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PRINCIPAL INVESTIGATOR: Peter J. Kushner, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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Peter J. Kushner, Ph.D.

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University of California, San Francisco
San Francisco, California 94143-0962

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Tamoxifen-liganded estrogen receptor (ER) activates target genes regulated by AP-1 sites in uterine cells, an action that may underlie the estrogen-like side effects of tamoxifen. We tested the ER functions needed for this AP-1 pathway. Point mutations in the ER AF-1 or AF-2 interfere with ER action at a classical estrogen response element (ERE), but do not hinder tamoxifen action at AP-1 reporter genes. Similarly a point mutation that blocks ER dimerization is without effect on an AP-1 target. Deletion of the ER hinge region, which contains nuclear localization functions, blocks tamoxifen, but not estrogen action at AP-1. Although deletion of the DNA binding domain blocks tamoxifen action at AP-1, substitution of this domain with the DNA binding domain of the yeast GAL4 protein leaves tamoxifen activation of AP-1 targets These results indicate that transcriptional activation, dimerization, and ERE binding functions of the ER are not needed for the tamoxifen-AP-1 pathway, and they reinforce the notion that this pathway operates by protein-protein interactions.

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INTRODUCTION

Tamoxifen and other related "antiestrogens" sometimes have paradoxical estrogen-like effects. For example, whereas tamoxifen does not act as an estrogen to stimulate growth of breast tissue or cultured breast cells, it does act as an estrogen to stimulate growth of uterine tissue and cultured cells (1). These effects are paralleled by the estrogen-like ability of tamoxifen to stimulate transcription of many estrogen-target genes in uterine cells including C3 component of complement, alkaline phosphatase, progesterone receptor, and IGF-I (2-5). These effects of tamoxifen may underlie an increase in the frequency of uterine cancer seen in tamoxifen treated patients, an estrogen-like effect that may limit the usefulness of tamoxifen in the prevention of breast cancer (6). In addition to its estrogen like action on the uterus, during the course of breast tumor progression tamoxifen may acquire distressing estrogen-like effects on tumor growth (7-9).

The origin of the estrogen-like effects of tamoxifen has been attributed to two mechanisms. In each mechanism tamoxifen is postulated to bind competitively with estrogen to the ligand binding domain (LBD) of the estrogen receptor (ER) and thereby allow the receptor to become free of the complex with heat shock proteins that keep it inactive. In the first postulated mechanism the tamoxifen bound ER then binds to its cognate estrogen response element (ERE) in target genes via its DNA binding domain (DBD). Although the ER so bound is limited in its ability to stimulate transcription because the tamoxifen bound LBD has an inactive activator function 2 (AF-2), the activator function 1 (AF-1) in the receptor amino terminal domain (NTD) has variable residual activity and can stimulate transcription (10). For this mechanism to explain the tissue specific estrogen-like agonism of tamoxifen the variable AF-1 activity would have to be high in the uterus and low in breast tissues. Recent reports (work done by our lab in the first year of this grant) indicate, however, indicate that AF-1 is weak in its ability to stimulate transcription of ERE regulated reporter genes in cultured cells of uterine origin, and, indeed, is stronger in breast cells (11). Thus this first mechanism is unlikely to explain the tissue tropism of tamoxifen. A second postulated mechanism is that the tamoxifen bound ER is efficiently able to stimulate a second set of target genes that have cognate response elements for the AP-1 family (Jun/Fos family) of transcription factors. It has been noted by several laboratories that ER can activate genes regulated by Jun/Fos through uncharacterized protein-protein interactions (12-14). The activation requires the presence of AP-1 proteins and a cognate AP-1 site to which Jun/Fos, but not the ER, bind. Tamoxifen is a potent ligand for this ER/AP-1 pathway (11). Furthermore, the ability of tamoxifen to stimulate this pathway is strong in cells of uterine origin, but appears weak, or nonexistent, in cultured cells of mammary origin (11). Thus stimulation of this ER/AP-1 pathway may underlie the tissue specific estrogen-like effects of tamoxifen.

The precise biochemical mechanism by which tamoxifen stimulates the ER/AP-1 pathway is itself unknown. The pathway requires both ER and AP-1 proteins and occurs even when the AP-1 proteins are supplied by expression vectors at optimal levels (11) . It is thus unlikely to occur via an induction of endogenous AP-1. Two ER domains are needed for the tamoxifen pathway, the amino terminal A/B domain and the DBD. It is believed that these domains are needed for critical protein-protein interactions and not for AF-1 activity or ERE binding function, but this remains unproven. There is indirect evidence, however, that the tamoxifen pathway may be independent of transcriptional activation functions because the fusion of the powerful VP16 activation domain to the ER N-terminus did not appear to increase tamoxifen action (11).

