biochemical analysis of six ER α splicing variants that arise by the deletion of one of its internal exons (ER Δ E2 through ER Δ E7, where the deleted exon is indicated numerically). In agreement with the functional organization of wild-type ER α (wt ER α), the loss of a particular exon results in the loss of activity ascribed to that exon (5). The deletion of an exon is also observed to disrupt activities attributed to other exons and to bestow novel function on the receptor isoform (5). Although most appear to be functionally impaired, analysis of receptor isoforms demonstrates that individual variants do retain wt ER α function with regard to protein-protein interaction, DNA binding, ligand binding and cellular localization. However, none of the variants effectively promotes gene expression from a consensus estrogen response element (ERE) (5). The traditional view of estrogen action involves hormone binding by ER to elicit a conformational change in the receptor allowing it to dimerize, bind DNA at an ERE and activate target genes (6-8). Recently a novel pathway for regulation of transcription by ER has been described that involves cooperation of the receptor with AP-1 factors (e.g., c-Jun and c-Fos) (5,9,10). An important distinction of this non-classical pathway for ER action is that functional domains within the receptor that are crucial for transactivation of an ERE appear to be dispensable for ER α activity on AP-1 directed promoters (5,9,10). This suggests a potential role for ER α splicing variants in regulation of non-classical EREs. Studies to investigate the activity of ER α variants on estrogen responsive promoters that lack a consensus ERE indicate that, depending on the promoter context, some of the splicing variants have the capacity to regulate gene expression (5, and A. Bollig and R.J. Miksicek, unpublished data). In addition to providing evidence that specific ER α splicing variants are transcriptionally active, results from studies assessing the activity of variant receptors (which can be viewed as deletion mutants of wt ER α) on noncanonical EREs may also offer insight into the mechanism of wt ER α activity in non-classical (AP-1 directed) pathways.

Summary of Research and Training Accomplishments

For the past year, the general focus of my research has been to investigate the role of ER α splicing variants in regulating gene transcription. Initial work indicated that ER α variants are ineffective at promoting reporter gene expression from an ERE; however, two of the splicing variants, ER Δ E3 and ER Δ E5, were observed to positively regulate gene expression from AP-1 dependent promoters that lack an ERE. In order to characterize the transactivation function of ER α splicing variants, many of my efforts have been concentrated on identifying responsive genes using a cell culture transfection system to test candidate promoters. With this same approach, I have also assessed ligand and AP-1 activator requirements for transactivation. With the evolution of my research, greater attention is being given to questions regarding the potential mechanisms of transactivation by ER α variants. In addition to further studies to determine which promoters are activated by the variants, I have completed *in vitro* protein binding studies to determine if the ER Δ E3 and ER Δ E5 variants retain the

ability to dimerize with wt ER α and if coactivators can interact with these variants, and thereby potentially effect their activity.

Identification of promoters activated by ER α splicing variants and assessment of AP-1 activation and hormone requirement

As reported, cDNA expression vectors containing the single exon-skipped ER α variants ER Δ E2 through ER Δ E7 were constructed to enable their functional characterization in a well defined cell transfection system (5). Each of these plasmids efficiently expresses a stable variant protein in Cos7 cells, as determined by western analysis, with a molecular weight consistent with its predicted translational reading frame (5). Expression vectors for wt ER α and ER α mRNA splicing variants were transiently coexpressed in HeLa cells with reporter gene constructs driven by the promoter of interest. Candidate promoters predicted to be part of a non-classical estrogen response pathway were selected based on information in the literature describing them to be regulated by AP-1 and ER α without having a consensus ERE. With this criteria I have identified four genes that are regulated by one or both of the wt ER α variants, ER Δ E3 and ER Δ E5. Expression of a reporter gene driven by the chicken ovalbumin promoter is induced by ER Δ E5 in cells treated with phorbol 12-myristate, 13-acetate (PMA, a phorbol ester that stimulates AP-1 via a direct activation of protein kinase C) and by ER Δ E3 and wt ER α with 17 β -estradiol (E₂) and PMA co-treatment (ref. 5 and Appendix A, fig. 7). Overexpression of cJun enhanced wt ER α , ER Δ E3 and ER Δ E5 activity (ref. 5 and Appendix A, fig. 8). Estrogen regulation of the ovalbumin promoter has been mapped to a critical AP-1 enhancer motif (9).

The human collagenase promoter also harbors an AP-1 element described to direct E₂ regulated gene expression (10). A short region of the collagenase promoter (-73 to +63 relative to transcription start site) containing the critical AP-1 motif is

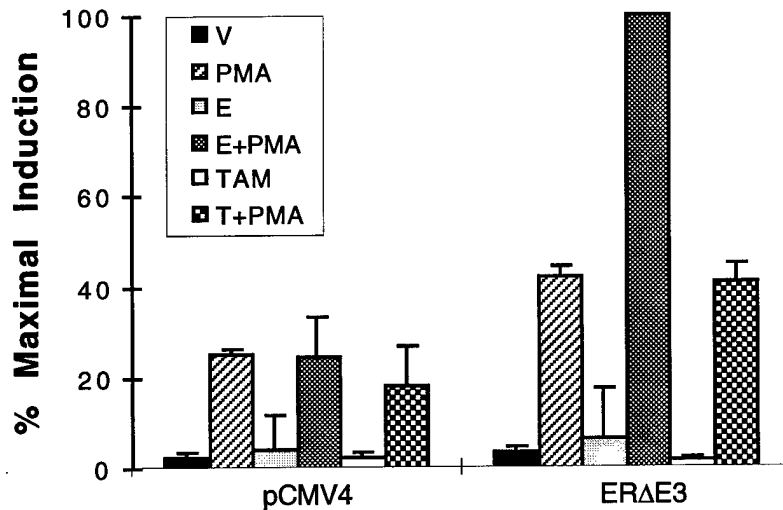


FIGURE 1. Treatment Breakdown for the Activation of Coll73-Luc by ER Δ E3
Maximal induction of the collagenase promoter by ER Δ E3 requires the combined presence of PMA and E₂. The promoter demonstrates a modest response to PMA that is enhanced with cotransfected ER Δ E3. Tamoxifen treatment does not support transactivation of the reporter gene. Values are expressed as a percentage of maximum induction (ER Δ E3, E+PMA). Error bars represent the standard error of the mean of three independent experiments.

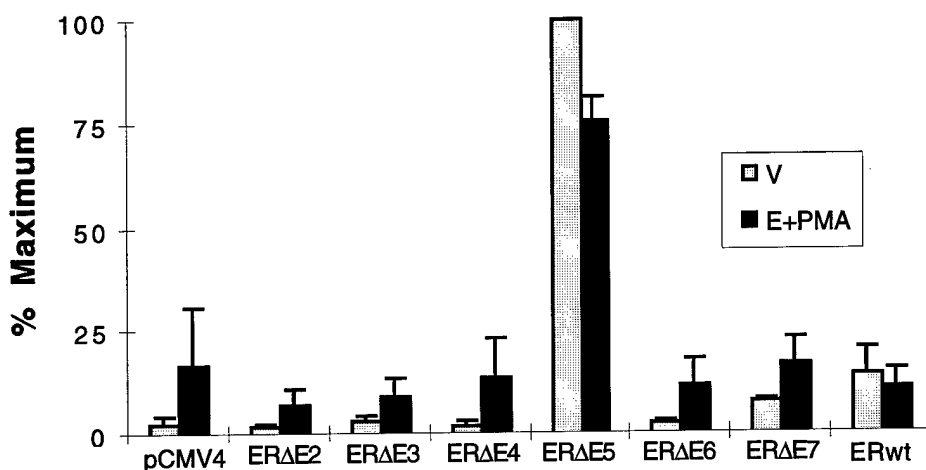


FIGURE 2. Cotransfection of wt ER α and ER α Splicing Variants with IGF-1(1630)Luc. A luciferase reporter gene construct driven by a region of the human IGF-1 promoter (-1630 relative to transcription start site) is transactivated with cotransfection of ER Δ E5. ER Δ E5 activity is not enhanced with PMA treatment. However, the promoter is modestly responsive to PMA in the absence of co-transfected receptor (pCMV4). Values are expressed as a percentage of maximum induction (ER Δ E5, vehicle). Error bars represent the standard error of the mean of 3 independent experiments.

activated by ER Δ E5 and E₂-liganded ER Δ E3 in the presence of PMA (see original proposal). In contrast to an earlier report from another laboratory (10), in our hands wt ER α demonstrates no activity on this promoter. Further analysis indicates that ER Δ E3 activation of the collagenase promoter requires PMA and E₂ co-treatment (fig. 1). Results from reporter gene experiments using these promoters provide compelling evidence that two of the ER α mRNA splicing variants, specifically ER Δ E3 and ER Δ E5, can exert positive effects on gene transcription. Furthermore, although E₂ regulation for both the collagenase and the ovalbumin promoter has been mapped to an AP-1 motif, the difference in the relative strength of activation by the various receptor isoforms suggests that the context of the AP-1 element, (i.e., the flanking sequence) may confer a difference in the mechanism and pattern of regulation, as well as the structural requirements for ER α .

Another promoter that has attracted my attention is the human IGF-1 promoter. Reporter gene expression from the human IGF-1 promoter (IGF-1(1630)Luc) is induced by PMA treatment (fig. 2). The promoter does not respond to wt ER α and is constitutively induced by ER Δ E5 (fig. 2). It is unclear where ER Δ E5 regulation is directed in the sequence of the IGF-1 promoter, since it lacks an ERE or consensus AP-1 element (11). Efforts to transfer ER Δ E5 responsiveness onto a heterologous promoter have been unsuccessful, showing that this effect requires the context of the promoter. In a further attempt to localize the ER Δ E5 response element, studies are planned to test ER Δ E5 activation of truncated and short deletions of the promoter.

The TGF β 3 gene is another potential target for non-classical transcriptional regulation by ER α and/or ER α splicing variants. Notably, the TGF β 3 promoter does not have an ERE, yet TGF β 3 gene expression is reportedly regulated by ER (12). A related report describes the presence of an unusual anti-estrogen (raloxifene) response element in this promoter that is active in MG63 osteosarcoma cells (13). In preliminary cotransfection experiments, PMA treatment induced E₂-bound wt ER α and ER Δ E3 transactivation of the TGF β 3 promoter (data not shown). For this reason, I plan to include the TGF β 3 reporter in future studies.

