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13. ABSTRACT *(Maximum 200 Words)*

This report presents results from functional analysis of estrogen receptor-alpha (ERα) mRNA splicing variants and offers insight into the nature of their transcriptional activity. Efforts to establish a cellular transactivating role for ERα mRNA splicing variants have identified three genes, which do not contain consensus EREs in their promoters, that are regulated by one or both of the isoforms, ERΔE3 and ERΔE5. Expression of a reporter gene driven by the chicken ovalbumin promoter is induced by ERΔE5 in cells treated with PMA, and by ERΔE3 and wt ERα with E_2 and PMA cotreatment. A short region of the collagenase promoter (-73 to +63 relative to the transcription start site) containing an AP-1 motif is activated by ERΔE5 and E_2-liganded ERΔE3 in the presence of PMA. The human IGF-1 promoter is constitutively induced by ERΔE5 but is not regulated by wt ERα or ERΔE3. Overexpression of c-jun enhanced receptor activities on both the ovalbumin and collagenase promoters. It is likely that the regulation of these noncanonical promoters by wt ERα and ERα splicing variants involves protein-protein interactions with co-regulatory proteins and upstream factors that in turn act through their cognate DNA-response elements (e.g., c-jun and c-fos). *In vitro* binding studies show that wt ERα directly binds c-jun and c-fos; also, ERΔE3, ERΔE5 and wt ERα bind the steroid receptor corepressor SMRT.

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Alicia Bolling
PI - Signature

Richard J. Mrozek
Mentor - Signature

June 12, 2001
Date

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Introduction

Numerous variant ERα cDNA have now been cloned and sequenced from a variety of breast tumors and established cell lines (Castles *et al.*, 1995: Gotteland *et al.*, 1995; Miksicek *et al.*, 1993; Murphy *et al.*, 1997). 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 ERα protein, suggesting that they arise as a result of imprecise splicing of the primary ERα mRNA transcript. ERα 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 (Castles *et al.*, 1995: Gotteland *et al.*, 1995; Miksicek *et al.*, 1993; Murphy *et al.*, 1997). These basic variants are referred to as ERΔE2 through ERΔE7, where the deleted exon is indicated numerically. Although there is no consistent ratio of relative expression, wt and variant ERα transcripts are coexpressed in all ERα-positive cell lines and tissues examined (Castles et al., 1995; Gotteland *et al.*, 1995; Hu *et al.*, 1996). Quantitation of individual variants shows that they generally represent a minority of ERα mRNA; however, as a population, splicing variants typically constitute as much as 50 percent of the total ERα mRNA (Castles *et al.*, 1995; Erenburg *et al.*, 1997; Gotteland *et al.*, 1995; Murphy *et al.*, 1997). .

While there has been extensive analysis of the pattern of expression and abundance of ERα splicing variants, limited information is available on their functional activity, particularly regarding their capacity to regulate gene expression. The prevalence of exon-skipped ERα variant expression indicates that further study is warranted to evaluate their function.

Biochemical Analysis of ERα mRNA Splicing Variants

The biochemical properties of ERα variants lacking a single internal coding exon have been characterized and compared to wt ERα function. These studies have shown that, while all of the variants can be expressed to give rise to stable proteins, ERΔE2, ERΔE4, ERΔE6, and ERΔE7 represent functionally impaired receptor isoforms that fail to localize to the nucleus and have no demonstrable effects (either stimulatory or inhibitory) on gene expression (Bollig and Miksicek, 2000). Whether these variants fulfill some as of yet undefined role within the cytoplasmic compartment is currently unknown. It should be stressed, however, that none of these cytoplasmic ERα variants can bind ligand and therefore can not be involved in mediating any of the biological effects of estrogens. The ERΔE3 and ERΔE5 variants, in contrast, represent nuclear isoforms that retain at least some aspects of receptor function. For ERΔE3, this includes the ability to bind ligand, to form homologous or mixed dimers with itself or wt ERα, respectively, and to interact with at least some of the same co-regulatory proteins (specifically, SRC-1e and SMRT) that cooperate with the wt ERα transactivating function (Bollig and Miksicek, 2000; Bollig and Miksicek, unpublished results). Although it is unable to bind ligand or dimerize, the ERΔE5 variant may bind weakly to

DNA and also interacts with the nuclear receptor coactivator SRC-1e and the nuclear corepressor SMRT. These properties enable both ERΔE3 and ERΔE5 to potentially exert complex effects on gene expression (Bollig and Miksicek, 2000; Bollig and Miksicek, unpublished data). In accordance with their inability to efficiently bind DNA, and in stark contrast with wt ERα activity, ERΔE3 and ERΔE5 do not effectively promote gene transcription from an ERE. The observation that ERΔE3 and ERΔE5 inhibit wt ERα activity on an ERE provided an indication that these variants modulate gene transcription (Bollig and Miksicek, 2000). More compelling evidence that ERΔE3 and ERΔE5 mRNA splicing variants may indeed be transcriptionally active is seen in transfection experiments involving reporter gene constructs containing nonconsensus regulatory elements.

Analysis of the Transactivating Function of ERα mRNA Splicing Variants

Early reports that ERΔE5 can support weak, cell type-dependent activity (Chaidarun and Alexander, 1998; Fuqua *et al.*, 1991) and that, when tested on an ERE, both ERΔE3 and ERΔE5 are dominant negative receptor forms in the presence of wt ERα (Bollig and Miksicek, 2000) indicate that splicing variants may be transcriptionally active. Fuqua and colleagues have reported that ERΔE5 (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 (Fuqua *et al.*, 1991). These authors also describe that overexpression of ERΔE5 in a stably transfected breast cancer cell line supported greater proliferation when compared with control cells. Furthermore, ERΔE5 imparted a tamoxifen-resistant phenotype to transfected cells (Fuqua and Wolf, 1995). In the human osteosarcoma cell line U2-OS, it has recently been reported that coexpression of ERΔE5 significantly enhances ERE-directed reporter gene expression induced by wt ERα (Chaidarun and Alexander, 1998). The existence of a constitutively active receptor variant 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 (Wiseman *et al.*, 1993; Wolf and Jordan, 1994). However, this model is challenged by conflicting observations that ERΔE5 and closely related, genetically engineered ERα mutants do not efficiently induce transcription from an ERE reporter in transiently transfected ER-negative HeLa or CEF cells, or promote proliferation in stably transfected breast tumor cells (Bollig and Miksicek, 2000; Kumar and Chambon, 1988; Rea and Parker, 1996).

Recent reports have identified an increasing number of genes with promoters lacking an ERE which show ERα-enhanced gene expression. For some of these promoters, transcriptional stimulation by ERα appears to be mediated by an indirect mechanism, involving synergistic protein-protein interactions with AP-1 transcription factors (Bollig and Miksicek, 2000; Gaub *et al.*, 1990; Webb *et al.*, 1995). AP-1 describes the ubiquitous jun/fos family of transcription factors whose activity is crucial

for the efficient expression of a wide variety of genes. As an important downstream target for the MAPK signaling cascades, AP-1 is a central player in mediating the effects of serum and growth factors on cellular proliferation (Boyle *et al.*, 1991; Westwick *et al.*, 1994). 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 (Tora *et al.*, 1988), and the insulin-like growth factor-1 (IGF-1) gene whose expression is stimulated by E_2 in the uterus of ovariectomized-hypophysectomized rats and in cultured rat osteoblast cells (Ernst and Rodan, 1991; Murphy and Ghahary, 1990). An AP-1 enhancer motif identified in the chicken IGF-1 promoter is essential for E_2 and phorbol ester-stimulated gene transcription (Umayahara *et al.*, 1994). Phorbol ester serves to stimulate increased AP-1 activity by directly activating PKC, which activates c-jun N-terminal kinase (JNK), and in turn increases AP-1 phosphorylation in treated cells (Boyle *et al.*, 1991; Westwick *et al.*, 1994). Like the rat IGF-1 gene, the chicken ovalbumin promoter does not have an ERE and the ERα regulatory element maps to a critical AP-1 site (Gaub *et al.*, 1990; Tora *et al.*, 1988). That this effect is mediated by AP-1 is supported by the observation that the synergistic induction of ERα-dependent expression by E_2 and phorbol ester cotreatment is further enhanced by cotransfection with c-fos or c-jun (Bollig and Miksicek, 2000; Gaub *et al.*, 1990).

Reporter gene cotransfection studies with expression vectors for AP-1 isoforms and ERα in HeLa cells indicate that a similar mechanism regulates the human collagenase promoter (Webb *et al.*, 1995). The minimal region of the collagenase promoter reported to be responsive to tamoxifen-liganded wt ERα — and to a variety of ERα mutants — harbors a critical AP-1 element and lacks a consensus ERE. The activity of ERα on the collagenase promoter was enhanced with AP-1 (c-jun or c-fos) overexpression (Webb *et al.*, 1995). Furthermore, evidence that ERα regulation converges with AP-1-directed gene transcription is provided by results from protein binding assays indicating that c-jun and c-fos is able to bind to wt ERα *in vitro* (see below). It is of interest that the structural requirements for ERα to regulate many noncanonical hormone response elements are less stringent than transcriptional stimulation through a consensus ERE (Gaub *et al.*, 1990; Webb *et al.*, 1995). For this reason, I have examined the behavior of ERα splicing variants on several reporter constructs containing noncanonical hormone response elements.

In agreement with previously published reports, I have demonstrated that wt ERα stimulates ovalbumin promoter activity. Expression of a reporter gene driven by the chicken ovalbumin promoter is also induced by ERΔE5 in cells treated with PMA, and by ERΔE3 with E_2 and PMA cotreatment (Bollig and Miksicek, 2000). Moreover, overexpression of c-jun enhanced wt ERα, ERΔE3 and ERΔE5 activities on the ovalbumin promoter (Bollig and Miksicek, 2000). The human collagenase promoter, which similarly harbors an AP-1 element described to direct E_2 regulated gene expression (Uht *et al.*, 1997), is activated by ERΔE5 and E_2-liganded ERΔE3 in the presence of

PMA (see DAMD Report, 2000; Bollig and Miksicek, unpublished data). Overexpression of c-jun enhances both ERΔE3 and ERΔE5 activity of a reporter gene driven by a portion of the collagenase promoter containing the ERα regulatory element (see below). Further analysis indicates that ERΔE3 activation of the collagenase promoter requires PMA and E_2 cotreatment (DAMD Report, 2000; Bollig and Miksicek, unpublished data). These results provide evidence that, like wt Eα, ERΔE3 and ERΔE5 can activate gene expression. Interestingly, these results also indicate that although estrogen regulation for both the collagenase and the ovalbumin promoter has been mapped to an AP-1 motif, these promoters are not activated with equal effectiveness by the same receptors. This distinction suggests that the AP-1 motif flanking sequence may confer a difference on the mechanism of regulation, as well as the structural requirements for ERα-induced activity. This difference is underscored by the observation that only ERΔE5 activated the $p(AP\text{-}1)_3$-TK-CAT (a construct with three AP1 motifs driving CAT expression) reporter gene construct in cell cultures treated with PMA (DAMD Report, 2000; Bollig and Miksicek, unpublished data).

Another promoter that has attracted my attention is the human IGF-1 promoter. Reporter gene expression from the human IGF-1 promoter is induced by PMA treatment (see DAMD Report, 2000). This promoter does not respond to wt ERα or ERΔE3 and is constitutively induced by ERΔE5 (DAMD Report, 2000; Bollig and Miksicek, unpublished data). It is unclear where ERΔE5 or PMA regulation is directed in the sequence of the IGF-1 promoter, since it lacks an ERE or consensus AP-1 element (Kim *et al.*, 1991). Testing ERΔE5 activity on a series of truncated IGF-1 promoter-driven reporter gene constructs demonstrates that, although it is dramatically attenuated with each successive loss of sequence, ERΔE5 is active on all lengths of the IGF-1 promoter tested (see below). Efforts to transfer ERΔE5 responsiveness onto a heterologous promoter were unsuccessful, showing that this effect requires support from the other regions of the promoter sequence (Bollig and Miksicek, unpublished data).

From these observations it is clear that ERα splicing variants, namely ERΔE3 and ERΔE5, like wt ERα can exert significant positive effects on gene transcription. Comparing the activities of ERΔE3 and ERΔE5 with wt ERα, it appears that these mRNA variants may target a unique, but overlapping subset of genes for transcriptional regulation. Considering that both ERΔE3 and ERΔE5 demonstrate the capacity to positively regulate a select number of promoters and also inhibit the activity of wt ERα on ERE-containing genes, it is reasonable to propose that *in vivo* coexpression of the nuclear splicing variants ERΔE3 and ERΔE5 with wt ERα has the potential to shift the pattern of gene expression away from ERE-containing genes to those that are regulated non-classically by estrogens.

With the characterization of the ERβ isoform and initial discovery of ERα mRNA splicing variants (Fuqua *et al.*, 1991; Mosselman *et al*,. 1996; Wang and Miksicek, 1991), the results described above call into question the appealing, but simplistic notion that all of the biologically important regulatory effects of ERα are mediated by a single 595 amino acid form of this receptor that was originally described by Chambon and colleagues in 1986 (Green *et al.*, 1986). Rather, there is growing evidence that ERα

splicing variants (most notably ERΔE3 and ERΔE5) may have a significant impact on the ability of the breast and perhaps other tissues to respond to estrogens.

Summary of Research and Training Accomplishments

Throughout the course of my graduate research, I have worked to characterize the biochemical activities of ERα mRNA splicing variants and I have investigated their potential to regulate gene transcription. Functional analysis of ERα exon-skipped splicing variants shows that, while many of the isoforms are functionally incapacitated by their deletions, ERΔE3 and ERΔE5 retain activities assigned to the full-length receptor. My efforts to study the role of variants in gene expression indicate that ERΔE3 and ERΔE5 positively regulate a select number of promoters and also inhibit the activity of wt ERα on ERE-containing genes. The results described below offer further insight into the nature of variant transcription activity and conclude my analysis of their biochemical function.