Because the tamoxifen-ER-AP-1 pathway appears unusual, we have proceeded to examine in more detail the ER requirements for tamoxifen activation by testing the ability of various mutant and chimeric ERs to support tamoxifen activation at AP-1. We find that ER activation functions, as defined at an ERE, are not required for the tamoxifen-AP-1 pathway, nor is the LBD dimerization function. Although, as previously noted, the ER DBD is required for the pathway, the DBD of the yeast GAL4 protein suffices for this function. Thus this pathway requires neither conventional activation functions, nor the ability to bind to an ERE.

BODY

MATERIALS AND METHODS

Plasmid Construction

The GAL4 fusion protein responsive luciferase reporter gene ∂GK1 was derived from a similar reporter gene in which five GAL-UAS 17-mers were cloned upstream of the adenovirus e1b minimal promoter 5XGal-e1b-Luc20. The promoter/UAS unit was isolated by digestion with HinDIII and BamH1 and cloned into the equivalent sites of the parental vector coll73-LUC, in order that coll73 and 5XGal-e1b promoters could be compared in isogenic plasmid backbones, lacking the pUC AP-1 site.

ER-S118A and HE15-S118A were constructed by replacing a not/PstI restriction fragment overlapping the codon for residue S118 with a similar fragment from an ER expression vector (ERGG-S118A) in which the serine to alanine switch had already been made.

SG5-AB was constructed from vector EGE, in which the ER-DBD had been replaced with that of the GR. To facilitate this swap a KpnI site had been introduced after codon 184. The GR DBD and ER-LBD coding sequences were removed by digestion with KpnI and BamHI and a short oligonucleotide containing stop codons in all three frames was introduced such that the AB domain terminated immediately after residue 184. The nature of the vector was confirmed by sequence analysis.

Vectors pLEN, HER-T3, GST-AB and GST-LBD (11) have been previously described. SG5 was purchased from stratagene. ER expression vectors HE0, HEG0, HE15, HE11, EGE, HE72, GAL-AB, HE241G, HE15B, HE16 and HE17 were gifts from Pierre Chambon, Strasbourg France. Vector 5XGal-e1b-Luc20 was a gift from Michael Karin, San Diego. ERGG-S118A was a gift from Geoffrey Greene, University of Chicago.

Construction of ER Mutants Using Site Directed Mutagenesis

ER-L547A,M548A and ER-I514R were generated by site directed mutagenesis as previously described (11). The nature of each plasmid was confirmed with sequence analysis.

Construction of E-Gal-E Using Splicing by Overlap Extension

E(GAL)E, in which the sequences encoding amino acids 185-250 of HE0 have been replaced by sequences encoding amino acids 1-147 of yeast GAL4, was constructed by Splicing by Overlap Extension-PCR (26, 27). Sequences 5' and 3' to those encoding the ER DBD were amplified, using primers whose 5' ends contained GAL4 sequences. Similarly, GAL4 -DBD cDNA was amplified using primers whose 5' ends contained HE0 sequences. The initial products were combined in subsequent rounds of PCR. The contents of the PCR reactions were as follows :-0.5 µM primers; 200 ng template DNA; 40 µM dNTP's; 10 µl 10X buffer for *Pfu* DNA polymerase (Stratagene); 2.5 *Pfu* DNA polymerase (Stratagene)
H_20 to 100 µl. The reactions were overlaid with mineral oil and set up for the following program: (95° 2'>95 ° 1'>60° 1'>74° 1'>(repeat 25X)>74° 7'). All PCR products were separated on an agarose gel and extracted using QIAEX beads (Qiagen) before use in subsequent reactions.

The region 5' to the ER DBD was amplified, using linearized pSG5-HEO as a template, with the primers E(GAL)E1: 5'-GTGTACAACTACCCCGAGG-3' and E(GAL)E2: 5'ACAGTAGCTTCATGTAGCGAGTCTCCTTGGCA-3'. The GAL4 DBD was amplified from the plasmid pGBT9 (Clontech), using the primers E(GAL)E5: 5'-GGAGACTCGCTACATGAAGCTACTGTCTTC-3' and E(GAL)E6: 5'-TTCGTATCCCACCTTTCGATACAGTCAACTGT-3'. The products of these reactions were then combined in a subsequent round of PCR, along with the primers E(GAL)E1 and E(GAL)E6, to yield the fragment E(GAL). The region 3' to the ER DBD was amplified using the primers E(GAL)E3: 5'-AGTTGACTGTATCGAAAGGTGGGATACGAAAAGACC-3' and E(GAL)E4: 5'-GTTGGTCAGTAAGCC-3'. The product of this reaction was combined with E(GAL) in another round of PCR, using primers E(GAL)E1 and E(GAL)E6, to yield the final product,

E(GAL)E. The final PCR product was digested with *Not*I and *Hind*III and cloned into pSG5-HE0 at these naturally occurring restriction sites. The construct was confirmed by sequencing.