Dimerization and Coactivator Binding Properties of ER Δ E3 and ER Δ E5

None of the ER α mRNA splicing variants effectively activate an ERE, however, ER Δ E3 and ER Δ E5 are observed to influence ERE-directed transcription by disrupting wt ER α transactivation when they are coexpressed in reporter gene experiments (5,14,15). Results from *in vitro* binding assays suggest that a direct interaction between the variants and factors responsible for ERE-directed transcription might explain the inhibitory effects of ER Δ E3 and ER Δ E5 on wt ER α activity. Specifically, I tested for a direct interaction of ER Δ E3 and ER Δ E5 with wt ER α and steroid receptor coactivator-1e (SRC-1e). The C-terminus of wt ER α and fragments of the SRC-1e protein were expressed as fusion proteins with glutathione S-transferase (GST) and attached to glutathione-Sepharose beads. Binding assays with GST fused to the C-terminus of wt ER α and ³⁵S-methionine labeled *in vitro* translated receptor demonstrate that ligand-bound wt ER α and ER Δ E3, but not ER Δ E5, dimerize with the ligand-binding domain of ER α in solution (ref. 5 and Appendix A, fig. 6A). In experiments with GST-SRC-1e fragments, both ER Δ E3 and ER Δ E5 were observed to bind to regions of the coactivator that also bind wt ER α (ref. 5 and Appendix A, fig. 6B).

Training and Academic Progress

In addition to planning and executing the experiments described above, my training activities include attending a weekly Molecular Biology Journal Discussion Group in which participants present and discuss recent journal articles with relevance to their research. I have also presented my data and findings to the Michigan State University department of Physiology during an annual Research Forum. I recently addressed a meeting of my Research Advisory Committee where I presented data and conclusions as well as leading a discussion about my preliminary findings and explained my progress, and the future experiments I am planning. This year I also completed work on a manuscript that was published in the journal Molecular Endocrinology (ref. 5 and Appendix A). Prior to receiving the U.S. Army BCRP Fellowship in June 1999, I successfully completed my comprehensive examinations and defended my thesis proposal and was named a candidate for Ph.D. in the Michigan State University Department of Physiology. Presently I am in my fifth year of graduate training and am in good academic standing.

Research Accomplishments

- Analyzed the biochemical behavior of ER α splicing variants
- Completed protein interaction studies between variants and the nuclear receptor coactivator, SRC-1e
- Initiated transfection experiments to assess the effect of ER variants on gene expression from the chicken ovalbumin, human collagenase, IGF-1 and TGF β 3 promoters.

Reportable Outcomes

- Publications:

Bollig A, Miksicek RJ 2000 An estrogen receptor- α splicing variant mediates both positive and negative effects on gene transcription. *Mol Endocrinol* 14:634-649

- Presentation of data and findings at a Michigan State University Department of Physiology Research Forum (January, 2000)
- Conducted a review of the progress of my research and graduate education at an annual Research Advisory Committee meeting (May, 2000)

Conclusions

- ER Δ E3 dimerizes with wt ER α
- Similar to wt ER α , ER Δ E3 and ER Δ E5 bind SRC-1e fragments
- c-Jun cooperates with ER Δ E3 and wt ER α to activate the chicken ovalbumin promoter
- ER Δ E5 constitutively activates the human IGF-1 promoter. The IGF-1 promoter is not induced by wt ER α in parallel reporter gene studies.
- Activation of a hormone responsive human collagenase promoter fragment by ER Δ E3 in transfected cells requires dual AP-1 activation by PMA and E₂ treatment.

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An Estrogen Receptor- α Splicing Variant Mediates Both Positive and Negative Effects on Gene Transcription

Aliccia Bollig and Richard J. Miksicek

Michigan State University
Department of Physiology
East Lansing, Michigan 48824-1101

Analysis of mRNA prepared from a variety of estrogen-responsive cell lines, breast tumor specimens, and normal breast tissue have established that estrogen receptor- α (ER α) mRNA is typically expressed as a mixture of transcripts. Using PCR amplification, this heterogeneity has been shown to result largely from an imprecise pattern of mRNA splicing that gives rise to a family of correctly processed and exon-skipped ER α transcripts. We have reconstructed ER α cDNAs representing the single exon-skipped variants ER Δ E2 through ER Δ E7 to enable their functional characterization in a well defined cell transfection system. All six of the ER α splicing variants support the efficient expression of stable proteins in Cos7 cells, and each shows a characteristic pattern of subcellular distribution. Each of the variants displays a dramatic reduction in DNA-binding activity with a consensus estrogen response element (ERE) in an *in vitro* gel mobility shift assay. While this DNA-binding defect appears to be complete for ER Δ E2, ER Δ E3, ER Δ E4, and ER Δ E6, weak DNA binding is observed for ER Δ E5 and ER Δ E7. Scatchard analysis of hormone binding demonstrates that among the variants, only ER Δ E3 binds 17 β -estradiol (E₂) and does so with an affinity similar to wild-type ER α (wt ER α). Individual variants cotransfected with the pERE-TK-CAT reporter plasmid [a consensus ERE-driven chloramphenicol acetyltransferase (CAT) reporter gene that is highly responsive to E₂-liganded wt ER α] were ineffective at inducing CAT expression in ER-negative HeLa cells. Only ER Δ E5 showed indications of positive transcriptional activity on the pERE-TK-CAT reporter, but this activity was limited to approximately 5% of the activity of wt ER α . When variants were expressed simultaneously with wt ER α , ER Δ E3 and ER Δ E5 were observed to have a dominant negative effect on wt ER α transcriptional activity. Like the wild-type receptor, both ER Δ E3 and ER Δ E5 interact with steroid receptor coactivator-1e

(SRC-1e) *in vitro*; however, only ER Δ E3 retained the ability to dimerize with wt ER α . Transcription from a region of the ovalbumin promoter, which contains an ERE half-site and an AP-1 motif, is positively regulated by liganded wt ER α and ER Δ E3 in phorbol ester-treated, transiently transfected HeLa cells. In both cases, this activity was enhanced by cotransfected cJun. These observations suggest that selected ER α splicing variants are likely to exert important transcriptional effects, especially on genes that are regulated by nonconsensus EREs and subject to complex hormonal control. (*Molecular Endocrinology* 14: 634-649, 2000)

INTRODUCTION

Binding of estrogen to the estrogen receptor (ER) elicits a change in receptor conformation that allows the receptor to bind DNA and enhance transcription from the promoters of regulated genes (1-3). ER-induced gene expression supports the proliferation and, ultimately, the differentiation of target cells (4, 5). Interference with these proliferative effects forms the basis for the chemotherapeutic actions of estrogen antagonists that are used to treat cancers of the breast and reproductive tract (6). The reported success of antiestrogens such as tamoxifen and raloxifene in preventing breast tumors emphasizes a crucial role for ER in mammary carcinogenesis (7-9).

The transcriptional effects of estrogens are mediated by two closely related receptor isoforms, ER α and the more recently described ER β (10, 11), each of which is encoded by a separate gene. While ER β is also being investigated for its potential role in various diseases, including cancer, this study focuses solely on the ER α isoform. Analysis of mRNA prepared from a variety of estrogen-responsive cells and tissues, including breast tumors, has established that ER α mRNA is typically expressed as a mixture of transcripts (12-15). This heterogeneity results largely from a pattern of alternative mRNA splicing that gives rise to a family of correctly pro-

cessed and exon-skipped ER α mRNAs. ER α mRNA comprises sequences from 8 coding exons and is translated to yield a protein with discrete functional domains. An N-terminal transactivation function (AF1) encoded by exon 1 and a portion of exon 2 is thought to promote gene transcription by interacting with nuclear receptor coactivators and also with proteins integral to the transcription initiation complex (1, 16, 17). Derived from exons 2 and 3 is a centrally located zinc-finger motif (commonly referred to as the DNA-binding domain or DBD) that is essential for sequence-specific DNA binding and transcriptional activation through canonical estrogen response elements (EREs) (18). Within the region encoded by exon 4 are the nuclear localization signals (NLS) and a hinge region that allows for receptor conformational flexibility (3, 19). A ligand-binding domain (LBD) confers regulatory function to the receptor and is encoded by the C-terminal exons 4 through 8 (20). This region also includes determinants for subunit dimerization and a well characterized C-terminal transactivation function (AF2), which promotes gene transcription by recruiting coactivators (1-3, 21). Like other nuclear receptors, ER α is a modular protein in that individual domains are capable of demonstrating autonomous function within receptor mutants, as well as when they are introduced into heterologous fusion proteins (1, 18). It can reasonably be assumed that the exclusion of a particular exon will predictably result in a protein lacking the function ascribed to that exon. Additionally, it is probable that the loss of a particular exon will result in unpredictable functional deficits or perhaps even bestow a novel function on the variant receptor. This study examines the function of ER α splicing variants from the vantage point of what is known about the functional organization of wt ER α . Concurrently, the process of examining splicing variants, like mutational studies, improves our understanding of wt ER α function. We report results from experiments designed to assess receptor capacity to translocate to the nucleus, bind to DNA, bind ligand, participate in protein complexes, and promote gene transcription.

Fuqua and colleagues (22, 23) have reported that ER Δ E5 (which contains the AF1 domain, but lacks AF2 and the regulatory functions imparted by the LBD), is constitutively active in promoting transcription from an ERE in a heterologous yeast reporter gene assay. These authors have also described that overexpression of ER Δ E5 in a stably transfected breast cancer cell line (MCF-7) supported greater proliferation compared with control cells, as well as imparting a tamoxifen-resistant phenotype (24). In the human osteosarcoma cell line U2-OS, it has recently been reported that coexpression of ER Δ E5 significantly enhances ERE-directed reporter gene expression induced by wt ER α (25). The existence of a constitutively active receptor variant (such as ER Δ E5) able to exert a mitogenic effect in breast tumor cells in the absence of E $_2$

or in the presence of tamoxifen is an appealing explanation for the acquisition of antiestrogen resistance observed in previously responsive tumors and cell lines (26, 27). However, this model is challenged by conflicting observations that ER Δ E5 and closely related, genetically engineered ER α mutants do not efficiently induce transcription from an ERE reporter in transiently transfected ER-negative HeLa or CEF cells (2, 28), or promote proliferation in stably transfected MCF-7 cells (28).