ERΔE3 and ERΔE5 Activity on the Collagenase Promoter is Enhanced by c-jun Overexpression

The collagenase promoter is dramatically activated by ERΔE3 and to a lesser extent by ERΔE5 in response to combined treatment with estrogen and PMA in transiently transfected HeLa cells. Additional analysis indicates that maximal activation of a collagenase reporter gene construct, pColl(-73)Luc, by ERΔE3 requires cotreatment with both E_2 and PMA (see DAMD Report, 2000; Bollig and Miksicek unpublished data). Phorbol ester alone (or in combination with ER ligands) supports a modest, but statistically significant induction of the collagenase reporter that once again involves activation of endogenous AP-1 and is independent of coexpressed receptor. Regulation of pColl(-73)Luc by ERΔE5 similarly required activation of endogenous AP-1 by PMA treatment, but was independent of E_2, consistent with the inability of ERΔE5 to bind ligand. Substantiating the necessity for PMA treatment and the role of AP-1 in receptor regulation of the collagenase promoter, coexpression of c-jun is observed to enhance both ERΔE3 and ERΔE5 induction of the pColl(-73)Luc reporter gene (Fig. 2).

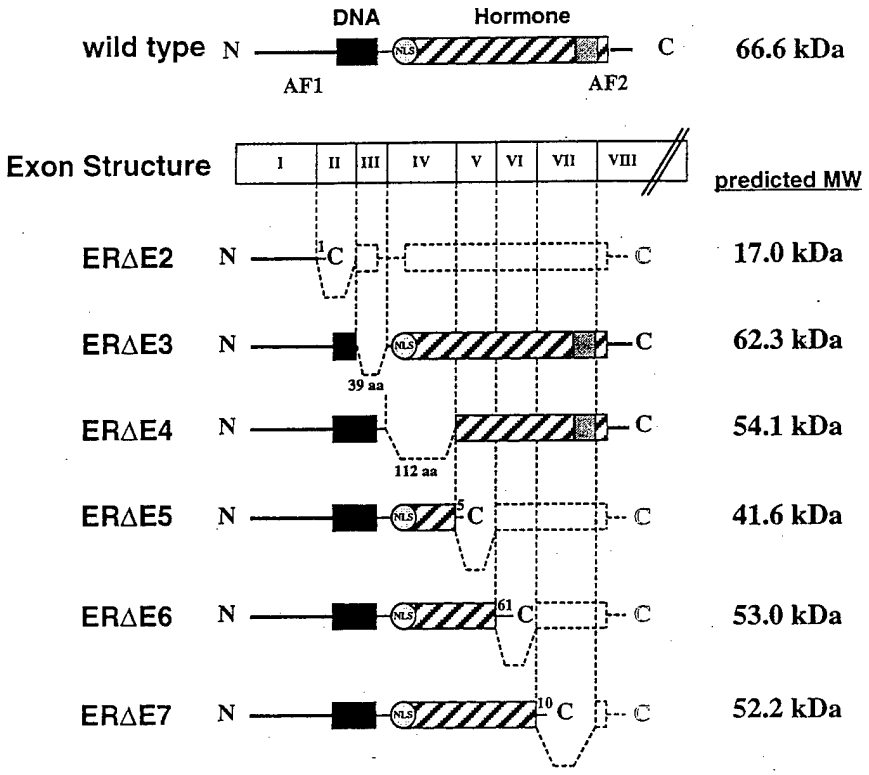

Fig. 1. **Comparison of ERα mRNA splicing variants and wt ERα structure.** The location of the various functional domains of ERα and the exon sequences from which they are derived are depicted. The variants are referred to by deleted exon. The size and molecular weight of each of the variants are predicted from the translational reading frame of the sequenced cDNA clones. *Dashed lines* indicate regions of the major open reading frame of the full-length ERα protein that are missing from each variant. The nuclear localization signal is *circled*. The regions encompassing the DNA- and ligand-binding domains are marked by *darkened* and *hatched boxes*, respectively. The AF1 and AF2 domains are indicated where they reside within the N- and C-termini of the wild type receptor.

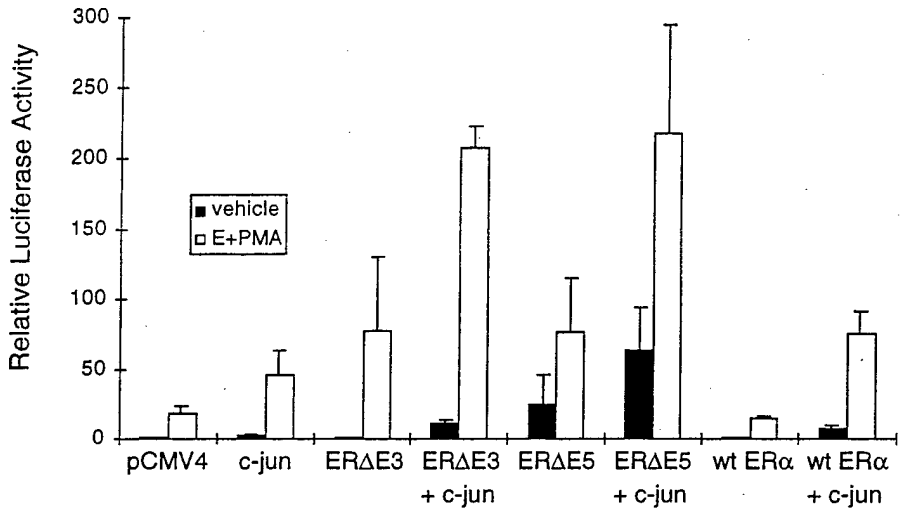

Fig. 2. **Coexpression of c-jun enhances both ERΔE3 and ERΔE5 activity on the collagenase promoter.** Cotransfection of 1 μg of c-jun expression vector with 0.5 μg of receptor isoform and 10 μg of pColl(-73)Luc demonstrates that c-jun cooperates with ERΔE3 and ERΔE5 to induce luciferase expression. Luciferase activities were normalized for protein and results are expressed relative to vehicle-treated empty expression vector (pCMV4) controls. *Error bars* represent the SEM of three independent experiments.

AP-1 factor binding properties of wt ERα

The observation that variant induced expression from the collagenase and ovalbumin promoters requires activation of both AP-1 and ERα isoform expression, strongly suggests that ERΔE3 and ERΔE5 are acting cooperatively with AP-1 on these promoters. Further consideration of the mechanism of activation by wt ERα and ERα splicing variants excludes a requirement for direct binding of receptor to the ovalbumin promoter. ERΔE3 is devoid of DNA-binding activity, arguing that activation of ovalbumin by these receptors must be mediated indirectly through specific protein-protein interactions. Figure 3 shows that ERα interacts with c-jun and c-fos in solution and indicates that the receptor may be recruited to the ovalbumin promoter by interacting with AP-1 factors. Binding assays with GST-fused ERα and ^{35}S-labeled c-jun and c-fos (translated individually or in combination *in vitro*) demonstrate that, independent of E_2 treatment, ERα binds c-jun and c-fos under all conditions tested. Although ERα variants

were not specifically included in these binding studies, Webb and coworkers have reported an interaction between c-jun and the isolated N-terminus of ERα, a region conserved in both ERΔE3 and ERΔE5 (Webb *et al.*, 1995).

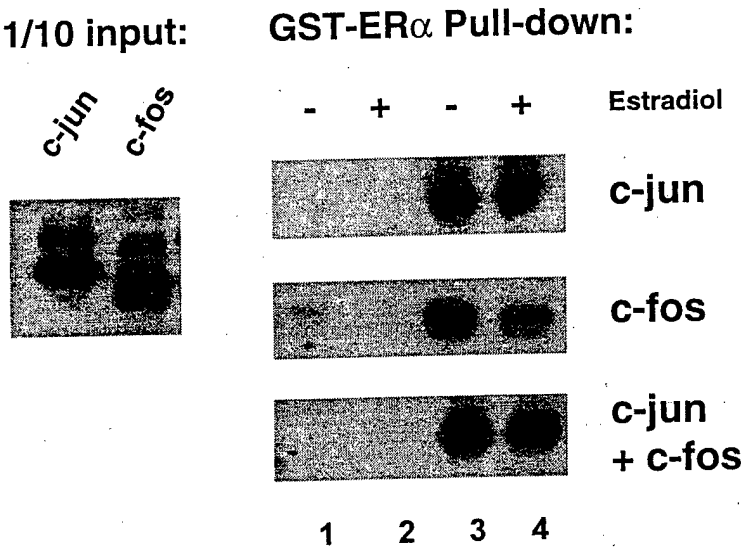

Fig. 3. **Binding of c-jun and c-fos with GST-fused wt ERα.** In the *right panel*, an autoradiograph demonstrates that *in vitro* translated ^{35}S-methionine-labeled c-fos and c-jun both bind wt ERα expressed as a GST-ERα fusion protein (*lanes 3 and 4*, respectively). The AP-1 factors do not interact with GST expressed without wt ERα (*lanes 1 and 2*). The interaction of c-fos and c-jun with GST-ERα did not require the presence of E_2. *Left panel* shows 10% of the radiolabeled input.

ERΔE3, ERΔE5 and wt ERα bind SMRT

Results from *in vitro* binding studies suggest that the regulation of noncanonical promoters by wt ERα and ERα splicing variants involves protein-protein interactions with coregulators. Overexpression of a coactivator enhances wt ERα transactivating function and ERΔE3 and ERΔE5 bind the steroid receptor coactivator SRC-1 (Bollig and Miksicek, 2000; Oñate *et al.*, 1995). Our understanding of wt ERα cooperation with co-regulatory factors has progressed with recent reports that wt ERα binds steroid receptor

corepressors (Smith *et al.*, 1997). In order to further evaluate the relationship between co-regulators and the splicing variants ERΔE3 and ERΔE5, we tested whether these variants, like wt ERα, also possess a corepressor binding function. *In vitro* binding studies show that wt ERα, ERΔE3, and ERΔE5 share a capacity to bind the corepressor SMRT (Fig, 4). For wt ERα and ERΔE3 this binding is observed with or without the addition of E_2 and in the presence of tamoxifen.

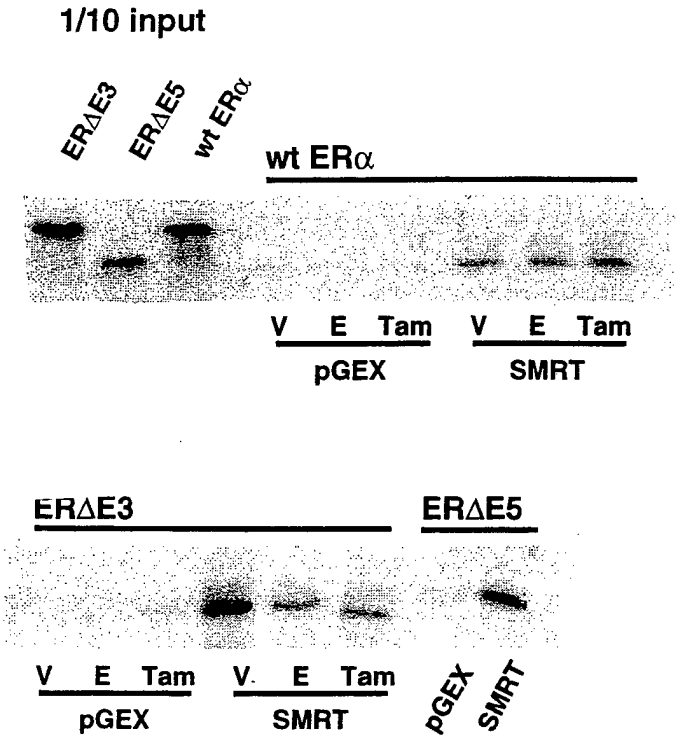

Fig. 4. **ERΔE3, ERΔE5, and wt ERα bind the corepressor SMRT.** Radiolabeled ERΔE3, ERΔE5 and wt ERα are retained by GST-fused SMRT protein bound to glutathione-Sepharose beads. The receptor isoforms were not retained by the GST protein expressed from the pGex vector without SMRT cDNA inserted. GST-SMRT association with each of the receptors was unaffected by the presence or absence of E_2 or tamoxifen. The three left lanes of the *top panel* represent 10% of the radiolabeled input.

Regulation of the IGF-1 Promoter by ERΔE5

IGF-1 promoter activity is strongly and constitutively induced by ERΔE5. The other exon-skipped variants and wt ERα do not activate this promoter. PMA treatment of ERΔE5 transfected cells has no additional stimulatory effect on ERΔE5 induction of this

reporter gene. In fact, PMA treatment slightly decreases ERΔE5 activity (DAMD Report, 2000; Bollig and Miksicek, unpublished data). This argues against the involvement of PKC or PKC-activated transcription factors such as AP-1 in the mechanism of ERΔE5 induction of human IGF-1 expression. Furthermore, unlike the ovalbumin and collagenase promoters described above, the IGF-1 promoter does not contain a consensus AP-1 element. In order to determine where within the IGF-1 promoter the ERΔE5 regulation is directed, transfections were performed with a series of 5'-deletion mutants of the human IGF-1 promoter. Relative to the 1630 sequence, activity was dramatically and progressively lost in mutants containing 926, 591 and 233 bp of the 5'-flanking sequence, while modest but significant ERΔE5-responsiveness persisted even in the shortest promoter construct (compare pCMV4 and ERΔE5 activity for each promoter, Fig. 5).