Cell Culture And Transfections
Hela cells were grown to a density of 4 million per 10 cm dish on Nunc tissue culture plastics as previously described (11). For transfection 1.5-2 million cells were electroplated with 10μg of reporter gene, 1μg of actin-β-hcg expression vector to normalize transfection efficiency and 0-5μg of ER expression vector normalized to 5μg of DNA by addition of control expression vector SG5 or pLEN where appropriate. Electroporations were carried out in standard buffers at 960μFd capacitance, 0.25KV. Each electroporation was plated on a six or twelve well tissue culture dish and treated with hormones as appropriate for forty hours.

Assays of Transfected Gene Products
Assays for Luciferase and CAT were carried out as previously described (11). Where appropriate, results were normalized to production of HCG in the medium.

In Vitro Translation
Labelled proteins were produced by incubation of plasmid DNA in a coupled in vitro-transcription/translation kit based on rabbit reticulocyte lysate (TNT, Promega).

Production of GST-ER Fusion Proteins and GST Binding Assays
Procedures were carried out as previously described (11). Fusions of GST to various domains of the human ER were prepared as follows. Briefly, bacteria expressing the fusion proteins were resuspended in buffer IPAB-80 (20 mM HEPES, 80 mM KCl, 6 mM MgCl2, 10% Glycerol, 1 mM DTT, 1 mM ATP, 0.2 mM PMSF and protease inhibitors; pH 7.9), sonicated mildly, and the debris was pelleted at 12,000 rpm for 1 hr in an ss34 rotor. The supernatant was incubated for 2 hrs. with 500 μL of glutathione sepharose 4B beads that were previously washed with 5 volumes of PBS 0.2 % Triton X-100 and equilibrated with 5 volumes of IPAB 80. GST-fusion proteins beads were then washed with 5 volumes of PBS 0.05% Nonidet P-40 and resuspended in 1 ml of IPAB-80 for storage at 4°C until use. All the above procedures were done in a cold room at 4°C.

Assays of GST-ER fusions were carried out in 100 μL volume that contained 40 μL of bead suspension (equivalent to 10 μL of compact beads volume) and 1 to 2 mL of 35S *in vitro* translated c-jun or c-fos in IPAB-80 2.5% non fat milk and incubated for 1.5 hr at 4°C. Beads were washed 5 to 6 times with IPAB-80 containing 0.05% Nonidet P-40. Input labelled proteins, proteins bound to GST, GST-hER and other ER fusion beads were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide and then to autoradiography.

RESULTS

Tamoxifen action at AP-1 is independent of ER AF-1 or AF-2, and is also independent of the dimerization function in the ER LBD.

Previous studies showed that tamoxifen bound ER activates a reporter gene with an AP-1 site only when the ER has a complete amino terminus and DNA binding domain (11).. However removal of the ligand binding domain yields a constitively active receptor that activates as strongly as the tamoxifen bound full length receptor. This suggests that functions in the ligand binding domain such the transcriptional activation function, AF-2, and the dimerization function are not needed for tamoxifen activation. To test this directly we examined the ability of tamoxifen to activate an AP-1 reporter gene in concert with ERs that had point mutations affecting AF-2, or the dimerization function. Mutations of two hydrophobic amino acids in the highly conserved activation helix of the mouse ER (15) and several other nuclear receptors abolishes AF-2 activity. When these amino acids were mutated to alanine in the human estrogen receptor (HER547,548A) activation at an ERE

reporter gene was abolished (Fig.1). Nonetheless, activation at an AP-1 reporter gene (collagenase promoter from -73 to + 63 encompassing the AP-1 site at -73 to -60) was, if anything, stronger in the case of the mutant ER than the wild type. Thus AF-2 is not needed for tamoxifen action at AP-1. This promoted us to also test the need for AF-1.
The full function of AF-1requires serine 118 (S118) a known site of phosphorylation (16). Mutation of S118 to alanine decreases AF-1 activity as measured on an ERE reporter gene 30% to 50% (17, 18). We observed such a decrease at an ERE reporter, but again the mutant receptor was, if anything, more potent than the wild type receptor in its ability to potentiate action at the AP-1 reporter (Fig.1). Similarly mutation S118A in the context of the constitutively active receptor missing the ligand binding domain only increased its activity on the AP-1 reporter.