Recently, a novel mechanism for mediation of an estrogen response has been reported to involve AP-1-directed regulation of transcription by ER (29-32). AP-1 describes the fos/jun family of transcription factors that play a key role in transducing the effects of growth factors to regulate cell proliferation (33, 34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements (5'-TGAG/CTCA-3'), with or without a degenerate ERE or ERE half-site (5'-GGTCA-3' or 5'-TGACC-3'). Examples of such genes include ovalbumin, which is induced by E $_2$ in chicken oviduct cells (35), and the insulin-like growth factor-I (IGF-I) gene whose expression is stimulated by E $_2$ in the uterus of ovariectomized-hypophysectomized rats and in cultured rat osteoblast cells (36, 37). An AP-1 enhancer motif identified in the chicken IGF-I promoter is essential for E $_2$ and phorbol ester-stimulated gene transcription (31). Phorbol esters act directly on protein kinase C to initiate a signal transduction cascade that ultimately activates AP-1 (33). Reporter gene cotransfection studies with expression vectors for AP-1 isoforms and ER α in HeLa cells indicate that a similar mechanism regulates the human collagenase promoter (32). The minimal region of the collagenase promoter reported to be responsive to tamoxifen-liganded wt ER α , and to a variety of ER α mutants, harbors a critical AP-1 element and lacks a consensus ERE. Additionally, the activity of ER α on the collagenase promoter was enhanced with AP-1 (*c-jun* or *c-fos*) overexpression (32). Further evidence that ER regulation converges with AP-1-directed gene transcription is provided by results from protein binding assays indicating that *c-jun* is able to bind to wt ER α *in vitro* (32).

Although evidence for function of ER α variants has been elusive, reports that ER Δ E5 can support weak, cell type-dependent activity (23, 25, 28), and that, when tested on an ERE, both ER Δ E5 and ER Δ E3 are dominant negative receptor forms in the presence of wt ER α (38, 39) indicate that it is inaccurate to label these variants as transcriptionally inert. To investigate the capacity for ER α splicing variants to regulate gene transcription, we have expanded our transcriptional focus to include the noncanonical ERE of the ovalbumin promoter in addition to the consensus vitellogenin A2 ERE. Here we present data indicating that individual variants display both similarities and differences compared with wt ER α , and that selected splicing variants (specifically ER Δ E3 and ER Δ E5) have the ca-

capacity to both positively and negatively regulate gene expression, depending on the promoter context.

RESULTS

Numerous variant ER α cDNAs have now been cloned and sequenced from breast tumors and established tumor cell lines (12–15). The most common variants harbor a precise deletion of one of the internal exons from the eight that contribute to the structure of the mature ER α protein, suggesting that they arise as a result of imprecise splicing of the primary ER α mRNA transcript. ER α cDNAs with sequence deletions corresponding to exons 2, 3, 4, 5, and 7 have been identified, along with a large number of more complex variants (12–15). These basic variants will be referred to as ER Δ E2 through ER Δ E7, where the deleted exon is indicated numerically. Although there is no consistent ratio of relative expression, wild-type and variant ER transcripts are always coexpressed in ER-positive tumor cell lines and normal and tumorous breast tissue. Quantitation of individual variants shows that they generally represent a minority of ER mRNA; however, as a population, splicing variants typically constitute as much as 50% of the total ER mRNA in the tissues and cell lines examined (Refs. 12, 13, and 15 and D. P. Ankrapp and R. J. Miksicek, unpublished observations). While there has been extensive analysis at the RNA level of the pattern of expression and abundance of ER α splicing variants, limited information is available on their functional activity. We have therefore constructed cytomegalovirus (CMV) promoter-driven ER α cDNA expression vectors representing the exon-skipped variants ER Δ E2 through ER Δ E7 to enable their functional characterization in a well defined cell transfection system. Our assembled pool of ER α splicing variants also includes the hypothetical receptor ER Δ E6, even though this variant is not readily identified *in vivo*. Figure 1A diagrams the ER α mRNA splicing variants examined, showing the positions of deleted exons and their consequences with respect to protein structure. Deletion of exon 2, 5, 6, or 7 all cause a frame-shift mutation resulting in premature termination of translation, thereby generating a diverse class of C-terminally truncated receptor forms. Omission of either exon 3 or 4 does not disrupt the mRNA reading frame, but produces a receptor protein with an internal deletion.

Transient expression in Cos7 cells demonstrates that each of these variants translates to a stable protein able to accumulate to readily detectable levels within transfected cells (Fig. 1B). Based on immunoblot analysis with an N-terminal monoclonal antibody (Mab-17), which recognizes an epitope within exon 1 common to all of the variants (40), we observe that the mobility of the six variant proteins is consistent with their predicted molecular weights. No immunoreactivity is observed in mock transfected cells, confirming the specificity of the Mab-17 antibody.

Measurement of the DNA-Binding Activity of the ER α Splicing Variants

Efficient DNA binding by ER α requires the cooperation of several functional elements within this protein, including the centrally located DBD and a ligand-inducible subunit dimerization motif located near the C-terminal end of the LBD (2, 18). It is also possible that additional subunit contacts occur elsewhere in the protein. Because all of the ER α splicing variants sustained deletions within various regions of this protein, it was of interest to systematically assess the DNA-binding ability of each variant. For this purpose, gel mobility shift assays were performed using extracts prepared from E₂-treated, transiently transfected Cos7 cells. Extracts were incubated with a ³²P-labeled oligonucleotide containing a consensus ERE (AGGTCACAGTGACCT) from the *Xenopus* vitellogenin A2 promoter. As expected, variants that harbor a mutation within the DBD (ER Δ E2 and ER Δ E3) are completely unable to recognize the ERE (Fig. 2, lanes 5–8). Less predictably, the loss of exons contributing to the LBD also result in a strong defect in ERE recognition (Fig. 2, lanes 9–16). For ER Δ E5 and ER Δ E7, however, this appears to be a quantitative defect in DNA binding. The addition of the monoclonal antibody, Mab-17, to the binding reactions consistently results in the recovery of weak DNA binding by ER Δ E5 (Fig. 2, lane 12). Presumably, the role of the bivalent antibody is to stabilize the interaction of receptor subunits with their palindromic binding site, mimicking the function of the missing dimerization motif present within the C terminus of the LBD. These results suggest the possible existence of cell-specific constituents that perform the same function *in vivo* and may account for the variable activity of ER Δ E5 and related constructs in different cell types (2, 25, 28). We have also observed the formation of a complex between ER Δ E7 and labeled ERE probe (Fig. 2, lane 16, and data not shown). Overall, however, the relative weakness of DNA-binding observed in these studies raises serious questions about the extent to which any of the variants, including ER Δ E5 and ER Δ E7, are able to recognize and bind to a consensus ERE, *in vivo*. Furthermore, that ER Δ E7 binds an ERE *in vitro* has little transcriptional relevance in light of the observation that ER Δ E7 is not translocated to the nucleus when expressed in Cos 7 cells (see below).

ER Δ E3, Like wt ER α , Binds Ligand

To test the ability of the ER α mRNA splicing variants to bind hormone, we performed a saturation binding assay on whole-cell extracts from Cos7 cells transiently transfected with wt ER α or the ER α variants. Only wt ER α and ER Δ E3 were able to bind ³H-labeled E₂, whereas all of the remaining variants demonstrated no specific ligand binding (Fig. 3A). The individual deletion of exons 2, 4, 5, 6, and 7 effectively eliminates all, or a significant portion, of the LBD (see Fig. 1A), consistent with their loss of hormone binding. We confirmed these results using an *in vivo* ligand-binding

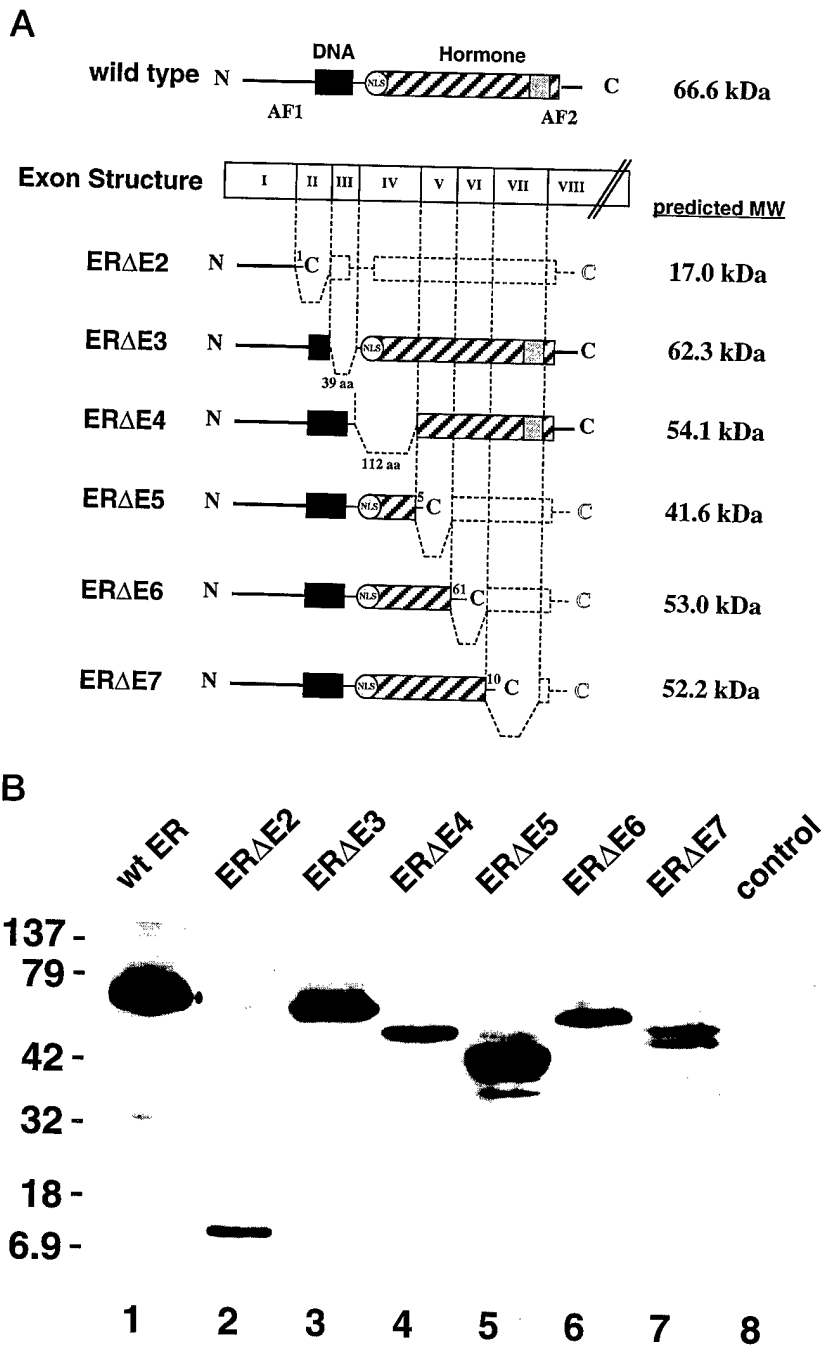


Fig. 1. Comparison of ER α mRNA Splicing Variants and wt ER α Structure

Panel A depicts the various functional domains of the receptor and the exon sequences from which they are derived. The variants are referred to by deleted exon. The size and mol wt of each variant are predicted from the translational reading frame of the sequenced cDNA clones. *Dashed lines* indicate regions of the major open reading frame of the full-length ER α protein that are missing from each variant. The nuclear localization signal is *circled*. The regions encompassing the DNA- and hormone-binding domains are marked by *darkened and hatched boxes*, respectively, where they are expressed. The AF-1 and AF-2 domains are indicated where they reside within the N and C termini, respectively, of the wild-type receptor. B, Immunoblot analysis indicates that the mol wt of each variant is consistent with its predicted translational reading frame. Samples containing 20 μ g of protein from extracts of transfected Cos 7 cells were analyzed on a 10% SDS-polyacrylamide gel probed with the ER α -specific antibody, Mab-17. The figure is representative of three independent transfection experiments.