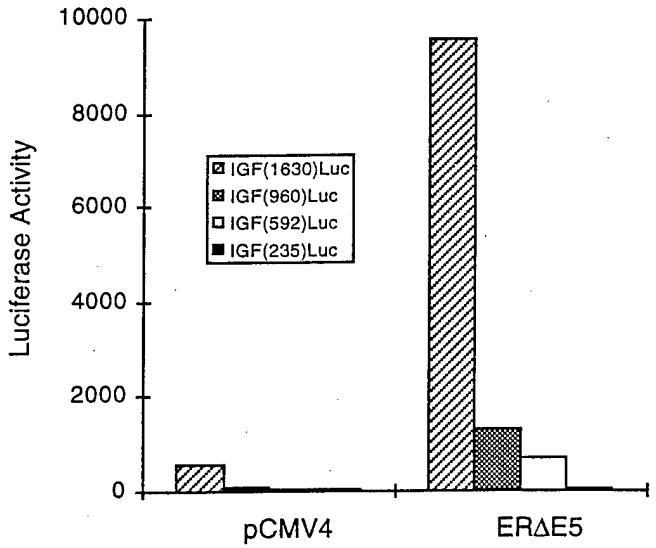

Fig. 5. **ERΔE5 induces the expression of a reporter gene driven by a series of truncated IGF-1 promoters.** Cotransfection of ERΔE5 demonstrates that, although it is dramatically attenuated with each successive loss of sequence, it is active on all lengths of the IGF-1 promoter tested. The length of IGF-1 promoter used in each luciferase reporter construct is specified by the number that counts base pairs upstream from the IGF-1 transcription start site. A representative assay for ERΔE5 activity on the IGF(960)Luc, IGF(592)Luc and IGF(235)Luc reporter genes is shown.

Training and Academic Progress

In the years prior to my receipt of the U.S. Army BCRP Fellowship in June 1999 I successfully completed requisite course work, passed my comprehensive examinations, defended my thesis proposal, and completed teaching assistant requirements. Last year I published a report in the journal Molecular Endocrinology (Bollig and Miksicek, 2000). This year, in addition to planning and executing the experiments described above, I have completed work on a second manuscript recently submitted for publication to the journal Molecular Endocrinology. With that and the publication of my dissertation, this year will mark the end of my graduate research and training in the Department of Physiology at Michigan State University. I have applied for graduation and am scheduled to defend my dissertation in July 2001. Therefore as of June 30, 2001, year 3 of this award will be declined.

Research Accomplishments

(2000)

- Analyzed the biochemical behavior of ERα splicing variants:
  - DNA-binding function
  - Cell localization
  - Ligand-binding capacity

- Completed protein interaction studies between variants and the nuclear receptor coactivator, SRC-1e

- Assayed for variant activity on a consensus estrogen response element

- Initiated transfection experiments to assess the effect of ERα mRNA splicing variants on gene expression from the chicken ovalbumin, human collagenase, IGF-1 and TGFβ3 promoters.

(2001)

- Completed protein interaction studies between variants and the nuclear receptor corepressor, SMRT

- Completed binding studies with wt ERα and AP-1 factors c-jun and c-fos

- Tested the effect of c-jun overexpression on variant activation of the collagenase promoter

- Completed studies designed to localize the ERΔE5 response element in the human IGF-1 promoter

Publications

Bollig A, Miksicek RJ (2000) An estrogen receptor-alpha splicing variant mediates both positive and negative effects on gene transcription. *Molecular Endocrinology*, 14, 634-649.

Bollig A, Miksicek RJ (2001) Estrogen receptor-alpha splicing variants lacking exons 3 or 5 display promoter-specific transcriptional effects through non-consensus estrogen response elements. Submitted for publication to *Molecular Endocrinology*, June 2001.

Conclusions

(2000)

- ERΔE3 dimerizes with wt ERα
- Similar to wt ERα, ERΔE3 and ERΔE5 bind SRC-1e fragments
- ERΔE3 and wt ERα cooperate with c-jun to activate the chicken ovalbumin promoter
- ERΔE5 constitutively activates the human IGF-1 promoter (The IGF-1 promoter is not induced by wt ERα or any other receptor variant tested in parallel reporter gene studies)
- Activation of a hormone responsive human collagenase promoter fragment by ERΔE3 in transfected cells requires dual AP-1 activation by PMA and E_2 treatment.
- Activation of the collagenase promoter by ERΔE5 requires activation of AP-1 by PMA treatment

(2001)

- wt ERα binds c-jun and c-fos
- ERΔE3 and ERΔE5 cooperate with c-jun to activate the collagenase promoter
- ERΔE3, ERΔE5 and wt ERα bind steroid corepressor SMRT in the presence or absence of E_2 and tamoxifen
- Although it was attenuated with the loss of sequence, ERΔE5 transactivates the IGF-1 promoter constructs: IGF(1630)Luc, IGF(960)Luc, IGF(592)Luc and IGF(235)Luc

References

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

Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, Hunter T (1991) Activation of protein kinase C decreases phosphorylation of cJun at sites that negatively regulate its DNA-binding activity. Cell, **64**, 573-584.

Castles CG, Klotz DM, Fuqua SAW, Hill SM (1995) Coexpression of wild-type and variant oestrogen receptor mRNAs in a panel of human breast cancer cell lines. Br J Cancer, **71**, 974-980.

Chaidarun SS, Alexander JM (1998) A tumor-specific truncated estrogen receptor splice variant enhances estrogen-stimulated gene expression. Mol Endocrinol, **12**, 1355-1366.

Erenburg I, Schachter B, Mira y Lopez R, Ossowski I (1997) Loss of an estrogen receptor isoform (ERαΔE3) in breast cancer and the consequences of its reexpression: interference with estrogen-stimulated properties of malignant transformation. Mol Endocrinol, **11**, 2004-2015.

Ernst M, Rodan GA (1991) Estradiol regulation of insulin-like growth factor-1 gene expression in osteoblastic cells: evidence for transcriptional control. Mol Endocrinol, **5**, 1081-1089.

Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Malley BW, McGuire WL (1991) Variant human breast tumor estrogen receptor with constititive transcriptional activity. Cancer Res, **51**, 105-109.

Fuqua SAW, Wolf DM (1995) Molecular aspects of estrogen receptor variants in breast cancer. Breast Cancer Res Treat, **35**, 233-241.

Gaub MP, Bellard M, Scheuer I, Chambon P, Sassone-Corsi P (1990) Activation of the ovalbumin gene by the estrogen receptor involves the Fos-Jun complex. Cell, **63**, 1267-1276.

Gotteland M, Desauty G, Delarue JC, Liu L, May E (1995) Human estrogen receptor messnger RNA in both normal and tumor breast tissues. Mol Cell Endocrinol, **112**, 1-13.

Green S, Walter P, Kumar V, Krust A, Bornet JM, Argos P, Chambon P (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-erbA. Nature, **320**,134-139.

Hu C, Hyder SM, Needleman DS, Baker VV (1996) Expression of estrogen receptor variants in normal and neoplastic human uterus. Mol Cell Endocrinol, **118**, 173-179.

Kim SW, Lajara R, Rotwein P (1991) Structure and function of a human insulin-like growth factor-1 gene promoter. Mol Endocrinol, **5**, 1964-1972.

Kumar V, Chambon P (1988) The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell, **55**, 145-156.

Miksicek RJ, Lei Y, Wang Y (1993) Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. Breast Cancer Res Treat, **26**, 163-174.

Mosselman S, Polman J, Dijkema R (1996) ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett, **392**, 49-53.

Murphy LJ, Ghahary A (1990) Uterine insulin-like growth factor-1: regulation of expression and its role in estrogen-induced uterine proliferation. Endocr Rev, **3**, 443-453.

Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH (1997) Oestrogen receptor variants and mutations in human breast cancer. Ann Med, **29**, 221-234.

Oñate SA, Boonyaratanakornkit V, Spencer TE, Tsai SY, Tsai M-Y, Edwards DP, O'Malley BW (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. J Biol Chem, **273**, 121010-12108.

Rea D, Parker MG (1996) Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. Cancer Res, **56**, 1556-1563.

Smith CL, Nawaz Z, O'Malley BW (1997) Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol, **11**, 657-666.

Tora L, Gaub MP, Mader S, Dierich A, Bellard M, Chambon P (1988) Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter. EMBO J, **7**, 3771-3778.

Uht RM, Anderson CM, Webb P, Kushner PJ (1997) Transcriptional activities of estrogen and gluccocorticoid receptors are functionally integrated at the AP-1 element. Endocrinology, **138**, 2900-2908.

Umayahara , Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y, Kamada T (1994) Estrogen regulation of the insulin-like growth factor 1 gene transcription involves an AP-1 enhancer. J Biol Chem, **269**, 16433-16442.

Wang Y, Miksicek RJ (1991) Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol, **5**, 1707-1715.

Webb P, Lopez GN, Uht RM, Kushner PJ (1995) Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol, **9**, 443-456.

Westwick JK, Weitzel C, Minden A, Karin M, Brenner DA (1994) Tumor necrosis factor α stimulates AP-1 activity through prolonged activation of the c-Jun kinase. J Biol Chem, **269**, 2639-26401.

Wiseman LR, Johnson MD, Wakeling AE, Lykkesfeldt AE, May FEB, Westley BR (1993) Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells. Eur J Cancer, **29A**, 2256-2264.

Estrogen Receptor-α Splicing Variants Lacking Exons 3 or 5 Display Promoter-Specific Transcriptional Effects Through Non-Consensus Estrogen Response Elements

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ABSTRACT

Studies of estrogen receptor-α (ERα) mRNA splicing variants have identified two exon-skipped variants that share significant residual function compared to their full-length counterpart, wild type ERα (wt ERα). Among the numerous ERα splicing variants observed in estrogen-responsive tissues, only the variants with sequence deletions corresponding to exon 3 or exon 5 (ERΔE3 and ERΔE5, respectively) display normal nuclear uptake and retain the ability to interact with transcriptional co-regulators. These properties confer on ERΔE3 and ERΔE5 the ability to inhibit the activity of some genes, but to stimulate the transcription of other genes. Genes that represent targets for induction by these ERα splicing variants appear to lack consensus DNA-binding sites for ERα, but instead are regulated indirectly through interactions with other transcription factors such as AP-1. Therefore, in contrast to what is understood to be the traditional mechanism of gene regulation by wt ERα, ERα splicing variants function primarily through a recently proposed non-classical pathway. A variety of potential gene targets for transcriptional regulation by the ERΔE3 and ERΔE5 variants are described.

INTRODUCTION

RNAs expressed from the ERα gene typically represent a mixed population of wild type and variant mRNAs that result from imprecise splicing of the primary transcript. A wide variety of variant ERα mRNAs have been described in the literature (1-6), but those most commonly observed harbor a precise deletion of one of the internal exons that contribute to the structure of the mature ERα protein. Despite their discovery nearly a decade ago (7, 8), relatively little is known about the functional role of ERα splicing

variants. The most prominent activity described for two of the splicing variants (ERΔE3 and ERΔE5) is to interfere with the transcriptional activity of wt ERα on ERE-containing genes, where they behave as dominant-negative mutants with modest potency (8-10). The inhibitory activity of ERΔE3 and ERΔE5 is likely to result in part from their ability to sequester nuclear receptor coactivators or other limiting transcription factors into a non-productive complex (10). In addition, ERΔE3 can block the binding of wt ERα to DNA by forming functionally inactive, mixed dimers (11).

The in-frame deletion of exon III harbored by ERΔE3 (Fig. 1) causes partial loss of its DNA-binding domain (DBD), but leaves the ligand-binding domain (LBD) and therefore its ability to respond to hormones intact. Conversely, the LBD is severely truncated in ERΔE5 resulting in the complete loss of hormone binding (10). Although ERΔE5 contains an intact DBD, its DNA-binding activity is impaired as a consequence of the loss of helix 11 within the LBD, which contains critical contact sites for subunit dimerization (12, 13). While the ERΔE5 splicing variant is reported to display modest transcriptional activity in certain cell- and promoter-specific contexts (7, 10, 14, 15), its relatively poor binding to the palindromic estrogen response element (ERE) suggests that it is unlikely to be a significant effector of transcription through a consensus ERE (10). Similarly, the remaining exon-skipped ERα splicing variants (ERΔE2, ERΔE3, ERΔE4, ERΔE6, and ERΔE7) appear to completely lack transcriptional stimulatory activity when tested in transient transfection assays with an ERE-containing reporter plasmid (10). For the studies detailed below, we focused our attention on the ERΔE3 and ERΔE5 splicing variants for the reason that these receptor isoforms continue to show efficient localization to the nucleus and despite their mutations, they retain the ability to bind to the steroid receptor coactivator

SRC-1e in an *in vitro* protein interaction assay (10).

In recent years, a novel pathway for regulation of transcription by estrogen receptors (both ERα and ERβ) has been described that involves cooperation of the receptors with the AP-1 transcription factors, c-jun and c-fos (16-20). This has been broadened to include additional genes for which estrogen regulation was mapped to binding sites for the Sp1 (21-23) and ATF2 transcription factors (24), which bind to GC-rich and CRE (cyclic AMP response element)-like motifs, respectively. An important feature of these non-classical pathways for estrogen action is that functional domains within the receptor that are crucial for transcriptional activation through a consensus ERE are in some cases dispensable for ERα activity on AP-1, Sp1, or CRE-regulated promoters (10, 16, 18-21, 24). This led us to consider the possibility that the nuclear ERα splicing variants ERΔE3 and ERΔE5 may function preferentially as transcriptional regulators through noncanonical hormone response elements as opposed to consensus EREs. Four promoters have been identified that are efficiently stimulated by either ERΔE3 or ERΔE5. In some instances, this activity is shared by wt ERα, while in other cases gene activation is limited to the ERΔE3 or the ERΔE5 variants alone.