To examine the role of the LBD dimerization function in the ER/AP-1 pathway we mutated isoleucine 514 to argenine. The analogous mutation in the mouse ER had previously been shown to block dimerization in vitro, and in transfected cells (19, 20). The human and mouse ERs are identical in this region, and it may be expected that a mutation in the human ER would also block dimerization. Consistent with this expectation, the I514R mutant was unable to activate transcription from the ERE reporter gene (Fig.2) The I514R mutant liganded to tamoxifen was, however, fully capable of activating the AP-1 reporter. These observations suggest that dimerization per se is not required for tamoxifen -ER activation at an AP-1 site.

The ability to bind to an ERE is not needed for tamoxifen-ER action at AP-1 sites.

Previous studies showed that deletion of the ER DNA binding domain from the full length ER eliminates tamoxifen action at AP-1 sites, but leaves residual estrogen activation (11). To examine the contribution of the DBD to tamoxifen action in more detail, we first dissected the ability of the truncated ER missing the LBD to activate the AP-1 reporter gene. As previously noted, this constitutively active truncated receptor activates as well as the tamoxifen liganded full length receptor, so we wished to determine whether it too required the DNA binding domain. Further deletion of the DBD, leaving only the A/B region, abolished the ability of this receptor to activate (Fig. 3). That the A/B fragment of the receptor was expressed well in the transfected cells was confirmed by its ability to interfere with expression of a wild type receptor at an ERE (data not shown). Similarly the ER DBD alone, without the A/B region, was unable to activate the AP-1 reporter. That this DBD fragment was expressed in the transfected cells was verified by its ability to interfere with wild type receptor at an ERE (not shown). These studies confirm the requirement for the ER DNA binding domain in activation of AP-1 regulated target genes by the ER.

To determine whether the requirement for the ER DNA binding domain is a specific requirement for ERE binding function, we tested a previously characterized chimera in which the A/B domain was fused to the DNA binding domain of the yeast GAL4 protein. This chimera had previously shown to be active in stimulating transcription from reporter genes with a response element for GAL4 (GAL-RE). We were surprised to observe that fusion of the DBD from the yeast GAL4 protein to the ER A/B domain forming GAL-AB (Fig.3) restored action at an AP-1 site. This activity required both the ER and GAL4 moieties because the GAL4 domain by itself, or fused to the activation domain of VP16 did not activate at an AP-1 site (Fig. 3), although the latter was a potent activator from a GAL-RE reporter (not shown). Since the GAL4 DBD can activate only at a cognate response element and not at an ERE, it appears that activation by the constitutively active truncated ER at AP-1 sites does not require the ability to bind to an ERE.

These observations prompted us to test whether tamoxifen activation in an ER derivative containing the LBD was also independent of the ability to bind to an ERE. We therefore constructed a chimera in which the DNA binding domain of the ER was exactly replaced with the DNA binding domain of GAL4. This receptor, E-GAL-E, was able to activate transcription in a hormone dependent manner from a GAL-RE but not from an ERE reporter (Fig. 3). The E-GAL-E receptor liganded to tamoxifen was also able to activate transcription from an AP-1 reporter. Indeed the spectrum of response of an AP-1 reporter to E-GAL-E with various ligands was indistinguishable from that of wild type ER (Fig.3). These studies together demonstrate that ER

action at AP-1 sites is basically independent of the ability to bind to an ERE. Although there may be a requirement for some sort of DNA binding domain, the requirement is non-specific.

The ER DNA binding domain contributes to binding interactions with Jun.

Previous studies using the GST "pull down" assay showed that ER, like other member of the steroid/thyroid family, binds to Jun, but not Fos in vitro (11). Jun binds to the ER A/B domain, but not to the ER LBD. In light of the above studies showing a contribution of the ER DNA binding domain to activation at AP-1 sites we examined in more detail the contribution of that domain to binding interactions with Jun. GST fusions of the ER A/B region were able to precipitate radiolabelled Jun (prepared by in vitro translation) from solution (Fig.4, lane 2) in conformity with previous studies. GST fused to the ER A/B+DBD regions showed a stronger interaction with radiolabelled Jun (Fig.4 lane 3). Thus, although the ER A/B region is able to interact with Jun on its own, the presence of the DBD strengthens the ER-Jun interaction.

A similar study with the c-fos gene revealed much weaker binding between the ER and the c-fos protein. We previously reported that binding of c-fos to ER was hard to detect. Fig. 4 B shows some very weak binding of c-fos to the ER, but at an overall level about ten times lower than that obtained with c-jun. Once again, this weak interaction was dependent upon the presence of the ER-DBD.