assay in which the binding of a fluorescent estrogen analog was visualized in cells cultured on cover slips. Cos7 cells transiently expressing the individual vari-

ants or wt receptor were treated with the fluorescent ligand, nitrile tetrahydrochrysen (nitrile THC) (41). Only those cultures transfected with wt ER α or ER Δ E3

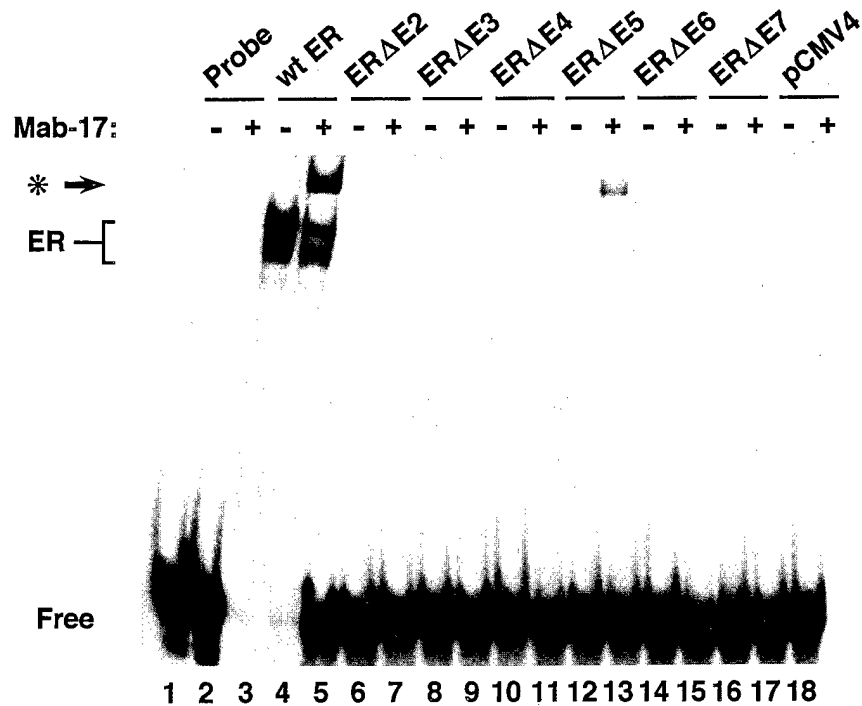


Fig. 2. Gel Mobility Shift Assay to Assess the DNA-Binding Activity of ER α mRNA Splicing Variants

A gel mobility shift assay of lysates from E₂-treated, transiently transfected Cos7 cells confirms that wt ER α binds efficiently to a ³²P-labeled consensus ERE. Confirmation that the indicated band represents an authentic ER α /DNA complex is provided by the ability of an ER α -specific monoclonal antibody (Mab-17) to supershift this complex (compare lane 4 with lane 3). In contrast, all of the splicing variants display strong defects in DNA binding. In this assay, which is representative of three equivalent and independent experiments, weak binding of ER Δ E5 and ER Δ E7 to the ERE can only be observed when their respective DNA complexes are stabilized by the addition of the ER α -specific antibody (lanes 12 and 16). Position of the antibody-supershifted complex is indicated by an asterisk.

were observed to stain with this ligand. In both cases, staining was localized tightly within the nucleus. This suggests that among the variants examined, wt ER α and ER Δ E3 exclusively bind ligand and in both cases, ligand-bound receptors are translocated normally to the nucleus of expressing cells (Fig. 3B). With Scatchard analysis we compared the affinity of ER Δ E3 and wt ER α for ³H-labeled E₂. The measured dissociation constants were 0.66 nM for wt ER α and 0.79 nM for ER Δ E3 (Fig. 3C).

Subcellular Localization of ER α Splicing Variants

To more carefully assess the subcellular localization of ER α splicing variants, including those that fail to bind ligand, Cos7 cells were transiently transfected with expression vectors encoding wt ER α or individual variants. These receptors were detected in transfected cells by indirect immunofluorescence staining (using the MAb-17 monoclonal antibody) and confocal microscopy. Similar to wt ER α , ER Δ E3 and ER Δ E5 localize to the nuclei of transfected cells, although ER Δ E5 showed perinuclear as well as nuclear staining (Fig. 4). These results are consistent with the fact that both ER Δ E3 and ER Δ E5 retain a NLS immediately downstream of the DBD (3, 19).

Subcellular localization studies have also been completed for the exon 2, 4, 6, and 7 deletion variants. Each of these proteins can be readily detected in transfected cells, but they all possess dramatic defects in nuclear targeting (Fig. 4). Nuclear targeting of wt ER α is governed, in large part, by a tripartite karyophilic signal present within exon 4 (19). Loss of this signal is therefore consistent with the cytoplasmic pattern of distribution of mutants such as ER Δ E2 and ER Δ E4, both of which lack protein corresponding to exon 4 sequences. Inappropriate presentation or folding of this signal must account for the defects in nuclear localization seen with ER Δ E6 and ER Δ E7, since the NLS is retained in these variants. Based on their subcellular distribution, we would predict that only ER Δ E3 and ER Δ E5, like wt ER α , would have the potential to exert nuclear effects, such as modulating gene transcription. Furthermore, the inability of the cytoplasmic variants ER Δ E2, ER Δ E4, ER Δ E6, and ER Δ E7 to dimerize with wt ER α predicts that their subcellular distribution will not be influenced by coexpression with the nuclear isoforms (wt ER α , ER Δ E3, and ER Δ E5). For ER Δ E4, this was confirmed with a cotransport assay using a dimerization-competent ER α (data not

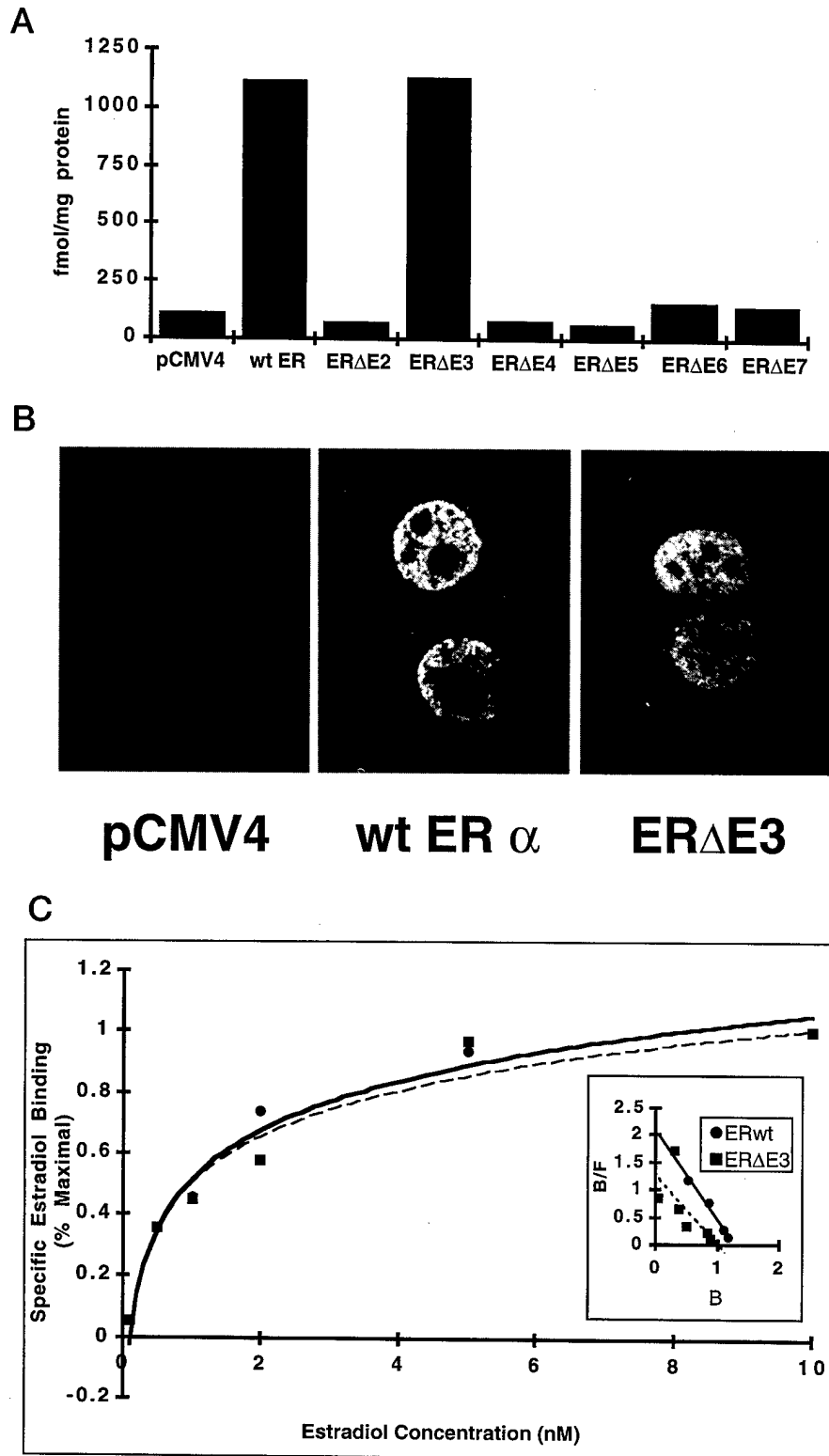


Fig. 3. Ligand Binding of ER α and ER α mRNA Splicing Variants

A, Ligand-binding capacity was assessed by measuring the specific association of 10 nM ^3H -17 β -E $_2$ with wt ER α and its splicing variants expressed in Cos7 cells. Only wt ER α and the ER Δ E3 variant demonstrate specific binding of E $_2$. B, Confirming the ligand binding results, ER Δ E3 and wt ER α are the only isoforms of the receptor observed to display specific cell staining using a fluorescent estrogen analog (nitrile THC) to treat transiently transfected Cos7 cells. C, Scatchard analysis shows that wt ER α and ER Δ E3 have similar affinities for 17 β -estradiol.