RESULTS

ERα splicing variants do not effectively promote gene expression from a palindromic ERE (8-11, 15). However, this understanding does not preclude the possibility that transcriptional effects of ERα variants may be directed at promoters with non-classical hormone response elements. Recent reports have identified an increasing

number of genes with promoters lacking an ERE which show ER-enhanced expression. For these genes, transcriptional stimulation by ER appears to be mediated by an indirect mechanism, involving synergistic protein-protein interactions with upstream transcription factors (such as AP-1, Sp1, and ATF2) (16-24) or nuclear receptor coactivators (including CBP/p300 or the p160 coactivators SRC-1, TIF-2/GRIP1, or AIB1/RAC3/ACTR) (25-29). The suggested mechanistic distinction between noncanonical- and ERE-directed transcription is indicated by ER mutational studies that demonstrate unique structural requirements for activation of noncanonical EREs compared to classical regulation through a palindromic ERE (10, 16, 18-21, 24). This observation suggests that a receptor isoform lacking particular domains may be transcriptionally active on certain promoters, but inactive on others. We have previously shown that ERΔE3, like wt ERα, activates the chicken ovalbumin promoter which lacks an ERE. This response is a complex one in that it requires not only the presence of E_2, but also activation of AP-1 by a phorbol ester (10). Phorbol ester serves to stimulate increased AP-1 activity by directly activating protein kinase C (PKC), which activates c-jun N-Terminal Kinase (JNK), and in turn increases AP-1 phosphorylation in treated cells (30, 31). That this effect is mediated by AP-1 is supported by our observation that induction of the ovalbumin reporter by both wt ERα and ERΔE3 is enhanced by cotransfection with c-jun (10). A requirement for direct binding of ERα to this promoter is barred by the fact that ERΔE3 is devoid of DNA-binding activity, arguing that activation of ovalbumin by these receptors must be mediated indirectly through specific protein-protein interactions. Moreover, the ovalbumin promoter does not have an ERE and the ER regulatory element maps to a critical AP-1 site (16, 32). Fig. 2 demonstrates that ER interacts with c-jun and c-fos in solution and suggests that ER may be recruited to the ovalbumin promoter by interacting with AP-1 factors. Binding assays with GST-fused ERα and ^{35}S-labeled c-jun and c-fos translated individually or in combination

in vitro demonstrate that, independent of E_2 treatment, ER binds c-jun and c-fos under all conditions tested. Although ER variants were not specifically included in these binding studies, Webb *et al.* (19) have reported an interaction between c-jun and the isolated N-terminus of ERα, a region conserved in both ERΔE3 and ERΔE5.

To further investigate the role of ERα splicing variants in the regulation of gene expression, we used a cell culture transfection system to assay for receptor activity on a variety of reporter gene constructs. The promoters tested in these studies attracted our interest because they are reported to be ERα regulated and/or to contain an AP-1 motif. These include the human collagenase and human IGF-1 promoters, and a contrived promoter consisting of three consensus AP-1 elements. For preliminary studies, HeLa cells were transfected with each of these reporters together with wt ERα or a variant ERα expression plasmid. HeLa cells express minimal endogenous ERα and yet possess a transcriptional milieu that enables reporter plasmids to respond efficiently to transfected receptor. Paired cultures were treated with 10 nM E_2 in combination with 20 nM PMA, or with vehicle alone. This was done to ensure that an activating ligand was present, if necessary, and that AP-1 factors were maximally stimulated in the event that promoter activation required stimulation of PKC.

Both ERΔE3 and ERΔE5 can activate the Coll(-73)Luc reporter

The promoter of the human collagenase gene has received considerable attention as a target for non-classical regulation by both ERα and ERβ. Collagenase is a member of the family of matrix metalloproteases, which are regulated similarly and demonstrate enhanced expression in response to injury and during inflammatory reactions (33-37). Collagenase gene expression is elevated in certain tumor cells and may contribute to advancement of

metastasis (36). In cultured fibroblasts and skin cells treated with carcinogens and tumor promoting agents, induction of collagenase is mediated by phorbol ester treatment (33, 37). A short region of the collagenase promoter (-73 to +63 relative to the transcription start site) which harbors an AP-1 element is described to direct estrogen- or tamoxifen-regulated gene expression of a luciferase reporter gene in a variety of cell lines (17-20). In contrast to published reports, we find that wt ERα behaves no differently on the pColl(-73)Luc reporter than the empty pCMV4 control vector (Fig. 3A). In both cases, a relatively modest (approximately 6-fold) increase is observed that depends on activation of endogenous AP-1 by PMA treatment. However, this promoter can be dramatically activated by ERΔE3 and to a lesser extent by ERΔE5 in response to combined treatment with estrogen and PMA (Fig. 3A). Like wt ERα, the remaining splicing variants (ERΔE2, ERΔE4, ERΔE6, and ERΔE7) were without significant effect. Substantiating the necessity for PMA treatment and the role of AP-1 in receptor regulation of the collagenase promoter, co-expression of c-jun is observed to enhance ERΔE3 and ERΔE5 induced pColl(-73)Luc reporter gene expression (Fig. 3B).

Additional analysis indicates that maximal activation of pColl(-73)Luc by ERΔE3 requires co-treatment with both E_2 and PMA (Fig. 3C). Phorbol ester alone (or in combination with ER ligands) supports a modest, but statistically significant induction of the collagenase reporter that once again involves activation of endogenous AP-1 and is independent of co-expressed receptor. Regulation pColl(-73)Luc by ERΔE5 similarly required activation of endogenous AP-1 by PMA treatment, but was independent of E_2 (data not shown), consistent with the inability of ERΔE5 to bind ligand. The partial agonist tamoxifen was without effect on induction of this reporter by ERΔE3, either alone

or in the presence of PMA. Inclusion of either E_2 (10 nM) or tamoxifen (100 nM) alone or together with PMA had no effect on the activity of pColl(-73)Luc co-transfected with wt ERα (or pCMV4) compared to PMA alone (data not shown). The human collagenase promoter therefore demonstrates a difference in behavior compared to the pOvalb-CAT reporter which was not induced by ERΔE5, but was activated by both ERΔE3 and wt ERα in cells co-treated with E_2 and PMA (10).

Due to the stimulatory behavior of ERΔE3 and ERΔE5 on the ovalbumin and collagenase promoters both of which contain AP-1 sites in their native promoter contexts, it was of interest to examine the effects of the various ERα splicing variants on a reporter plasmid containing an AP-1 site in the context of a promoter not otherwise regulated by ERα or estrogens. For this purpose, we chose $p(AP\text{-}1)_3$-TK-CAT, which contains three consensus AP-1 sites in tandem upstream of a minimal thymidine kinase promoter from *Herpes simplex* virus. Somewhat unexpectedly, ERΔE5 (but not wt ERα, ERΔE3, or any of the remaining splicing variants) was able to induce this promoter approximately seven-fold in HeLa cells (Fig.4). Like the pColl(-73)Luc reporter described above, ERΔE5 activity on the $p(AP1)_3$TK-CAT reporter required PMA treatment and did not depend on the presence of E_2 (data not shown). It is clear that by some reports ERα is an estrogen-dependent inducer of transcription through a consensus AP-1 element (16-20). These results demonstrate that the activity of ERα on AP-1 containing promoters is a complicated and conditional effect. The data presented here agree well with a previous report demonstrating that a C-terminally truncated receptor lacking the LBD is more active than E_2-liganded wt ERα on an analogous AP-1/TK reporter construct (18). Also, it is evident that the context of an AP-1 site within a promoter is critical for confering its ability to respond,

and the degree of that response, to wt ERα or any of the ERα splicing variants.

ERΔE5 activates the human IGF-1 promoter

Insulin-like growth factor I (IGF-1) is expressed in and effects cellular growth in many tissues. In general, growth hormone acts as the major regulatory factor for the IGF-1 gene; however, several other factors (including thyroid hormone, epidermal growth factor, parathyroid hormone and estrogen) function to modulate IGF-1 expression (38-43). Induction of IGF-1 is stimulated by E_2 in the uterus of ovariectomized / hypophysectomized rats (40, 41) and in cultured rat osteoblast cells (38). In transient cotransfection studies a defined region of the chicken IGF-1 promoter directed reporter gene induction by liganded ER (44). Like the ovalbumin and collagenase promoters mentioned above, the IGF-1 promoter lacks a consensus ERE sequence (45, 46). Further complicating this picture are independent data showing that in primary fetal rat osteoblasts E_2 can block the increase in IGF-1 synthesis caused by parathyroid hormone or prostaglandin E_2 (47). It has been shown that this hormonal regulation of IGF-1 expression is mediated by a binding site for CCAAT/enhancer-binding protein-delta (C/EBP-δ) located between positions +202 and +209 within exon 1 of this gene (48). For the chicken promoter, the positive ER regulatory domain was mapped to an AP-1 motif essential for both E_2 and phorbol ester-stimulated gene transcription (44). Although there is a great deal of homology among IGF-1 promoters of various species, this site is not conserved in mammals (44-46). The human IGF-1 promoter is thus different from the promoter examples described above in that it lacks any consensus binding sites for either AP-1 or Sp1 that might represent likely targets for regulation by ERα. However, Nagaoka *et al.* report that PKC activation by PMA treatment increases the rate of IGF-1 transcription in the human macrophage-like cell line U937 (49). This suggests that AP-1 factors or

another PKC-responsive transcription factor positively regulate the human IGF-1 gene. Furthermore, they may be functioning at a nonconsensus AP-1 or ATF2 binding site.

When co-transfected into HeLa cells, activation of PKC by treatment with PMA modestly induced expression of a luciferase reporter plasmid containing 1.63 kb of sequence upstream from the human IGF-1 transcription start site (pIGF1-1630-Luc) (Fig. 5A). This same reporter was unresponsive to wt ERα transfection. IGF-1 promoter activity was strongly and constitutively induced by ERΔE5, greater than 30-fold compared to cells transfected with control plasmid. PMA treatment of ERΔE5 transfected cells had no additional stimulatory effect on ERΔE5 induction of this reporter gene. In fact, PMA treatment slightly decreased ERΔE5 activity (Fig 5A). This argues against the involvement of PKC or PKC-activated transcription factors such as AP-1 in the mechanism of ERΔE5 induction of human IGF-1 expression. In order to determine where within the IGF-1 promoter the ERΔE5 regulation is directed, transfections were performed with a series of 5'-deletion mutants of the human IGF-1 promoter. Relative to the 1630 sequence, activity was dramatically and progressively lost in mutants containing 926, 591 and 233 bp of the 5'-flanking sequence, while modest but significant ERΔE5-responsiveness persisted even in the shortest promoter construct (compare pCMV4 and ERΔE5 activity for each promoter, Fig 5B). Efforts to transfer ERΔE5-responsiveness onto a heterologous promoter were unsuccessful (Fig. 5C), suggesting that activation of IGF-1 expression by ERΔE5 may require complex interactions between transcription factor binding sites scattered throughout the IGF-1 promoter.

DISCUSSION

As we and others have shown, the ERα gene (ESR-1) gives rise to a markedly heterogeneous population of RNA transcripts that includes exon-skipped, as well as correctly spliced ERα mRNAs. Splicing variants of ERα are present in all estrogen-responsive tissues and exist in both normal and transformed cells (1-8, 50-52). The biochemical properties of ERα variants lacking a single internal coding exon have been characterized in model transfection systems (10). These studies have shown that, while all of the variants can be expressed to give rise to stable proteins, ERΔE2, ERΔE4, ERΔE6, and ERΔE7 represent functionally impaired receptor isoforms that fail to localize to the nucleus and have no demonstrable effects (either stimulatory or inhibitory) on gene expression (10). Whether these variants fulfill some as yet undefined role within the cytoplasmic compartment is currently unknown. It should be stressed, however, that none of these cytoplasmic ERα variants can bind ligand and therefore can not be involved in mediating any of the biological effects of estrogens. The ERΔE3 and ERΔE5 variants, in contrast, represent nuclear isoforms that retain at least some aspects of receptor function. For ERΔE3, this includes the ability to bind ligand, to form homologous or mixed dimers with itself or wt ERα, respectively, and to interact with at least some of the same co-regulatory proteins that enable wt ERα to function (10). Although it is unable to bind ligand or to dimerize, the ERΔE5 variant may bind weakly to DNA and also interacts with nuclear receptor coactivators, albeit not as efficiently as either ERΔE3 or wt ERα. These

properties enable both ERΔE3 and ERΔE5 to exert complex effects on gene expression.

The traditional view of estrogen action depicts ERα as the central player in target gene selection by virtue of its ability to bind to DNA containing a close match to the ERE consensus sequence (53). It has been necessary to modify this view to accommodate the newly discovered ERβ, which binds to the same palindromic ERE sequence (54). Recent evidence also indicates that many estrogen-regulated genes may contain noncanonical hormone response elements organized around AP-1 or SP1 sites. Among these targets are genes such as IGF-1, cyclin D1, c-fos, c-myc, RARα, collagenase, and cathepsin D that play important roles in controlling cell growth and tissue remodeling (16-24, 56). It is of interest that the structural requirements for ERα to regulate many noncanonical hormone response elements are less stringent than transcriptional stimulation through a consensus ERE (10, 16, 18-21, 24). For this reason, we have examined the behavior of ERα splicing variants on several reporter constructs containing noncanonical hormone response elements. In agreement with published reports, our previous studies indicate that wt ERα stimulates ovalbumin promoter activity (10). The dual requirement for estradiol and a phorbol ester suggest that this regulation occurs through an AP-1 site identified in the upstream region of the ovalbumin promoter and involves a cooperative interaction between ERα and AP-1 factors (10, 16, 32). A significant estradiol- and phorbol ester-dependent induction of ovalbumin activity is supported by ERΔE3, despite its lack of an intact DNA-binding domain (10). ERΔE5 also supports a modest stimulation of ovalbumin activity that was phorbol ester-dependent, but estradiol-independent. These observations reveal a complex mechanism by which ERα stimulates the ovalbumin promoter that includes both a

phorbol ester-sensitive pathway involving the amino terminal activation domain (AF1) and an estradiol-sensitive pathway involving the agonist-regulated carboxy terminal activation domain (AF2). Moreover, while it is clear that neither of these splicing variants is as active as wt ERα on this promoter, the capacity for ERΔE3 and ERΔE5 to activate the ovalbumin promoter implies that direct binding of the receptor to DNA is clearly not required for this regulation.