It has previously been reported that apparent interactions between DNA binding proteins may be obtained on GST columns that depend, not on protein-protein interactions, but rather upon spurious colocalization of both proteins upon sheared chromosomal DNA that may persist in bacterial extracts. This type of interaction is sensitive to ethidium bromide in the binding buffer. Figure 4 c reveals that interaction of GST-HE15 and c-jun protein is completely insensitive to 10μg/ml ethidium bromide suggesting that the ER-DBD mediates protein-protein contacts between the ER and c-jun.

The region spanning the constitutive nuclear localization signals of the ER contributes functions for activation at AP-1.

Although we previously tested each of the major domains of the ER for their ability to contribute to ER action at AP-1 sites, we had not tested the contribution of the hinge region between the DNA and ligand binding domains. This region has been shown to contain important elements for ER nuclear localization, a function that is distributed between three constitutive and cooperative nuclear localization sequences (NLS1-3, Fig.5) and an inducible nuclear localization function in the ligand binding domain (NLS-i, Fig.5) (21). Because, as noted above, ER absent the ligand binding domain activates at AP-1 in a manner similar to the tamoxifen liganded full length ER, we first examined the contribution of the hinge region to this activation. This permits an analysis that is not complicated by contributions of the inducible nuclear localization function. Deletion of NLS-1 (HE15, Fig.5) does not seriously impair ER action either at an ERE or an AP-1 site. Deletion of NLS1 and NLS 2 (HE15B, HE16 Fig. 5), or all three NLSs (HE17, Fig. 5), abolishes ER action at both an ERE and an AP-1 site. These observations suggest that nuclear localization function is needed for ER action at AP-1 as it is for ER action at an ERE.

We attempted to confirm the role of the previously identified nuclear localization sequences in the context of ER with the ligand binding domain. Deletion of most of NLS1 and 2 (HE12, Fig. 5) did not decrease estrogen action at either an ERE or AP-1 site. Tamoxifen action at an AP-1 site in HE12 was also retained. A longer deletion that removes all three NLS sequences (HE241G, Fig. 5) did block tamoxifen activation at AP-1 but left estrogen action at AP-1 unaltered. Unfortunately NLS3 is within the functionally defined DNA binding domain ,and it is not possible to determine from these observations whether loss of the ability of the tamoxifen bound ER after deletion of NLS3 reflects simple loss of nuclear localization ability or some other function. Nonetheless, these observations suggest that there is a requirement for some function in the hinge region and surround for tamoxifen-ER action at AP-1 sites (discussed below).

DISCUSSION

Previous studies suggested that the functions in the ER ligand binding domain were not needed for the tamoxifen-ER-AP-1 pathway because ER derivatives that were missing the LBD constitutively activated AP-1 reporters in parallel to the tamoxifen liganded full length receptor. We have confirmed that two of the known functions in this domain, the AF-2 transcriptional activation function and the dimerization function are not required for tamoxifen response. Point mutations in the activation helix in the ER LBD abolish AF-2 activity and interfere with activation at an ERE. Such mutations leave tamoxifen (and other ER ligands) action at AP-1 unaffected or indeed magnified. A mutation that interferes with AF-1 has a similar effect. Thus the conventionally defined AF-1 and AF-2 functions are not needed for tamoxifen action at AP-1. The same may be said for the dimerization function in the ER LBD. A point mutation in the ligand binding domain (I514R) that is known to block dimerization in vitro abolishes action at an ERE without decreasing action at AP-1 regulated target genes.

Several previous studies showed that the DNA binding domain of the ER was partly dispensable for estrogen-ER activation of AP-1 regulated target genes (11, 14). This suggested that such activation was through ER-target protein interactions rather than through interactions between ER and specific elements on DNA. When it was found that tamoxifen also activates the ER-AP-1 pathway and in a cell specific manner it was natural to assume that ER-protein interactions were also responsible. However, unlike estrogen activation at AP-1 reporters, tamoxifen activation was reported to require the presence of the ER DBD. This complication raised the faint possibility that tamoxifen activation occurs through ER -ERE interactions of some sort. The observations in this study show that the ability to bind and activate at an ERE is not essential for tamoxifen-ER action at AP-1 regulated target genes. Replacement of the DNA binding domain of the ER by the DNA binding domain of the yeast protein GAL4 leaves intact the ability of the receptor to activate AP-1 regulated reporters in the presence of tamoxifen. Since this chimeric receptor is unable to activate transcription from classical EREs it is clear that such activation is not essential for tamoxifen action at AP-1. Thus the tamoxifen stimulated ER pathway to AP-1 is likely to involve ER-protein rather than ER - DNA interactions, and the requirement for the DNA binding domain may reflect a subtle feature of those protein interactions.