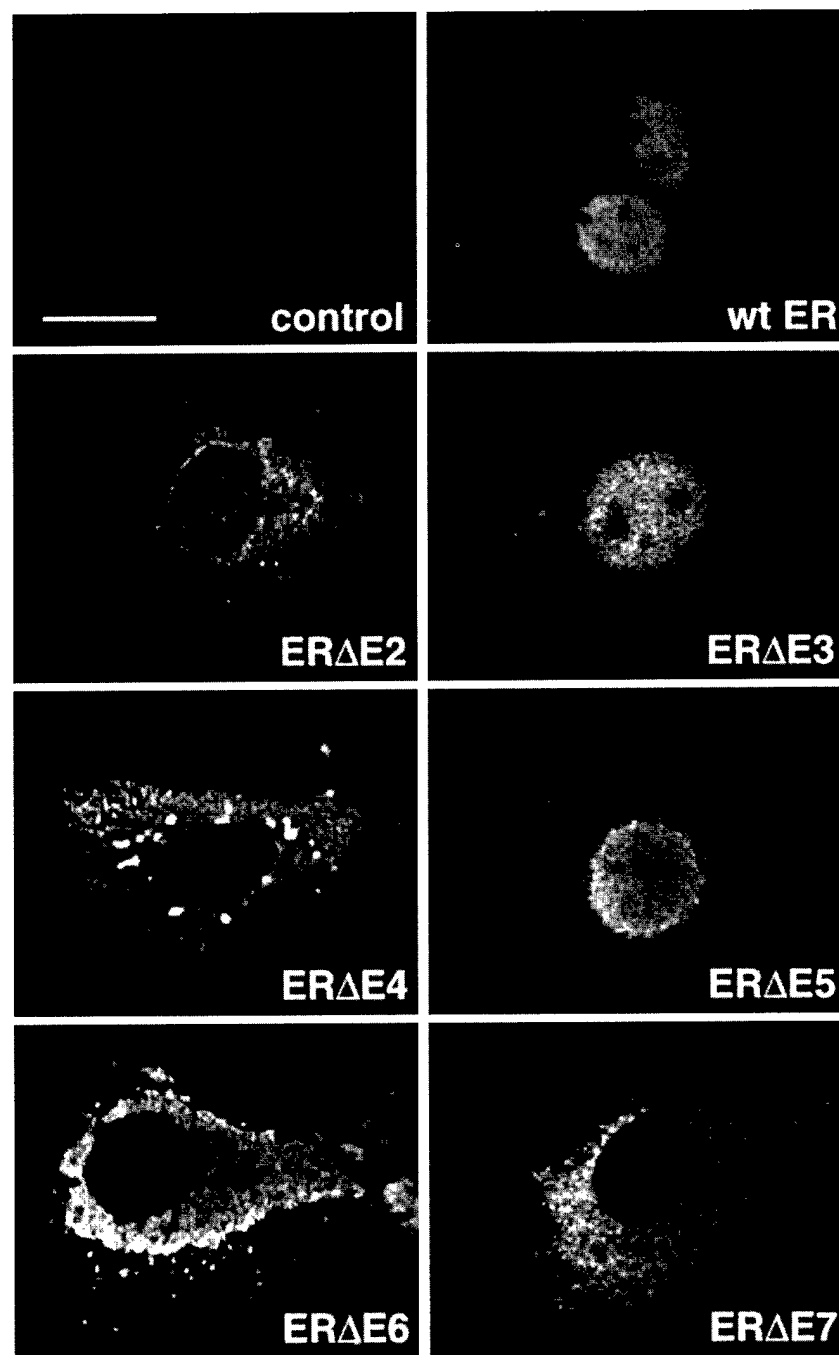


Fig. 4. Localization of ER α and ER α Splicing Variants by Confocal Microscopy

Receptor isoforms were detected by immunofluorescence staining with an ER α -specific monoclonal antibody (Mab-17) and a rhodamine-conjugated secondary antibody. The *upper left panel* shows a representative field from control cells transfected with empty expression vector (pCMV4) displaying minimal nonspecific background. Specific immunoreactivity can be observed in all other frames. Cells expressing wt ER α , ER Δ E3, and ER Δ E5 demonstrate strong nuclear staining in confocal sections. ER Δ E2, ER Δ E4, ER Δ E6, and ER Δ E7 are predominately localized to the cytoplasm. *Bar*, 10 μ m.

shown). It is worth noting, moreover, that the existence of translationally stable, cytoplasmic splicing variants such as these may provide an explanation for cytoplasmic staining that is commonly observed during immunohistochemical analysis of breast biopsy specimens to assess ER α status.

Characterization of the Transactivation Function of ER α Splicing Variants on the Vitellogenin ERE

HeLa cell cotransfection experiments designed to assess the transcriptional activity of individually expressed ER α splicing variants have failed to demon-

strate any significant ability of variant receptors to support gene activation through an ERE, with the possible exception of the ER Δ E5 variant, which is reported to display a low level of constitutive transcriptional activity on an ERE-driven reporter in some, but not all, cell types examined (23, 25, 28). In our hands none of the variants were effective transcriptional activators of an ERE-containing promoter. ER Δ E5 repeatedly showed only modest constitutive activity (~5% of wt ER α induction) on an ERE-directed reporter plasmid cotransfected into HeLa cells (see Fig. 7, inset).

It is important to recognize that the tissues and cell lines that express these variants also express wt ER α . We have previously reported that the ER Δ E3 variant acts as a dominant negative mutant when it is coexpressed with wt ER α in HeLa cells treated with E₂ (39). In the human breast epithelial cell line HMT-3522S1, ER Δ E5 has also been reported to disrupt transactivation by agonist-bound wt ER α of an ERE reporter gene (38). To clarify whether this is a function unique to these variants, we completed a series of experiments to test whether the remaining exon-skipped ER α variants also support transcriptional inhibitory effects. When examined in a HeLa cell cotransfection assay in which the expression of pERE-TK-CAT was driven by E₂-bound wt ER α , a 5-fold molar excess of any of the splicing variants lacking exons 2, 4, 6, or 7 failed to inhibit the E₂-dependent induction of chloramphenicol acetyltransferase (CAT) gene expression by intact receptor (data not shown). In agreement with previously published results, the ER Δ E3 and ER Δ E5 variants both demonstrated a dominant inhibitory activity at all molar ratios tested (Fig. 5). With the caveat that equal amounts of plasmid DNA support similar levels of variant receptor expression (see Fig. 1B), it appears that ER Δ E3 and ER Δ E5 are approximately equivalent in their inhibitory activity in HeLa cells.

Dimerization and Coactivator Binding Properties of ER Δ E3 and ER Δ E5

We next questioned whether a direct interaction between the variants and factors responsible for ERE-directed transcription might explain the inhibitory effects of ER Δ E3 and ER Δ E5 on wt ER α activity; specifically, we tested for a direct interaction of ER Δ E3 and ER Δ E5 with wt ER α and steroid receptor coactivator-1 α (SRC-1 α). The C terminus of wt ER α and fragments of the SRC-1 α protein were expressed as fusion proteins with glutathione S-transferase (GST) and attached to glutathione-Sepharose beads. Binding assays with GST fused to the C terminus of wt ER α (GST-AF2) and ³⁵S-methionine labeled *in vitro* translated receptor demonstrate that ligand-bound wt ER α and ER Δ E3, but not ER Δ E5, dimerize with the LBD of ER α in solution (Fig. 6A). In experiments with GST-SRC-1 α fragments, both ER Δ E3 and ER Δ E5 were observed to bind to regions of the coactivator that also bind wt ER α (Fig. 6B). ER Δ E3 and wt ER α bind the SRC-1 α fragments comprising amino acids 570-780

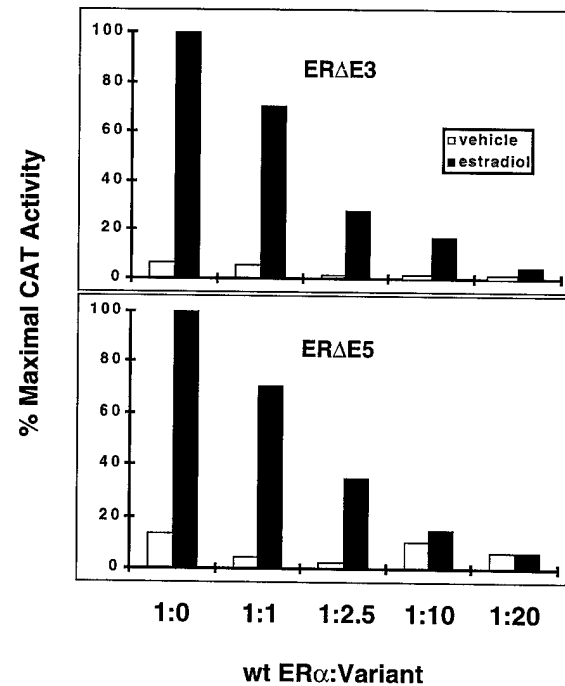


Fig. 5. ER Δ E3 and ER Δ E5 Inhibit Transactivation of a Consensus ERE Reporter by wt ER α in a Dose-Related Fashion

HeLa cells were cotransfected with 16 μ g of pERE-TK-CAT reporter gene, 1 μ g of wt ER α expression vector, and increasing amounts of expression vectors for ER Δ E3 or ER Δ E5 (from 0-20 μ g). The ratios of wt ER α to variant expression plasmid used in each transfection are indicated. The total amount of DNA in each transfection was held constant with the addition of empty expression vector, pCMV4. Reporter gene expression was normalized by measuring CAT activity in aliquots representing 100 μ g of soluble protein.

and 989-1240 and do so only in the presence of E₂. In contrast, binding of ER Δ E5 to the 989-1240 amino acid fragment is constitutive (Fig. 6B).