In contrast to the situation with pOvalb-CAT, the activity of wt ERα on the remaining three noncanonical hormone response element-containing promoters examined here was either non-existent or insignificant compared to that of the ERΔE3 or ERΔE5 splicing variants. Specifically, both ERΔE3 and ERΔE5 effectively enhanced expression of the pColl(-73)Luc reporter, and ERΔE5 alone strongly activated pIGF(-1630)Luc and $p(AP1)_3$TK-CAT. The behavior of these reporters in response to the various receptor isoforms was not readily predicted from their known biochemical properties and significant differences are evident for each of the reporters tested. In the case of the collagenase promoter fragment, induction by ERΔE3 required both estradiol and phorbol ester treatment. Activation of the promoters by ERΔE5 showed no estrogen responsiveness, consistent with the inability of this variant to bind ligand. The stimulatory activity of ERΔE5 required phorbol ester treatment for the $p(AP1)_3$TK-CAT reporter. ERΔE5 induction of the IGF-1 promoter was not enhanced by phorbol ester treatment, although the IGF promoter was positively regulated by PMA treatment in the absence of co-transfected ERΔE5. This suggests that activation of AP-1, presumably by stimulation of PKC and phosphorylation of AP-1 factors (e.g., c-jun and c-fos), plays different roles in the stimulation of these various reporter constructs. These data also suggest that the presence

of a functional AF2 domain is detrimental for activation of some (but not all) noncanonical hormone response elements by ERα since ERΔE5, but not wt ERα or ERΔE3 stimulated the $p(AP1)_3$TK-CAT and pIGF(-1630)Luc reporter plasmids. In contrast to the reporter plasmids described above, neither wt ERα, ERΔE3, or ERΔE5 stimulated reporter gene expression from a variety of control promoters representing the HSV thymidine kinase or c-Met genes, or the early region of Simian Virus 40 (Bollig and Miksicek, unpublished data).

Mechanistically, we believe that the regulation of noncanonical promoters by wt ERα and ERα splicing variants involves protein-protein interactions with jun, fos, Sp1, or other unidentified upstream factors that in turn act through their cognate DNA-response elements. Among the genes examined, the ovalbumin and collagenase promoters (as well as the $p(AP1)_3$TK-CAT reporter) contain at least one consensus AP-1 element. While estrogen regulation of the chicken ovalbumin promoter was originally believed to involve cooperative binding of ERα to low affinity sites resembling portions of a palindromic ERE, these "half-sites" have been reinterpreted to represent an AP-1 motif. Indeed, induction of this promoter was clearly shown not to require the direct binding of ERα to DNA (16). Consistent with this fact, we observe that ERΔE3 (which lacks a DNA binding domain) is nonetheless active on this reporter plasmid. Even though ERΔE5 may possess very low affinity for a consensus ERE, binding of ERΔE5 to DNA is unlikely to occur under physiologic conditions. Certainly, the ability of ERΔE5 to stimulate the collagenase and IGF-1 promoters does not involve DNA-binding, as these promoters also lack consensus ERE motifs. The differences observed in the behavior of these promoters indicate that the context of the AP-1 motif (or other noncanonical ERE) can exert a significant effect on how

these promoters respond to ERα and its variants. Since only some of these differences can be ascribed to structural differences between the variants, differences in the identity of the transcription factors or co-regulators involved in the expression of each of these genes must also be involved.

As noted above, stimulation of transcription by ERΔE3 appears to require dual activation by both estradiol and phorbol ester, in clear contrast to wt ERα which can induce ERE-containing genes in response to estradiol alone. The situation for ERΔE5 is less clear, since efficient induction of the collagenase and AP-1 reporters by this splicing variant requires simultaneous treatment with phorbol ester, while stimulation of the IGF-1 promoter does not. We interpret these findings to imply that gene activation by ERΔE3 (and in some, but not all cases by ERΔE5) involves the recruitment of transcriptional regulatory proteins through their intrinsic activation domains. Indeed, in this context ERΔE3 and ERΔE5 themselves appear to be acting in the role of transcriptional co-regulators for other DNA-interacting transcription factors. Two activation functions have been described in wt ERα, one within the amino-terminus (commonly referred to as AF1) that is shared by both ERΔE3 and ERΔE5. A second estrogen-inducible activation function (AF2) resides within the carboxy-terminal ligand-binding domain and is therefore present in ERΔE3, but not in ERΔE5. Studies performed using intact ERα demonstrate that these activation functions correspond to binding sites for various co-regulatory proteins including CBP/p300 or p160 coactivators such as SRC-1. In fact, we have demonstrated that both ERΔE3 and ERΔE5, like wt ERα, can interact with the nuclear receptor coactivator SRC-1 (10). For ERΔE3, this interaction involves at least two SRC-1

interaction sites and is dramatically stimulated by ligand. Only the constitutive amino-terminal site (AF1) is present in ERΔE5. Whether transcriptional stimulation of reporters containing noncanonical hormone response elements (such as pOvalb-CAT or pColl(-73)Luc) involves a direct interaction between ERα or its isoforms and the AP-1 components jun and fos, or an indirect interaction mediated through a bridging factor such as CBP/p300 or SRC-1 needs to be further clarified. Our experiments showing that GST-ERα fusion constructs can co-precipitate both c-jun and c-fos doesn't fully address this issue since they were performed in reticulocyte lysates that also contain nuclear receptor coactivators. Additionally, the identities of the downstream targets for activation by phorbol ester, while presumed to involve the PKC pathway, remain to be identified. Targets that must be considered include AP-1, nuclear receptor co-regulators, or the ERα isoforms themselves. A general model comparing ERα action through consensus and non-consensus hormone response elements is presented in Fig. 6.

While our transfection studies fail to directly address the impact that ERΔE3 and ERΔE5 have on the transcription of endogenous genes in normal tissues or in tumors, these results suggest that they nonetheless have the potential to make distinctive and possibly unique contributions to patterns of gene regulation. As an extracellular matrix protease, collagenase (along with other metalloproteases) is likely to play an important role in the tissue remodeling and stromal invasion that occurs in metastatic disease. The IGF-1 gene is similarly expressed in a variety of tissues, including the liver, bone, uterus and breast where it contributes to the normal growth and differentiation of these structures (39-41, 44, 57). In the breast, some uncertainty remains regarding the actual source of IGF-1 (i.e., the cell of origin) and with respect to its precise role in breast tumor growth. It is likely that the mammary epithelium is exposed to IGF-1 produced locally by the breast

stroma as well as from systemic sources such as the liver. In addition, some breast tumors produce IGF-1 and other growth factors of their own (57). Expression of IGF-1 (and other growth factors) appears to be deregulated in some tumors, contributing an autocrine component to the tumor cell growth. The ability of ERΔE5 to stimulate transcription of the IGF-1 gene is therefore of potential clinical significance. Interestingly, we observe that ERΔE5, but not wt ERα promotes expression of a co-transfected IGF-1 reporter plasmid, predicting that IGF-1 production by breast tumors that over-express ERΔE5 will be insensitive to tamoxifen treatment.

Stimulation of IGF-1 transcription by ERΔE5 is attenutated, yet preserved on promoter fragments containing as little as 592 bp of upstream sequence. As with the $p(AP1)_3$TK-CAT reporter shown above, the activity of ERΔE5 implicates the N-terminal activation domain (AF1) or the DNA-binding domain in this effect, while the inactivity of wt ERα and ERΔE3 suggest that the presence of the C-terminal activation domain (AF2) prevents ERα from stimulating IGF-1 expression. However, in contrast with the $p(AP1)_3$TK-CAT reporter, the failure of phorbol ester to stimulate the activity of ERΔE5 on the IGF-1 promoter suggests that AP-1 is unlikely to be involved and that transcription factors other than jun or fos must also represent targets for ERΔE5 interaction.

Our observations provide further evidence that two ERα splicing variants, namely ERΔE3 and ERΔE5, like wt ERα can exert significant positive effects on gene transcription and raise the intriguing possibility that each one of these receptor isoforms may target a unique, but overlapping subset of genes for transcriptional regulation. In addition to these stimulatory effects on gene expression, both ERΔE3 and ERΔE5 have

been shown to inhibit the activity of wt ERα on ERE-containing genes (8-11). It is therefore reasonable to propose that over-expression of the nuclear splicing variants ERΔE3 and ERΔE5 relative to wt ERα has the potential to shift the pattern of gene expression away from ERE-containing genes to those that are regulated non-classically by estrogens. These results call into question the appealing, but simplistic notion that all of the biologically important regulatory effects of ERα are mediated by a single 595 amino acid form of this receptor that was originally described by Chambon and colleagues in 1986 (58). Rather, there is growing evidence that ERα splicing variants (most notably ERΔE3 and ERΔE5) may have a significant impact on the ability of the breast and perhaps other tissues to respond to estrogens.

EXPERIMENTAL METHODS

Expression Vectors

Expression plasmids derived from pCMV4 that contain ERα cDNAs corresponding to wt ERα, ERΔE2, ERΔE3, ERΔE4, ERΔE5, ERΔE6, and ERΔE7 have been previously described (10). Mouse c-jun and c-fos cDNA cloned into the pCMV2 expression vector were provided by L. McCabe (Michigan State University, East Lansing, MI).

Transient Transfection Analysis

Transcriptional activity of receptor variants was assessed by measuring their effect on the activity of chloramphenicol acetyltransferase (CAT) or luciferase (Luc) reporter genes expressed in HeLa cells (or in stable MDA MB231 transformants) using the calcium phosphate coprecipitation technique, as previously described (10). Plasmids expressing wt or variant ERα cDNAs were transfected into recipient cells along with the indicated estrogen-responsive reporter plasmids (see below). Hela cells were plated at 1.0×10^6 cells per 60 mm dish 12 hours prior to transfection. Cells were transfected with 0.5 μg of receptor expression plasmid and 10 μg of reporter plasmid. Total input DNA was normalized using an empty pCMV4 vector, which also served as a negative control. Following overnight incubation with DNA, transfected cells were cultured for 24 hours in Dulbecco's Minimum Essential Medium (DMEM) containing 5% charcoal-treated calf serum, in the absence and presence of 5 nM 17β-estradiol (E_2), or 20 nM phorbol 12-myristate, 13-acetate (PMA). CAT and Luc activities (corrected for protein concentration) were normalized to the basal level of expression seen for untreated pCMV4 control transfections. The reporter plasmids used for these experiments included pERE-TK-CAT

(59), p(AP1)$_3$TK-CAT kindly provided by L. McCabe (Dept. of Physiology, MSU), pColl(-73)Luc received from P. Kushner (18-20); and pIGF(-1630)Luc, pIGF(-926)Luc, pIGF(592)Luc, and pIGF(235)Luc obtained from P. Rotwein (45).

Protein-interaction assays

The full-length ER cDNA was engineered into the pGEX vector (Pharmacia Biotech, Uppsala, Sweden) and GST-fusion proteins were bacterially expressed. Radiolabeled c-jun and c-fos were expressed by *in vitro* translation using the TNT-coupled transcription translation system (Promega, Madison, WI) in the presence of ^{35}S-Methionine. GST-pull down assays were performed as previously described (10). Radiolabeled proteins were incubated with GST-ER complexed glutathione Sepharose 4B beads (Pharmacia) with or without 2.5 μM E_2. The samples were boiled and resolved by SDS-polyacrylamide gel electrophoresis (60).

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We are grateful to L. McCabe for kindly providing us with the c-jun and c-fos pCMV2 expression vectors, to P. Kushner for the pColl(-73)Luc construct, and to P. Rotwein for the pIGF(-1630)Luc, pIGF(-926)Luc, pIGF(-592)Luc, pIGF(-235)Luc reporter plasmids. We would also like to thank M. Morrison and D. Ankrapp for helpful suggestions during the course of this work.

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REFERENCES

1. Dowsett M, Daffada A, Chan CM, Johnston SR 1997 Oestrogen receptor mutants and variants in breast cancer. Eur J Cancer 33:1177-1183
2. van Dijk MA, Floore AN, Kloppenborg KI, van't Veer LJ 1997 A functional assay in yeast for the human estrogen receptor displays wild-type and variant estrogen receptor messenger RNAs present in breast carcinoma. Cancer Res 57:3478-3485
3. Fuqua SAW, Chamness GC, McGuire WL 1993 Estrogen receptor mutations in breast cancer. J Cell Biochem 51:135-139
4. Miksicek R J 1994 Steroid receptor variants and their potential role in cancer. Sem Canc Biol 5:369-379
5. Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH 1997 Oestrogen receptor variants and mutations in human breast cancer. Trends Mol Med 29:221-234
6. Sluyser M 1995 Mutations in the estrogen receptor gene. Human Mut 6:97-103
7. Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Malley BW, McGuire WL 1991 Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 51:105-109
8. Wang Y, Miksicek RJ 1991 Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol 5:1707-1715
9. Ohlsson H, Lykkesfeldt AE, Madsen MW, Briand P 1998 The estrogen receptor variant lacking exon 5 has dominant negative activity in the human breast epithelial cell line HMT-3522S1. Cancer Res 58:4264-4268
10. 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

11. Miksicek RJ, Lei Y, Wang Y 1993 Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. Breast Cancer Res Treat 26:163-174

12. Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engström O, Öhman L, Greene GL, Gustafsson J- Å, Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753-758

13. Kumar V, Chambon P 1988 The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-156

14. Chaidarun SS, Alexander JM 1998 A tumor-specific truncated estrogen receptor splice variant enhances estrogen-stimulated gene expression. Mol Endocrinol 12:1355-1366

15. Rea D, Parker MG 1996 Effects of an exon 5 variant of the estrogen receptor in MCF7 breast cancer cells. Cancer Res 56:1556-1563

16. Gaub MP, Bellard M, Scheuer I, Chambon P, Sassone-Corsi P 1990 Activation of the ovalbumin gene by the estrogen receptor involves the Fos-Jun complex. Cell 63:1267-1276

17. Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson JA, Kushner PJ, Scanlan TS 1997 Differential ligand activation of estrogen receptors ER-α and ER-β at AP1 sites. Science 277:1508-1510

18. Uht RM, Anderson CM, Wevv P, Kushner PJ 1997 Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. Endocrinology 138:2900-2908