The target of the ER in these hypothesized protein interactions is uncertain. A candidate is Jun itself. In this regard, we observed that the DNA binding domain of the ER cooperates with the A/B domain in binding interactions with Jun. Curiously, the GAL4 DNA binding domain, which can replace the ER domain for tamoxifen activation has been reported to bind to Jun (22). These observations are consistent, but not compelling, for a role for the ER-Jun interaction in tamoxifen action at AP-1 sites. However, the notion of a direct contribution of the ER-Jun binding to tamoxifen activation should be approached with caution . Whereas the interaction between steroid receptors and Jun has been detected in several labs, none have as yet confirmed that mutations that disrupt this interaction disrupt modulation of AP-1 target genes by steroids. It remains possible the ER-Jun interaction is fortuitous, and not part of the mechanism of ER action at AP-1 sites.

We also explored the contribution of the nuclear localization sequences distributed in and around the hinge domain of the ER to the receptor's ability to activate at AP-1. In the absence of both the ligand binding domain, with it inducible NLS, and NLS1, which borders the LBD, the residual nuclear localization functions allow the ER to activate at both an ERE and AP-1 site. Further deletion of NLS2, or both NLS2 and 3, which are known to eliminate nuclear localization (21), abolish action at both an ERE and AP-1 site. Thus nuclear localization may be needed for ER action at AP-1.

In the context of the full length ER the region spanning NLS3 to NLS1 (amino acids 250-300) is needed for tamoxifen, but not estrogen action at AP-1. The 250-300 region is needed for action at an ERE presumably because it contains several of the distributed and cooperative nuclear localization signals in the estrogen receptor (21). It is uncertain, however, whether the requirement for this domain in the tamoxifen- AP-1 pathway similarly reflects a requirement for nuclear localization. Other functions besides nuclear localization have been identified in this region. In particular, the 250-300 region is needed for ER repression of gene expression from a truncated

prolactin promoter (23). In this repression pathway tamoxifen acts as an agonist (24). This region also appears to play a role in ER interactions with NFκB and CEBP on the IL-6 promoter in bone cells (25). Thus the 250-300 region may have some, as yet unidentified, function involved in each of these pathways.

Together these observations indicate that the tamoxifen liganded ER activates target genes regulated by AP-1 sites by an unusual mechanism that, with the possible exception of nuclear localization, does not exploit the functions of the ER that are integral to action at an ERE. The nature of this unusual mechanism is unclear. We previously speculated that ER action at AP-1 occurs through two formally distinguishable pathways. One is a DBD requiring, tamoxifen activated, pathway that is restricted to uterine cells but is not operative in breast cells. The other is a DBD independent pathway that is not cell specific and appears to require estrogen or another agonist. We speculated that this latter, more universal, pathway represented tethering of the ER on Jun/Fos at AP-1 sites, and synergy between ER and Jun/Fos activation functions. We offered no more detailed account of the tamoxifen stimulated pathway than to say it did not occur through such tethering. The present observations that neither AF-1 nor AF-2 are needed for tamoxifen action at AP-1 reinforce the notion that such activation does not occur by tethering. We elsewhere, based on studies of AP-1 activation functions, suggest a different mechanism for tamoxifen activation (in preparation).

CONCLUSIONS

Tamoxifen-liganded estrogen receptor (ER) activates target genes regulated by AP-1 sites in uterine cells, an action that may underlie the estrogen-like side effects of tamoxifen. We tested the ER functions needed for this tamoxifen AP-1 pathway. Point mutations in the ER AF-1 or AF-2 interfere with ER action at a classical estrogen response element (ERE), but they increase rather than hinder tamoxifen action at AP-1 reporter genes. A point mutation that blocks ER dimerization and action at an ERE does not hinder tamoxifen action on an AP-1 target. Although deletion of the DNA binding domain blocks tamoxifen action at AP-1, substitution of this domain with the DNA binding domain of the yeast GAL4 protein restores tamoxifen activation of AP-1 targets. Deletion of the ER hinge region and surrounding nuclear localization signals does interfere with tamoxifen, action at AP-1. These results indicate that transcriptional activation, dimerization, and ERE binding functions of the ER are not needed for the tamoxifen-AP-1 pathway, and they reinforce the notion that this pathway operates by, possibly nuclear, protein-protein interactions. These results suggest that the tamoxifen bound estrogen receptor exerts estrogen-like effects through an unusual mechanism which may be a potential target for drug therapy to block these effects.

Relationship to the Statement of Work

With the work described above and the work described in annual report for 1995 we consider that Task 1 is substantially complete. We have been working hard on Task 2 and have made some unexpected findings that cast a new light on the role of AP-1 proteins in the tamoxifen-ER pathway. Although this work is far advanced, we do not have figures or text in a form ready for this report. We would, however, like to include the following abstract summary.