ER Δ E3 Is a Positive Regulator of Gene Expression on an AP-1 Reporter

The results presented above indicate that ER α splicing variants are either inactive (ER Δ E2, ER Δ E4, ER Δ E6, and ER Δ E7) or largely inhibitory (ER Δ E3 and ER Δ E5) in their effect on reporter constructs that contain a consensus ERE. Recent reports suggest that wt ER α is also able to transactivate genes whose promoters do not contain an obvious ERE. In particular are promoters for the human collagenase, chicken IGF-I and ovalbumin genes that are regulated by wt ER α and contain a critical AP-1 element (29, 31, 32). Mutational analysis revealed that the DBD was not required for ER α -dependent expression of these genes. Clearly, the mechanisms of ER α transcriptional activity and DNA targeting are complicated by these reports. We propose that to assess the transactivating potential of ER α variants, the promoter focus must be expanded to include promoters that contain noncanonical regu-

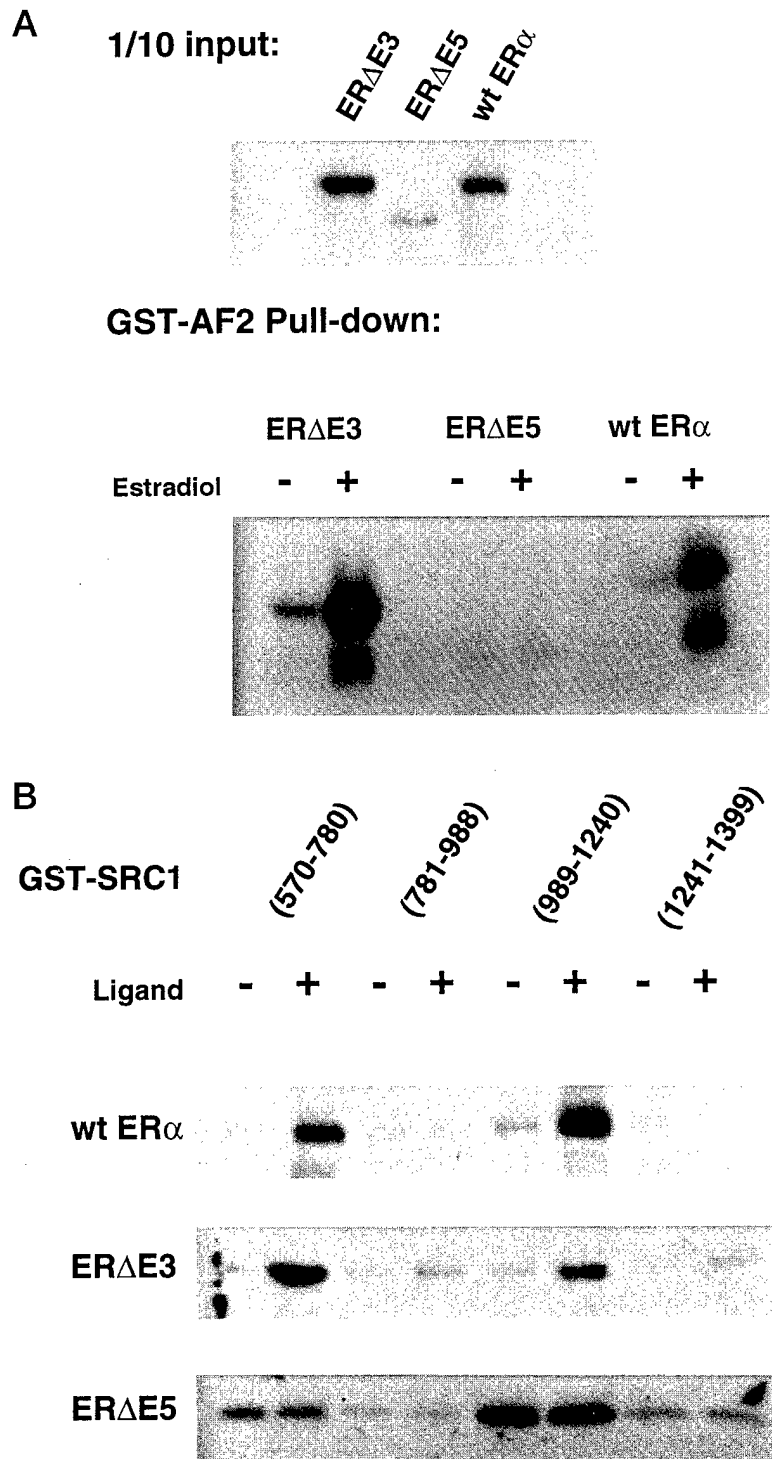


Fig. 6. *In Vitro* Binding of Radiolabeled Receptor Variants with GST-Fused wt ER α and SRC-1e Fragments

A, An autoradiograph showing *in vitro* translated, ³⁵S-methionine-labeled wt ER α and ER Δ E3 retained by the AF2 domain of wt ER α receptor fused to GST (GST-AF2) and complexed to glutathione-Sepharose beads. Dimer formation requires the presence of 2.5 μ M E₂. ER Δ E5 does not bind to the AF2 domain of ER. *Top panel* represents 10% of the radiolabeled input. B, In the presence of ligand, wt ER α and ER Δ E3 bind to SRC-1e fragments comprising amino acids 570-780 and 989-1240. The *lower panel* shows a strong constitutive interaction between radiolabeled ER Δ E5 and SRC-1e fragment (989-1240).

latory elements. These thoughts prompted us to test the activity of the ER α variants on the ovalbumin promoter that contains a complex hormone response element. We performed cotransfection experiments in HeLa cells using vectors expressing wt ER α or the exon-skipped variants ER Δ E2 through ER Δ E7 and a CAT reporter gene construct, pOvalb-CAT, driven by a fragment of the ovalbumin promoter (-1342 to +7 relative to the transcription start site) described to encompass much of the regulatory sequence of this gene (35, 42). Results from these experiments indicate that both wt ER α and ER Δ E3 support inducible gene expression from the ovalbumin promoter (Fig. 7) and that all of the remaining single exon-skipped variants are transcriptionally inactive on this reporter construct (data not shown). For wt ER α , this corroborates previously published reports (29, 35). Maximal activity was measured in cultures treated with both phorbol 12-myristate, 13-acetate (PMA, a phorbol ester) and E₂, where a 16-fold induction was observed. Like wt ER α , ER Δ E3 reproducibly induced this reporter, despite its lack of an intact DBD. While the induction shown in Fig. 7 for ER Δ E3 (averaging 9-fold) is less than that supported by wt ER α , the activity of ER Δ E3 equaled and occasionally exceeded that of the intact receptor in several individual experiments, confirming that this variant can be a potent inducer of transcrip-

tion. In both cases cotreatment with PMA and E₂ is highly synergistic as E₂ treatment alone has no significant effect, and PMA treatment alone supports only modest induction for wt ER α (2.5-fold relative to vehicle control, $P < 0.001$). Tamoxifen treatment of wt ER α - or ER Δ E3-transfected cultures, either alone or together with phorbol ester, had no significant effect on pOvalb-CAT expression. This contrasts with the stimulatory activity of tamoxifen observed on other AP-1 containing estrogen-responsive reporter genes (32). In control cells transfected with an empty CMV expression vector, treatment with PMA yielded negligible reporter gene activity. This suggests that, in the absence of ER α , activation of endogenous AP-1 alone is not an effective inducer of transcription from the ovalbumin promoter in these cells. To confirm that wt ER α and ER Δ E3 cooperate with AP-1 factors to regulate transactivation of the ovalbumin promoter, we measured pOvalb-CAT expression in HeLa cells cotransfected with both a receptor isoform and cJun. Transcriptional activity of wt ER α and ER Δ E3 supported by PMA and E₂ cotreatment was enhanced by cJun overexpression. While the presence of endogenous AP-1 tended to obscure the synergism between cJun and wt ER α in this system, the combined effects of these transcription factors were slightly more than additive. A greater than additive activation was also

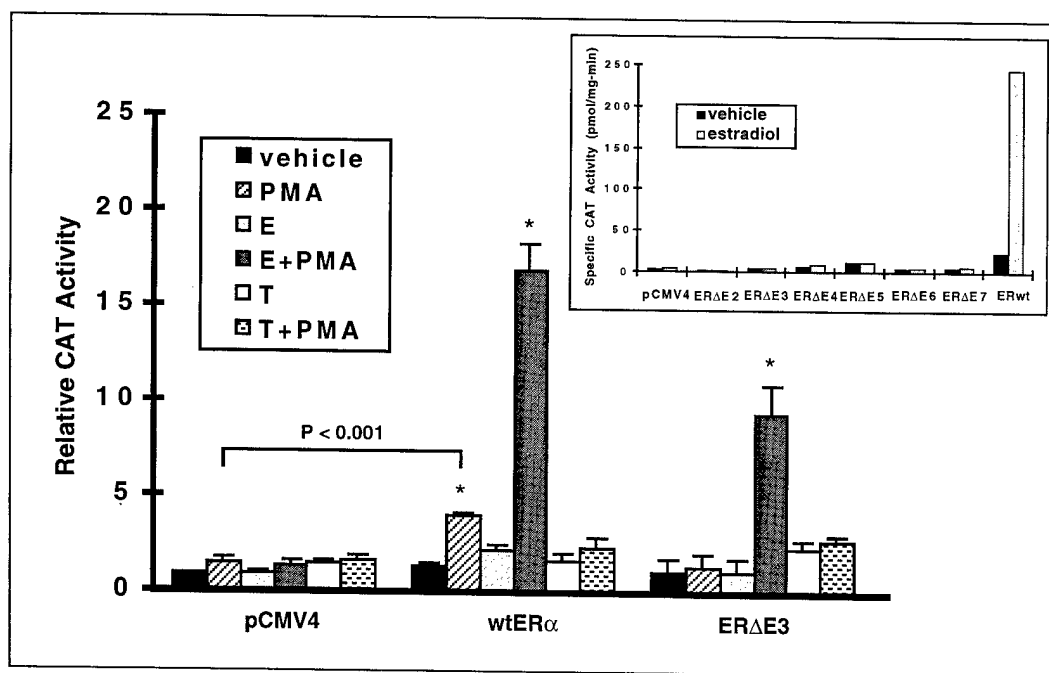


Fig. 7. Transcriptional Activity of wt ER α and Receptor Variants

Only ER Δ E3 supports CAT gene expression from the ovalbumin promoter, similar to wt ER α . HeLa cells were cotransfected with 16 μ g of reporter (pOvalb-CAT) and 0.5 μ g of the indicated ER α expression vector followed by 24 h of hormone treatment in the presence of 5% charcoal-treated calf serum. Cultures were treated as indicated: vehicle control; 2×10^{-8} M PMA; 10^{-8} M 17 β -estradiol (E); E + PMA; and 10^{-7} M tamoxifen (T) or T + PMA. CAT assays were normalized for equal amounts of protein. Values are expressed relative to vehicle-treated empty expression vector, pCMV4. Error bars represent the SEM of three independent experiments (*, $P < 0.001$). As shown in the inset, aside from wt ER α itself, none of the ER α splicing variants tested are strong activators of the ERE-driven CAT reporter gene (pERE-TK-CAT) in HeLa cells.

observed when cJun and ERΔE3 were coexpressed (Fig. 8). Exogenous cJun alone elicited only a modest response to PMA and E₂ treatment. These observations, combined with the dual requirement for activating both AP-1 and ERα, strongly suggest that these factors are acting cooperatively on the ovalbumin promoter.