19. Webb P, Lopez GN, Uht RM, Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9:443-456

20. Webb P, Nguyen P, Valentine C, Lopez GN, Kwok GR, McInerney I,

Katzenellenbogen BS, Enmark E, Gustafsson J-Å, Nilsson S, Kushner PJ 1999 The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 13:1672-1685

21. Duan R, Porter W, Safe S 1998 Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of extrogen receptor Sp1 complex formation. Endocrinology 139:1981-1990

22. Krishnan V, Wang X, Safe S 1994 Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. J Biol Chem 269:15912-15917

23. Sun G, Porter W, Safe S 1998 Estrogen-induced retinoic acid receptor alpha 1 gene expression role of estrogen receptor-Sp1 complex. Mol Endocrinol 12:882-890

24. Sabbah M, Courilleau D, Mester J, Redeuilh G 1999 Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. Proc Natl Acad Sci USA 96:11217-11222

25. Cavaillès V, Dauvois S, Danielian PS, Parker MG 1994 Interaction of proteins with transcriptionally active estrogen receptors. Proc Natl Acad Sci USA 91:10009-10013

26. Chen H, Lin RJ, Schiltz L, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM 1997 Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90:569-580

27. McInerney EM, Tsai M-J, O'Malley BW, Katzenellenbogen BS 1996 Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. Proc Natl Acad Sci USA 93:10069-10073

28. Voegel JJ, Heine MJS, Zechel C, Chambon P, Gronemeer H 1996 TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of

nuclear receptors. EMBO J 15:3667-3675

29. Yao T-P, Ku G, Zhou N, Scully R, Livingston DM 1996 The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. Proc Natl Acad Sci USA 93:10626-10631

30. Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, Hunter T 1991 Activation of protein kinase C decreases phosphorylation of cJun at sites that negatively regulate its DNA-binding activity. Cell 64:573-584

31. Westwick JK, Weitzel C, Minden A, Karin M, Brenner DA 1994 Tumor necrosis factor a simulates AP-1 activity through prolonged activation of the c-Jun kinase. J Biol Chem 269:2639-26401

32. Tora L, Gaub MP, Mader S, Dierich A, Bellard M, Dhambon P 1988 Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter. EMBO J 7:3771-3778

33. Angel P, Pöting A, Mallick U, Rahmsdorf HJ Schorpp M, Herrlich P 1986 Induction of metallothionein and other mRNA species by carcinogens and tumor promoters in primary human skin fibroblasts. Mol Cell Biol 6:1760-1766

34. Herrlich P, Angel P, Rahmsdorf HJ, Mallick U, Pöting A, Hieber L, Lücke-Huhle C, Schorpp M 1986 The mammalian genetic stress response. Adv Enzyme Reg 25:485-504

35. Whitham SE, Murphy G, Angel P, Rahmsdorf H-J, Smith BJ, Lyons A, Harris TJR, Reynolds JJ, Hierrlich P, Docherty AJP 1986 Comparison of human stromelysin and collagenase by cloning and sequence analysis. Biochem J 240:913-916

36. Liotta LA 1986 Tumor invasion and metastases-role of the extracellular matrix: rhoads memorial award lecture. Cancer Res 46:1-7

37. Angel P, Rahmsdorf HJ, Pöting A, Lücke-Huhle C, Herrlich P 1985 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced gene sequences in human primary

diploid fibroblasts and their expression in SV40-transformed fibroblasts. J Cell Biochem 29:351-360

38. Ernst M, Heath JK, Rodan GA 1989 Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insullin-like growth factor-I, and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. Endocrinology 125:825-833

39. McCarthy TL, Centrella M, Canalis E 1989 Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures from fetal rat bone. Endocrinology 124:1247-1253

40. Murphy LJ, Ghahary A 1990 Uterine insulin-like growth factor-1: regulation of expression and its role in estrogen-induced uterine proliferation. Endocrine Rev 11:443-453

41. Murphy LJ, Friesen HG 1988 Differential effects of estrogen and growth hormone on uterine and hepatic insulin- like growth factor I gene expression in the ovariectomized, hypophysectomized rat. Endocrinology 122:325-332

42. Stewart CEH, Rotwein P 1996 Growth, differentiation, and survival: multiple physiological function for insuling-like growth factors. Physiological Rev 76:1005-10026

43. Wolf M, Ingbar SH, Moses AC 1989 Thyroid hormone and growth hormone interact to regulate insulin-like growth factor-I messenger ribonucleic acid and circulating levels in the rat. Endocrinology 125:2905-2914

44. Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Moishima T, Yamasaki Y, Dajimoto Y, Kamada T 1994 Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem 269:16433-16442

45. Kim SW, Lajara R, Rotwein P 1991 Structure and function of a human insulin-like growth factor-I gene promoter. Mol Endocrinol 5:1964-1972

46. Hall LJ, Kajimoto Y, Bichell D, Kim S-W, James PL, Counts D, Nixon LJ, Tobin G, Rotwein P 1992 Functional analysis of the rat insulin-like growth factor I gene and identification of an IGF-I gene promoter. DNA Cell Biol 11:301-313

47. McCarthy TL, Ji C, Shu H, Casinghino S, Crothers K, Rotwein P, Centrella M 1997 17β-estradiol potently suppresses cAMP-induced insulin-like growth factor-I gene activation in primary rat osteoblast cultures. J Biol Chem 272:18132-18139

48. Umayagara Y, Billiard J, Ji C, Centrella M, McCarthy TL, Rotwein P 1999 CCAAT/enhancer-binding protein δ is a critical regulator of insulin-like growth factor-I gene transcription in osteoblasts. J Biol Chem 274:10609-10617

49. Nagaoka I, Trapnell BC, Crystal RG 1990 Regulation of insulin-like growth factor I gene expression in the human macrophage-like cell line U937. J Clinical Invest 85:448-455

50. Castles CG, Klotz DM, Fuqua SAW, Hill SM 1995 Coexpression of wild-type and variant oestrogen receptor mRNAs in a panel of human breast cancer cell lines. Br J Cancer 71:974-980

51. Gotteland M, Desauty G, Delarue HC, Liu L, May E 1995 Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues. Mol Cell Endocrinol 112:1-13

52. Friend KE, Resnick EM, Ang LW, Shupnik MA 1997 Specific modulation of estrogen receptor mRNA isoforms in rat pituitary throughout the estrous cycle and in response to steroid hormones. Mol Cell Endocrinol 13:147-155

53. Truss M, Beato M 1993 Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocrine Rev 14:459-479

54. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-Å 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925-5930

55. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguère V 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353-365

56. Chien CH, Wang FF, Hamilton TC 1994 Transcriptional activation of c-myc proto-oncogene by estrogen in human ovarian cancer cells. Mol cell Endocrinol 99:11-19

57. Pilihowska M, Kimua N, Fujiwara H, Nagura H 1997 Immunohistochemical study of TGF-alpha, TGF-beta, EGFR, and IGF-1 expression in human breast carcinoma. Mod Pathol 10:9699-9675

58. Green S, Walter P, Kumar V, Krust A, Bornet JM, Argos P, Chambon P 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erbA. Nature 320:134-139

59. Klock G, Strähle U, Schütz G 1987 Oestrogen and glucocorticoid responsive elements are closely related but distinct. Nature 329:734-736

60. Laemmli UK 1970 Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680-685

FIGURE LEGENDS

Fig. 1. Protein reading frames predicted from the sequences of cloned wt ERα splicing variants.

The variants are referred to by the deleted exon (exon 2 through exon 7). Abbreviations are N, amino terminus, C, carboxy terminus, NLS, nuclear localization signal, DBD, DNA-binding domain, and LBD, ligand-binding domain. The black rectangle at the end of the LBD represents the primary dimer contact site between ERα subunits. Cross-hatched lines indicate portions of the ERα reading frame that are not translated in each variant.

Fig. 2. Binding of c-jun and c-fos with GST-Fused wt ERα.

In the right panel, an autoradiograph demonstrates that *in vitro* translated, ^{35}S-methionine labeled c-fos and c-jun both bind wt ERα expressed as a GST-ERα?fusion protein. The AP-1 factors do not interact with GST expressed without wt ERα. The interaction of c-fos and c-jun with GST-ERα did not require the presence of 2.5 μM 17β-estradiol. Left panel shows 10% of the radiolabeled input.

Fig. 3. Transcriptional stimulatory activity of ERα splicing variants on a co-transfected human collagenase reporter plasmid, pColl(-73)Luc.

Hela cells were transiently transfected with 16 μg of reporter gene and 0.5 μg of receptor expression vector. Luciferase activities were normalized for protein and results are expressed relative to vehicle-treated empty expression vector (pCMV4) controls. Error

bars represent the standard error of the mean of three independent experiments. Panel A shows ERα and individual ERα splicing variant activity on pColl(-73)Luc. B, Cotransfection of c-jun expression vector (1 μg) enhances both ERΔE3 and ERΔE5 induction of reporter gene expression. Panel C demonstrates a breakdown of treatment conditions: 20 nM PMA with or without 10 nM 17β-estradiol (E) or or 100nM tamoxifen (Tam) for pColl(-73)Luc cotransfections with the pCMV4 or pCMV-ERΔE3 expression plasmids.

Fig. 4. Transcriptional activity of ERα splicing variants on cotransfected $p(AP1)_3$TK-CAT reporter plasmid.

HeLa cells were transiently transfected with 0.5 μg of pCMV vectors expressing the indicated receptor isoforms along with 10 μg of reporter gene. Transfected cells were treated with vehicle (ethanol + DMSO, 1% each) or 10 nM 17β-estradiol + 20 nM PMA (E + PMA) for 24 hours. Chloramphenicol acetylase (CAT) activities were normalized for protein and results are expressed relative to vehicle treated empty expression vector (pCMV4) controls. Error bars represent the standard error of the mean of three independent experiments.

Fig. 5. Transcriptional activity of ERα splicing variants on a cotransfected human IGF-I reporter plasmid.

A, HeLa cells were transiently transfected with 10 μg of pIGF-1(-1630)Luc and 0.5 μg of individual receptor isoforms. Luciferase activities were normalized for protein

and results are expressed relative to vehicle-treated empty expression vector (pCMV4) controls. Transfected cells were treated for 24 hours with ethanol + DMSO vehicle (V) or 10 nM 17β-estradiol + 20 nM PMA (E + PMA). Error bars represent the standard error of the mean of three independent experiments. B, ERΔE5 induces the expression of a reporter gene driven by a series of truncated IGF-1 promoters. Cotransfection of ERΔE5 demonstrates that, although it is dramatically attenuated with each successive loss of sequence, it is active on all lengths of the IGF-1 promoter tested. The length of IGF-1 promoter used in each luciferase reporter construct is specified by the number that counts base pairs upstream from the IGF-1 transcription start site.

Fig. 6. A model for the mechanism of transcriptional activation by ERα and its splicing variants through consensus and non-consensus estrogen regulatory elements.

FIGURE 1

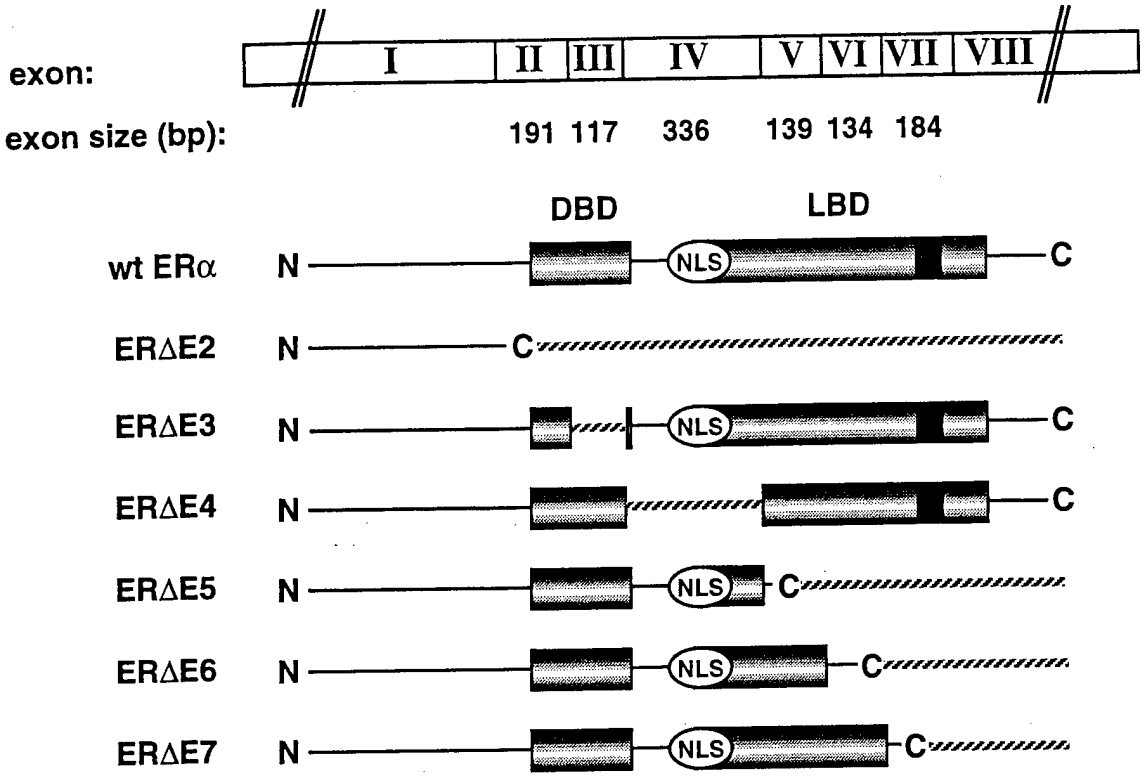

exon:
I
II
III
IV
V
VI
VII
VIII
exon size (bp):
191 117 336 139 134 184
DBD
LBD
wt ERα
N
NLS
C
ERΔE2
ERΔE3
ERΔE4
ERΔE5
ERΔE6
ERΔE7