The motive for surveying Jun-Fos family members for their ability to contribute to ER action at AP-1 was that such a survey might give clues as to why the estrogen-like effects of tamoxifen at AP-1 sites was tissue specific. We decided, in parallel, to explore which functions of the AP-1 proteins were required for tamoxifen action. In particular, we explored whether the ER could engage the activation functions of AP-1 proteins when they were presented as fusions to a heterologous DNA binding domain. We examined ER action on chimeras in which the yeast GAL4 DBD was fused to the activation domains of Jun, Fos and Elk 1 (a major activator of the fos gene and a well characterized target of signal transduction pathways that activate AP-1). We tested the ability of ER to activate a reporter gene with binding sites for GAL4. Tamoxifen bound ER potentiated the Jun, Fos and Elk-1 activation functions and the activation was comparable to that achieved by extracellular signals that are known to potentiate those activation functions through signal transduction cascades. Like pathways from extracellular stimuli, ER activation of Jun, Fos, and Elk requires the integrity of MAP kinase pathways and can be disrupted with either pharmaceutical or genetic reagents that block these pathways. However, our biochemical studies show that the ER does not directly activate these pathways. Moreover, ER activates GAL4 fused to v-Jun a viral derivative of Jun that does not require nor is subject to phosphorylation by extracellular signal transduction pathways for activity. Thus the ER activates AP-1 by directly engaging Jun, Fos, and Elk activation functions. This requires the activity of MAP kinase pathways, but the ER does not act by itself activating classical MAP kinase pathways.

Most surprising, the ability of ER to engage the activation functions of AP-1 proteins was tissue specific. For example, ER activated GAL-Jun in several uterine derived cell lines, but in none of the breast cell lines tested. These observations suggest that the tissue specificity of tamoxifen action may reflect a basic feature of ER's ability to engage the activation functions of AP-1 proteins, and not, as we had previously suspected, tissue specific variation in the composition of AP-1 proteins in the cell. We have some ideas as to the nature of this tissue specific variation and we are exploring them.

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APPENDICES

FIGURE LEGENDS

Fig 1. Tamoxifen activation at AP-1 is enhanced, not reduced, by mutations that interfere with the activity of ER Transactivation Functions at an ERE.

A) Mutations in the context of full length ER. Hela cells were transfected with 5 μg of the indicated AP-1 reporter gene driving luciferase expression and an isogenic ERE regulated reporter promoter driving CAT. Also included was 5μg of the indicated expression vectors for ER derivatives. Reporter gene activities were measured in extracts prepared from cells that were treated either without hormone or with saturating concentrations of ICI 164,384 (ICI, 1μm), tamoxifen (5 μm), or estradiol (100nm). Reporter activities are normalized to a transfection control reporter in which βhCG is driven by the actin promoter.

B) Effects of the AF-1 serine 118 mutation in truncated ER devoid of the ligand binding domain, which activates as well, or better than, tamoxifen liganded full length ER. SG5 is the expression vector devoid of ER sequences.

Fig.2 A point mutation that interferes with ER dimerization blocks action at an ERE, but leaves activation at AP-1 intact.

Hela cells were transfected with the an AP-1 or ERE responsive isogenic reporter based upon the collagenase minimal promoter. Also included in the transfection were 5μg of empty expression vector pLEN, HER-T3 or HER-I514R. Hormones were added as described for Fig. 1.

Fig.3 ERE binding function is not required for tamoxifen-ER action at AP-1.