DISCUSSION

Our efforts to functionally characterize exon-skipped ERα mRNA splicing variants have identified two receptor isoforms that possess the ability to modulate estrogen signaling on genes that are targeted by the ER. Although their protein structure is significantly altered, the ERΔE3 and ERΔE5 splicing variants retain many of the activities attributed to the full-length receptor. Loss of exon 3 results in a receptor protein with an internal deletion that lacks a major portion of the DBD and therefore prevents ERΔE3 from binding to a consensus ERE, as confirmed by gel mobility shift analysis. However, ERΔE3 retains the LBD and NLS, thereby allowing it to bind hormone with an affinity similar to wt ERα and translocate to the nucleus. The deletion of exon 5 causes a frame-shift mutation and results in a C-terminally truncated form of the receptor. Loss of the LBD predictably renders ERΔE5 unable to bind E₂. Nonetheless, ERΔE5 still retains the NLS, and immunofluorescence analysis shows nu-

clear staining in Cos7 cells transfected with this variant.

Rather than serving to stimulate transcription on a consensus ERE, results from transient transfection experiments in HeLa cells that combine either ERΔE3 or ERΔE5 with wt ERα and an ERE-driven reporter gene indicate that these isoforms actually function to inhibit transcriptional activation by wt ERα. These observations agree with our previous results and with those reported by others from similar experiments using HMT-3522S1 cells (38, 39). A 70% inhibition of transcriptional activation by E₂-liganded wt ERα on an ERE-driven CAT reporter gene was observed in HeLa cells when ERΔE3 and wt ERα expression vectors were cotransfected at a ratio of 5:1 (39). In the ER-negative cell line HMT-3522S1, coexpression of an equal amount of ERΔE5 significantly inhibited stimulation of an ERE reporter construct by wt ERα (38). Increasing the ratio of transfected variant to wt ERα demonstrates that the repression of wt ERα by ERΔE3 and ERΔE5 is dose-related and becomes nearly complete when the variants are present in sufficient excess (38, 39). This observation has physiological significance in the case of breast tumor cells that predominantly express one of these splicing variants (12, 22). Castles *et al.* (22) report that ERΔE5 is the major ER transcript in BT-20 and MDA MB 330 breast tumor cell lines. In BT-20 cells the ERΔE5 variant comprises 68% of the ER mRNA population while wt ERα measures 8%. Studies by Erenburg *et al.* (43) indicate that, while ERΔE3 tends to be underrepresented in breast tumors and tumor cell lines, it typically constitutes 50% or more of ERα mRNA in both stromal fibroblasts and epithelial cells isolated from reduction mammoplasty specimens. These authors further demonstrated that stable overexpression of ERΔE3 in MCF-7 cells to levels seen in normal mammary epithelial cells dramatically reduced the expression and estrogen inducibility of endogenous pS2 mRNA, as well as reducing their anchorage-independent growth and *in vivo* invasiveness (43).

The dominant negative character of ERΔE3 and ERΔE5 suggests that, like wt ERα, these variants are able to interact with at least one component of the ERE-directed transcription complex in a manner that disrupts positive gene regulation by wt ERα. Based on gel mobility shift assay analysis, it is unlikely that transcriptional interference by these variants involves binding to an ERE to the exclusion of wt ERα. Our DNA binding analysis indicates that ERΔE5 can bind only weakly to DNA, and only when the formation of this complex is stabilized by the addition of a bivalent antibody. The role of the antibody in this case is presumably to substitute for the missing dimerization interface and to tether receptor subunits together in a form more able to interact with DNA. DNA binding by ERΔE7 similarly requires the addition of antibody, but this binding is even less efficient than binding by ERΔE5. Interestingly, a correlation exists among the ERα variants between their ability to translocate to the

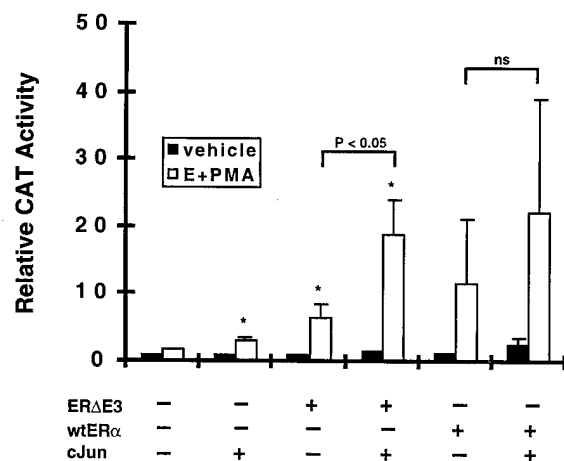


Fig. 8. Effect of cJun Overexpression on wt ERα and ERΔE3 Activation of the Ovalbumin Promoter

Cotransfection of a cJun expression vector enhances both wt ERα and ERΔE3 transactivation of the pOvalb-CAT reporter gene in HeLa cells cotreated with vehicle or 2×10^{-8} M PMA and 10^{-8} M 17β-estradiol (E + PMA). The addition of cJun increased the induced activity of the ovalbumin promoter approximately 2-fold relative to either receptor isoform alone. CAT assays were normalized for equal amounts of protein. Values are expressed relative to vehicle-treated empty expression vector, pCMV4. Error bars represent the SEM of at least four independent experiments (*, $P < 0.05$).

nucleus and their transcriptional inhibitory effect on wt ER α activity in mammalian cells. As of yet, no clear function has been established for the ER Δ E7 variant in mammalian cells, despite an earlier report that ER Δ E7 is a dominant inhibitor of wt ER α function in yeast (44). This is noteworthy since a number of quantitative studies have indicated that, as a rule, ER Δ E7 represents the most abundant of the ER α splicing variants in breast tumors (summarized in Ref. 15).

We have previously reported that although ER Δ E3 is unable to bind to an ERE itself, it can prevent wt ER α from binding to DNA (39). That ER Δ E3 inhibits both DNA complex formation and transactivation by wt ER α suggests that the potential targets of interaction by ER Δ E3 may include protein-protein contacts with wt ER α itself or interactions with nuclear receptor coactivators or other receptor-associated factors. ER α function may be disrupted when ER Δ E3, which lacks the DBD but retains the hormone-inducible dimerization domain, forms mixed dimers with wt ER α that are inefficient at binding stably to DNA. We are able to show that, in the presence of E₂, ER Δ E3 (but not ER Δ E5) can form a stable complex with the LBD of ER α fused to GST attached to glutathione-Sepharose beads. This is consistent with a model for direct inhibition of the DNA binding activity of full-length receptor by ER Δ E3. Experiments using fragments from SRC-1e fused to GST indicate that both ER Δ E3 and ER Δ E5 can bind a nuclear receptor coactivator. Similar to the pattern of wt ER α interaction with SRC-1e, *in vitro* translated ER Δ E3 is able to associate in an E₂-dependent manner with two regions of the steroid coactivator SRC-1e (amino acids 570–780 and 989–1240). This agrees with previous reports that also describe three conserved nuclear receptor-binding motifs (LXXLL) within the 570–780 amino acid region and a distinct site for AF-1 interaction within the 989–1240 amino acid fragment (17, 45). A site for SRC-1 interaction within ER α corresponds with the AF2 domain (46), a region that is retained in the ER Δ E3 variant. Isoforms of SRC-1 are potent enhancers of agonist-bound ER α and are required for its full transcriptional activity (47, 48). Transfection experiments in E₂-treated HeLa cell cultures demonstrate that coexpression of mutants containing the C terminus of ER α can attenuate ER α -dependent gene expression and that this decreased activity can be overcome with simultaneous overexpression of the SRC-1-related coactivator transcriptional intermediary factor 2 (TIF2) (49). These results suggest that coactivators are limiting factors for which the receptors are competing and that ER Δ E3, like wt ER α , is a target for SRC-1 binding.

In a surprising result from cotransfection studies using engineered mutants of ER α , maximal expression of an ERE-containing reporter gene could be observed when SRC-1 was transfected simultaneously with separate N- and C-terminal fragments of ER α , containing the AF-1/DBD and the LBD/AF2 regions, respectively (50). These results suggest that separate AF1- and AF2-containing ER α polypeptides can inter-

act in a transcriptionally productive manner, provided they are brought together by SRC-1. Furthermore, they provide an initial indication that SRC-1 interacts separately and perhaps directly with both the AF1 and AF2 domains. More support for this notion is provided by our observations that ER Δ E5 binds to the SRC-1e amino acid fragment 989–1240 in solution. These results suggest the possibility that the inhibitory function of ER Δ E5, which itself is relatively inefficient at binding DNA or activating transcription through an ERE, most likely results from competition with wt ER α for interaction with SRC-1 or other cellular factors.

The most compelling evidence that some of the ER α mRNA splicing variants may indeed be transcriptionally active is seen in transfection experiments involving ER Δ E3 and reporter gene constructs containing a non-consensus hormone-regulatory element. Recently, a novel mechanism for mediation of an estrogen response has been reported to involve AP-1-directed regulation of transcription by ER α (29–32). AP-1 and its isoforms represent a family of nearly ubiquitous transcription factors whose activity is crucial for the efficient expression of a wide variety of genes. As an important downstream target for the mitogen-activated protein kinase (MAPK)- and Jun kinase signaling cascades, AP-1 is a central player in mediating the effects of serum and growth factors on cellular proliferation (33, 34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements that often occur near a degenerate ERE or ERE half-site (29, 31, 32). It should be noted that these imperfect EREs may in some cases serve dual function as cryptic AP-1 response elements whose consensus sequence (5'-TGAG/CTCA-3') bears superficial similarity to an ERE half-site (5'-GGTCA-3' or 5'-TGACC-3').

An important observation from the analysis of genes regulated by noncanonical EREs is that the structure-activity requirements for activation by ER α (both for the ligand and for the receptor) are different than those for transcriptional activation through a palindromic ERE. Using a region of the collagenase gene promoter (-73/+63) that lacks an ERE but harbors an essential AP-1 element, Kushner and co-workers (32) demonstrated that a DBD-deleted mutant of ER α was significantly more effective at supporting E₂-induced reporter gene expression than wt ER α in transfected HeLa cells. Similar to collagenase gene expression, ER α -dependent activation of the chicken ovalbumin promoter, which lacks a palindromic ERE, does not require an intact DBD (29). Furthermore, an ERE half-site was determined to be the site for synergistic regulation of ovalbumin gene expression by AP-1 and ER α (29). Our studies involving cotransfection of an ER α splice variant with the ovalbumin promoter construct, pOvalb-CAT, agree with these findings. Results from Fig. 7 demonstrate that, compared with mock-transfected HeLa control cultures, pOvalb-CAT is strongly activated by either wt ER α or ER Δ E3. A breakdown of treatments indicates that maximal activity of

both wt ER α and ER Δ E3 clearly requires E₂ in addition to an AP-1 activator. *In vitro* assays demonstrate an interaction between cJun and the N terminus of ER α fused to GST (32). Additional evidence suggesting that ER Δ E3 and wt ER α cooperate with activated AP-1 to maximally transactivate the ovalbumin promoter is provided by our observation that receptor activity is enhanced by simultaneous cJun overexpression. In our studies tamoxifen treatment had little or no effect on the activity of wt ER α or ER Δ E3, either with or without PMA cotreatment. This contrasts with results observed when wt ER α was cotransfected with a collagenase reporter construct in HeLa cells, where tamoxifen supported a significant induction of reporter gene expression (32).