FIGURE 2

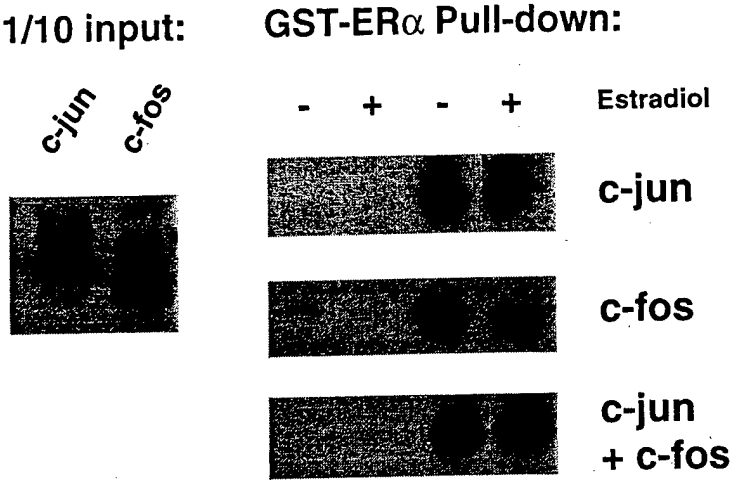

1/10 input:
GST-ERα Pull-down:
c-jun
c-fos
- + - +
Estradiol
c-jun
c-fos
c-jun
+ c-fos

FIGURE 3A

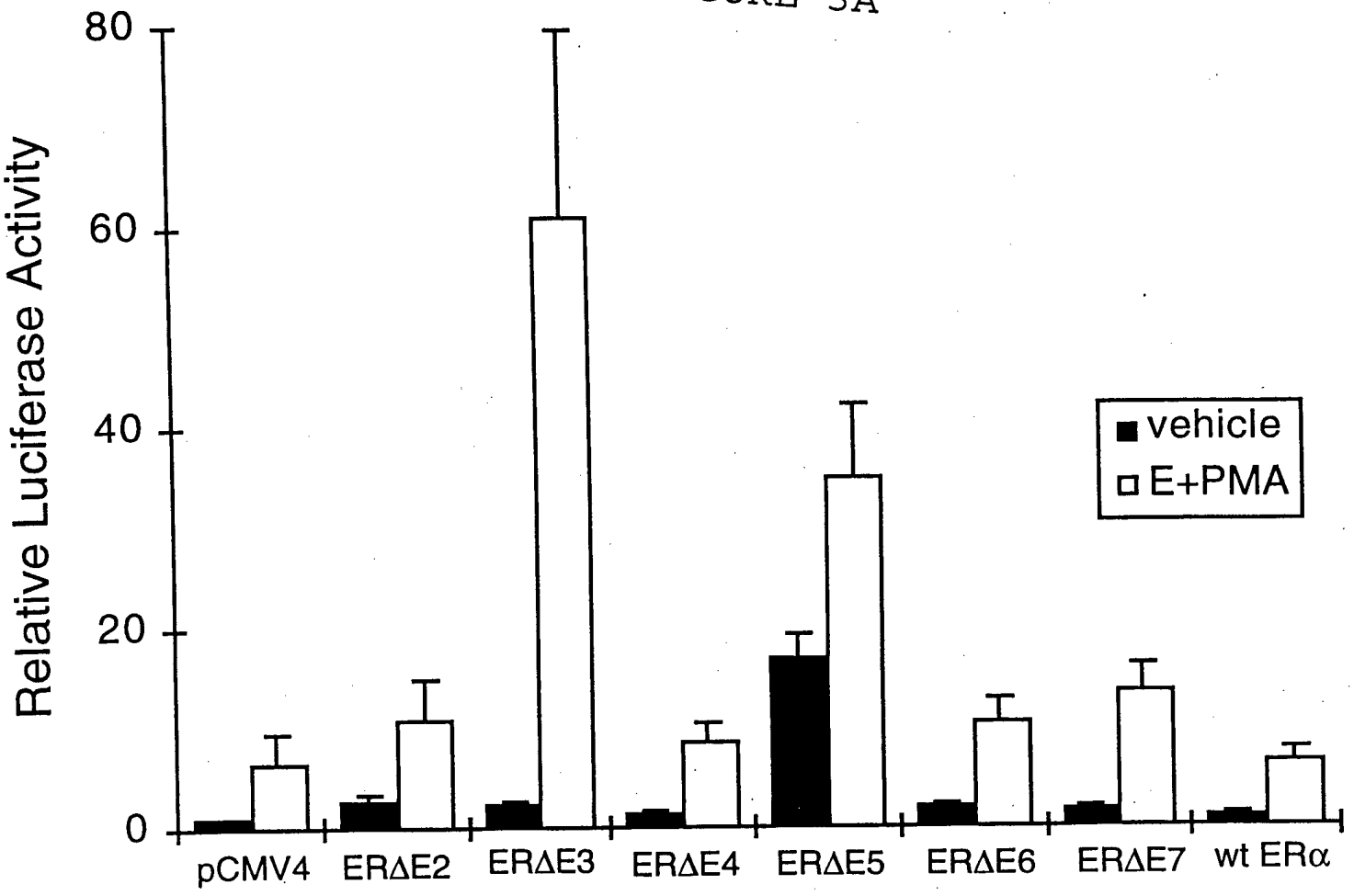

Relative Luciferase Activity
80
60
40
20
0
vehicle
E+PMA
pCMV4
ERΔE2
ERΔE3
ERΔE4
ERΔE5
ERΔE6
ERΔE7
wt ERα

FIGURE 3B

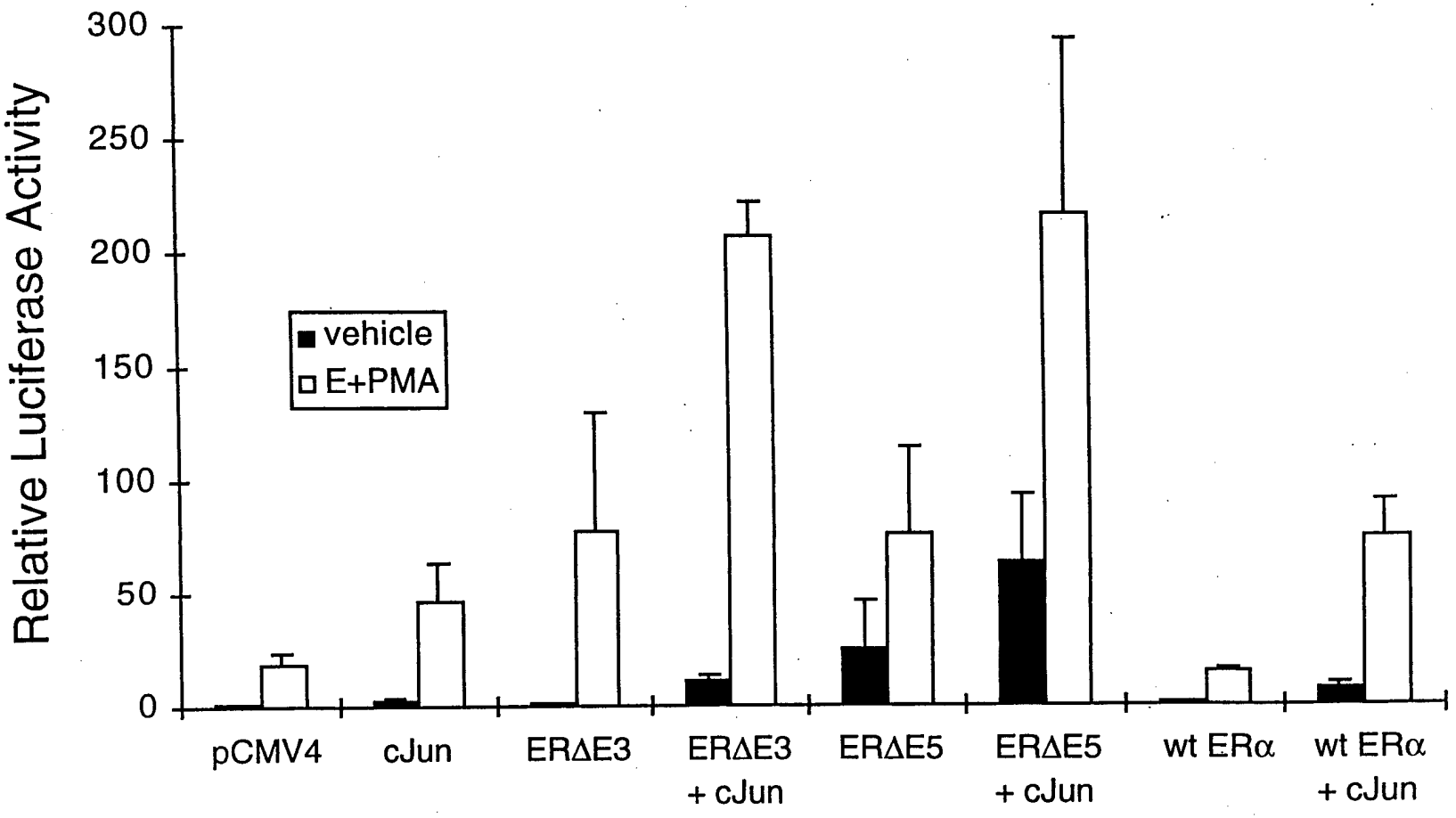

Relative Luciferase Activity
300
250
200
150
100
50
0
vehicle
E+PMA
pCMV4
cJun
ERΔE3
ERΔE3 + cJun
ERΔE5
ERΔE5 + cJun
wt ERα
wt ERα + cJun

FIGURE 3C

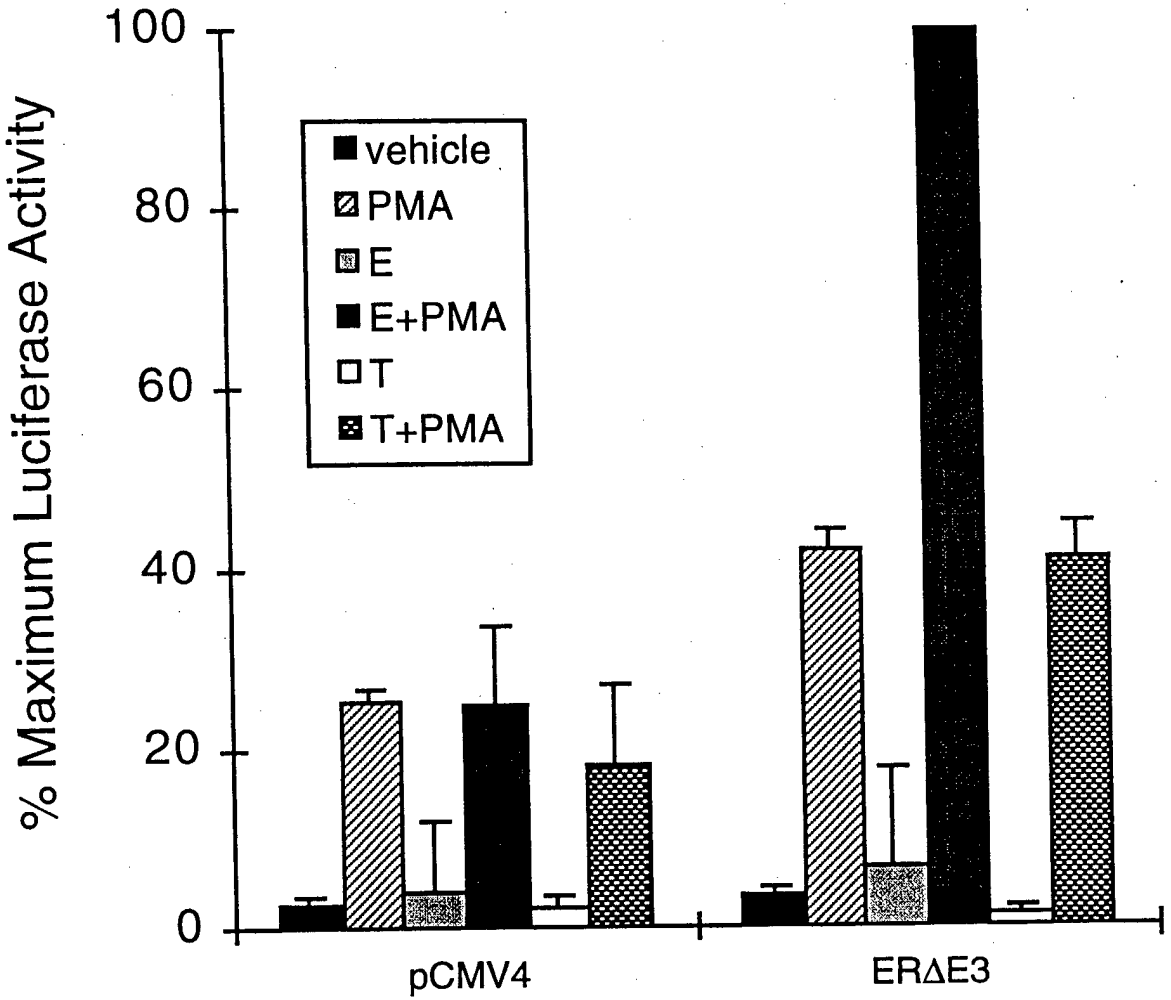

% Maximum Luciferase Activity
100
80
60
40
20
0
vehicle
PMA
E
E+PMA
T
T+PMA
pCMV4
ERΔE3