Our transfection results show that, while several of the ER α splicing variants are functionally incapacitated by their deletions, two of the variants clearly retain significant transcriptional activity. For the ER Δ E3 and ER Δ E5 variants, this activity is quite complex. Both of these variants represent stable receptor isoforms that, like the full-length receptor, localize efficiently to the nucleus where they can interact with the transcription apparatus. However, when acting through a consensus ERE, these variants completely lack (ER Δ E3) or show only weak (ER Δ E5) transcriptional stimulatory activity, consistent with their poor DNA binding ability. On the contrary, both variants serve to blunt the ability of coexpressed wt ER α to promote transcription of ERE-containing genes. At the same time, the ability of ER Δ E3 (and presumably also ER Δ E5) to interact productively with nuclear receptor coactivators or other transcription factors gives these ER α splicing variants the potential to stimulate or otherwise modulate gene expression through nonconsensus hormone response elements that are targeted by AP-1 motifs or other DNA-binding sites. We have clearly shown this to be true for ER Δ E3 and the chicken ovalbumin promoter and believe that this is also likely to be true for many other genes, such as those encoding collagenase, cathepsin D, IGF-I, transforming growth factor- β , c-fos, heat shock protein-27, and retinoic acid receptor- α , all of which lack an obvious ERE and yet still respond to estrogen. In this respect, ER α splicing variants may actually serve to redirect transcription away from ERE-containing genes to genes such as these that appear to be regulated nonclassically by estrogens.

MATERIALS AND METHODS

Expression Vectors

Plasmids for ER α mRNA splicing variant cDNAs were generated as derivatives of pCMV4 (51) and pcDNA3.1 (Invitrogen, San Diego, CA), which support high levels of receptor expression in HeLa and Cos7 cell lines (41). Plasmids expressing ER Δ E4, ER Δ E5, and ER Δ E6 were generated using synthetic oligonucleotides to construct the variant splice junctions within an otherwise wt ER α cDNA expression plas-

mid. The remaining plasmids were constructed with the use of flanking restriction sites to shuttle cloned cDNAs (39) into the appropriate expression vectors. Mouse cJun cDNA cloned into the pCMV2 expression vector was provided by L. McCabe (Michigan State University, East Lansing, MI).

Cell Culture, Transfection, and CAT Assays

Cos7 and HeLa cells were grown in phenol red-free DMEM supplemented with 10% calf-serum, 5 mM HEPES (pH 7.4), 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μ g/ml). Cells were transfected by the CaPO₄ method, as previously described (52). HeLa cells ($\sim 2 \times 10^6$ cells per 100-mm dish) were transfected with 1 μ g of the indicated ER α expression plasmid, 2 μ g of the cJun expression plasmid (where indicated) and 16 μ g of the estrogen-responsive reporter plasmid, pERE-TK-CAT (53) or pOvalb-CAT (a reporter gene construct containing -1342 to +7 bp of the chicken ovalbumin promoter relative to its transcription start site) (42). Calf thymus DNA (10 μ g) was added as carrier. After overnight incubation with DNA, culture medium was replaced with 5% charcoal-treated serum-supplemented DMEM containing the indicated hormones. After a 24-h incubation, cells were harvested and CAT assays were performed as previously described (54) using 100 μ g protein. Quantification of CAT activities was performed by phosphorimage analysis of thin layer chromatographs (ImageQuANT, Molecular Dynamics, Inc., Sunnyvale, CA). For experiments involving biochemical or cytochemical analysis of ER α variants, Cos7 cells were similarly transfected with 10 μ g of the indicated expression plasmid and 10 μ g of calf thymus carrier DNA. After overnight exposure to DNA, cells were cultured for 48 h in 10% calf serum-supplemented DMEM. All experiments involving extracts from transfected cells were normalized with respect to protein, as measured using the method of Lowry *et al.* (55). Two-way ANOVA and comparison with Student's *t* test were used to assess statistical differences between groups. Statistical significance was set at the $P < 0.05$ or $P < 0.001$ level as indicated in Figs. 7 and 8.

E₂ Binding Analysis

Ligand-binding assays were performed as previously described (40). Whole-cell extracts were prepared from transfected Cos7 cells that were resuspended and sonicated in extraction buffer (20 mM HEPES, pH 7.4, 20% glycerol, 0.4 M KCl, 1 mM MgCl₂) supplemented immediately before use with protease inhibitors (0.05 mg/ml each of chymostatin, trypsin inhibitor, antipain, leupeptin, aprotinin, and pepstatin). Aliquots containing 200 μ g of protein were incubated overnight at 4 C with various concentrations (0.1 nM–10 nM) of ³H-labeled E₂ (NEN Life Science Products, Boston, MA) in the presence or absence of a 200-fold molar excess of unlabeled E₂. Free ligand was separated from bound ligand by treatment with dextran-coated charcoal. For determination of equilibrium binding constants, these data were plotted according to the method of Scatchard (56).

DNA Binding Assays

DNA binding assays were performed as previously described (40). Aliquots containing 30 μ g of protein from extracts prepared as above from transfected Cos7 cells were preincubated for 15 min at room temperature in 10 μ l binding buffer [10 mM HEPES (pH 7.4), 1 mM MgCl₂, 1 mM dithiothreitol, and 20% glycerol] containing 1 μ g poly (dl-dC), with or without 1 μ l of added human ER-specific monoclonal antibody (Mab-17), generated as described by Neff *et al.* (40). Approximately 6 fmol (40,000 cpm) of a ³²P-labeled double-stranded ERE oligonucleotide (39) were added to the samples and incubated for 30 min at room temperature, followed by an addi-

tional 5-min incubation at 4 C. Samples were then loaded on a preelectrophoresed nondenaturing 5% polyacrylamide gel that was run in 0.5 \times Tris-Borate-EDTA at 275 V for 2 h. The gel was dried and exposed for autoradiography.

Immunoblot Analysis

Discontinuous 12% SDS-PAGE was carried out as previously described (57). After electrophoresis of 30 μ g of whole-cell protein from extracts of transfected Cos7 cells, proteins were electrophoretically transferred to nitrocellulose filters with a Trans Blot apparatus (Bio-Rad Laboratories, Inc. Richmond, CA) using the procedure of Erickson *et al.* (58). Immunoblots were probed with the ER-specific monoclonal antibody, Mab-17, obtained from a hybridoma culture supernate that was diluted with an equal volume of PBS (40). Immunoreactive protein was visualized by enhanced chemiluminescence using a horseradish peroxidase-conjugated goat antimouse IgG, following manufacturer's instructions (Amersham Pharmacia Biotech, Arlington Heights, IL).

In Vitro Protein-Protein Interaction Assays

Variant and wt ER α receptor protein was translated in the presence of [³⁵S]methionine using the TNT Coupled Reticulocyte System (Promega Corp., Madison, WI). GST-fusion proteins were expressed in the pGEX system (Pharmacia Biotech, Uppsala, Sweden) (45, 59). Overnight cultures of transformed bacteria were diluted 1:20 and cultured for 2 h before protein expression was induced with the addition of isopropyl β -D-thiogalactoside (IPTG, 0.2 mM final concentration). Bacteria were collected by centrifugation 2 h following IPTG induction, and pellets were resuspended in 400 μ l of extraction buffer supplemented with protease inhibitors. Cells were sonicated briefly, and the resulting lysates were centrifuged for 20 min at 20,000 rpm, 4 C. Protein concentrations were determined (55) and extracts were diluted to 2 μ g/ μ l extraction buffer and stored at -70 C until binding assays were performed.

Before use in protein interaction assays, 25 μ l of glutathione-Sepharose 4B beads (Pharmacia Biotech) were washed three times in 100 μ l NETN [0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris (pH 8.0) 100 mM NaCl] and suspended in 100 μ l NETN, 0.5% powdered milk. Washed beads were incubated with 40 μ g of GST-fusion protein for 2 h, rotating at room temperature. Beads complexed with GST-fusion proteins were washed three times with 100 μ l NETN, 4 C. For protein-protein interaction assays, 5 μ l of *in vitro* translated receptor were added to washed complexed beads resuspended in 100 μ l NETN supplemented with protease inhibitors (as above) with and without 2.5 μ M E₂. After a 2-h incubation during which the samples were rotated at room temperature, the beads were pelleted and washed four times with 100 μ l NETN, 4 C. Bound proteins were separated on a discontinuous 10% polyacrylamide SDS-PAGE gel (57). The gels were dried and exposed for autoradiography.

Immunohistochemical and Cytochemical Analysis

Indirect immunofluorescence analysis was performed as previously described (40) using Cos7 cells that were plated and transfected on glass cover slips. On the second day after transfection, cells were washed three times with Tris-buffered saline (TBS), fixed for 3 min in cold 95% methanol, rehydrated by three washes with TBS, and incubated 30 min at 37 C with primary antibody (Mab-17 hybridoma supernate used at a 1:10 dilution in TBS). Bound antibody was detected by staining with a rhodamine-conjugated affinity-purified goat antimouse IgG (Roche Molecular Biochemicals, Indianapolis, IN) diluted 1:2000 in TBS, and incubating for 30 min at 37 C in the presence of 0.02 μ g/ml of 4',6-diamidino-2-phenylindole di-

hydrochloride. Confocal images were recorded using the Odyssey system (Noran Instruments, Middleton, WI) on an Optiphot 2 Nikon (Melville, NY) microscope. Fluorescent ligand staining of transfected Cos7 cells was performed as described by Miksicek *et al.* (41) on live, whole-cell mounts treated in DMEM with 10⁻⁷ M nitrile THC. For these studies, cells were visualized using a Nikon UFX microscope equipped with a 100 watt mercury lamp for fluorescence excitation, and a 40 \times 0.7 numerical aperture Plan objective.

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Address requests for reprints to: Richard J. Miksicek, Department of Physiology, 108 Giltner Hall, Michigan State University, East Lansing, Michigan 48824-1101. E-mail: miksicek@msu.edu.

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