FIGURE 4

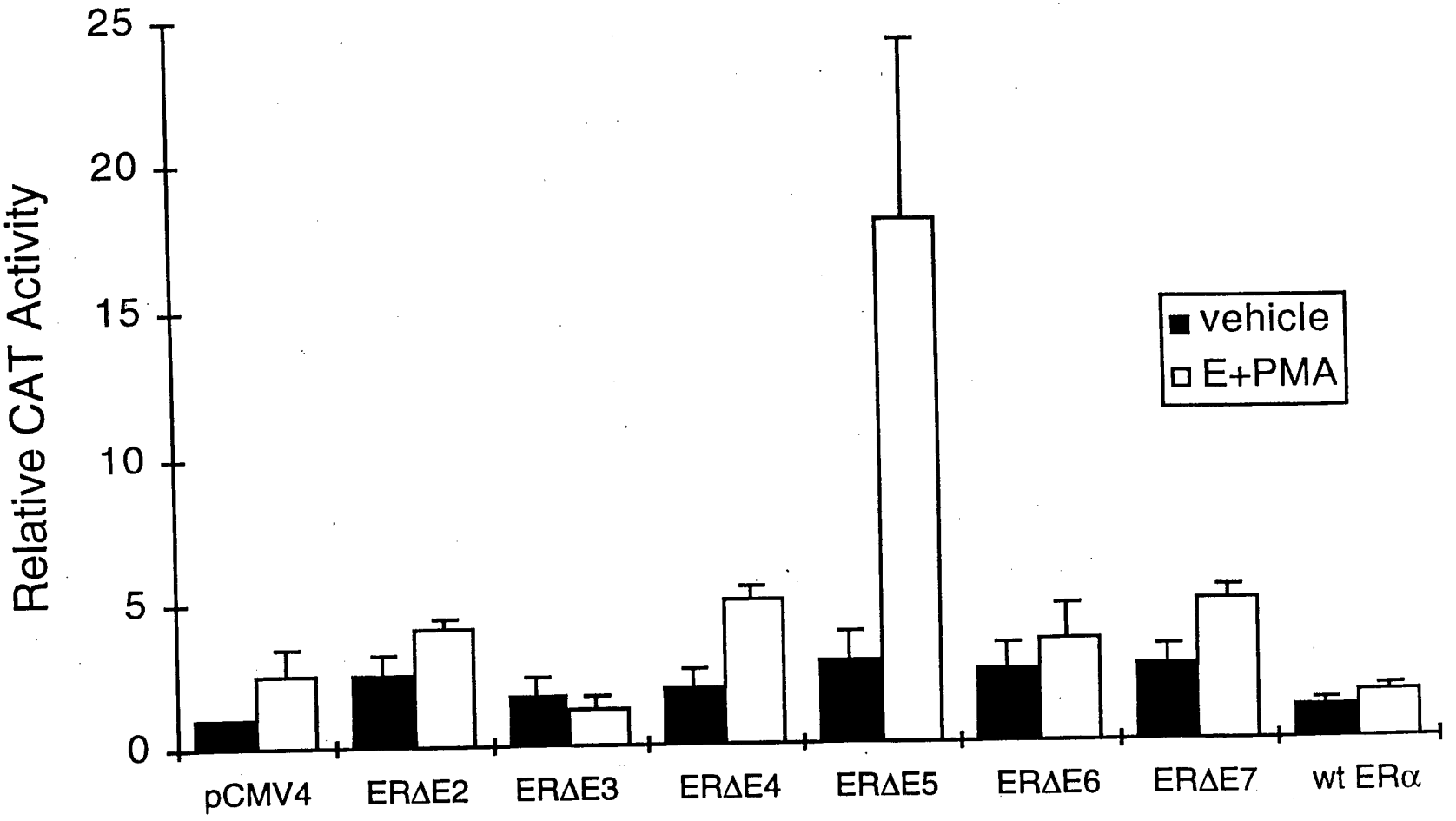

Relative CAT Activity
25
20
15
10
5
0
vehicle
E+PMA
pCMV4
ERΔE2
ERΔE3
ERΔE4
ERΔE5
ERΔE6
ERΔE7
wt ERα

FIGURE 5A

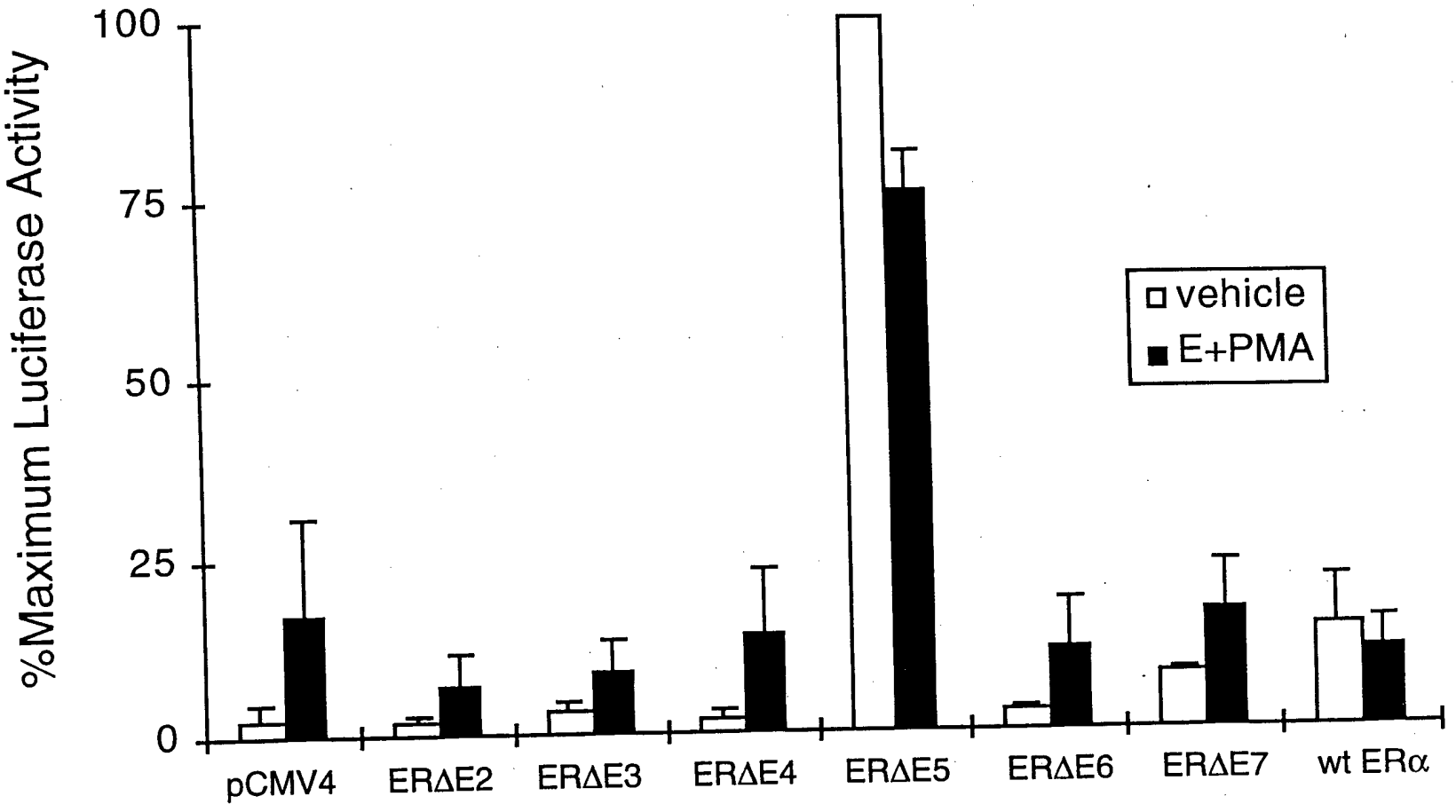

%Maximum Luciferase Activity
100
75
50
25
0
vehicle
E+PMA
pCMV4
ERΔE2
ERΔE3
ERΔE4
ERΔE5
ERΔE6
ERΔE7
wt ERα

FIGURE 5B

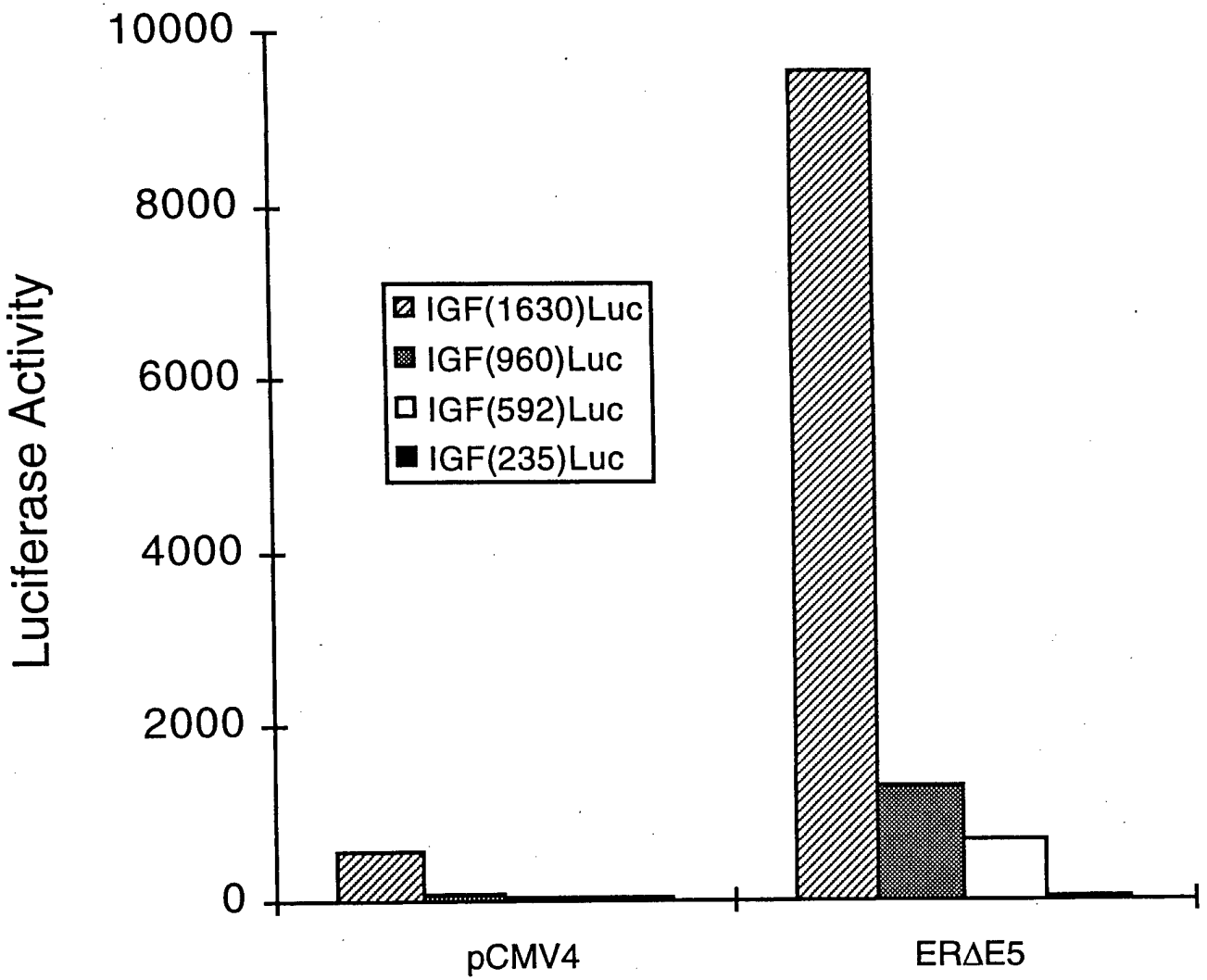
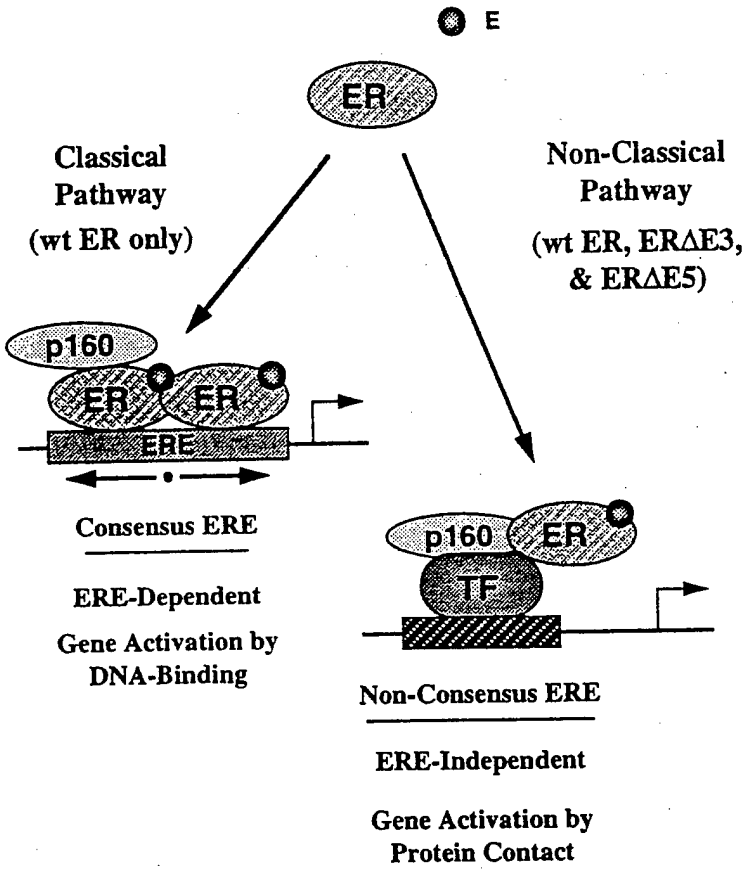

10000
8000
6000
4000
2000
0
Luciferase Activity
IGF(1630)Luc
IGF(960)Luc
IGF(592)Luc
IGF(235)Luc
pCMV4
ERΔE5

FIGURE 6


E
ER
Classical
Pathway
(wt ER only)
Non-Classical
Pathway
(wt ER, ERΔE3,
& ERΔE5)
p160
ER
ER
ERE
Consensus ERE
ERE-Dependent
Gene Activation by
DNA-Binding
p160
ER
TF
Non-Consensus ERE
ERE-Independent
Gene Activation by
Protein Contact