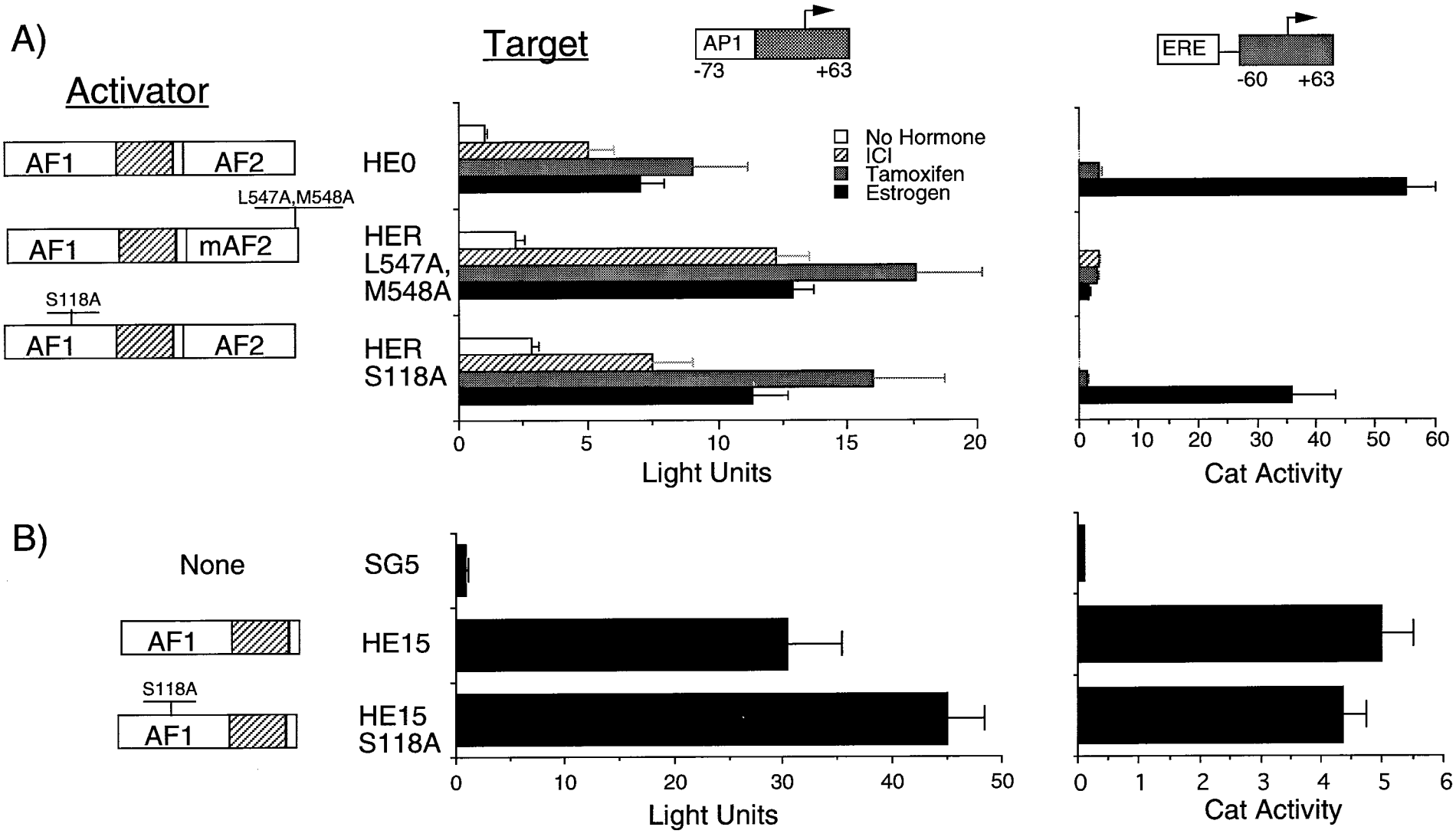
A, Fusing the DNA binding domain of the yeast GAL4 protein to the ER A/B domain allows activation of AP-1 reporter genes. Hela cells transfected as in Fig. 1 with the AP-1 reporter gene and the indicated activator proteins, derived from a truncated ER missing the LBD.

B, Substituting the GAL4 DNA binding domain for that of the ER allows tamoxifen activation at AP-1 sites without ER activity at an ERE. Response of reporter regulated by an AP-1 site to depicted derivatives of full length ER with deletions or substitutions in the DNA binding domain. The reporters are depicted above their plotted response. Hela cells transfected as in Fig. 1.

Fig. 4 The DNA binding domain of the ER contributes to binding interactions with Jun.

A) Autoradiogram of the products of a binding reaction between labelled Jun and GST fusions to ER proteins. The fusion proteins are depicted above the photograph. Lane 1 represents 20% of the input Jun protein. Lane 2 is binding to control GST beads, lane 3 to GST-HE15; A/B + DBD (amino acids 1-281), lane 4 to GST ER A/B (amino acids 1-184), lane 5 and 6 to GST-LBD (amino acids 282-595), in the absence and presence, respectively, of estrogen. The position of molecular weight markers is noted. B) A parallel study with labelled Fos. C) Interaction between the ER-DBD and Jun is insensitive to ethidium bromide. An autoradiogram of binding reaction products between GST-HE15 and radiolabelled c-jun. Lane 1, 20% of the input Jun protein, Lane 2 and 3 GST control beads without and with 10μg/ml ethidium bromide, Lane 4 and 5 GST-HE15 without and with ethidium bromide.

Fig. 5. The region spanning the constitutive nuclear localization signals of the ER contributes functions for activation at AP-1. A) A comparison of the ability of full length receptors with deletions extending through the hinge region to activate target reporter genes regulated by AP-1 or ERE sites. Reporters were transfected as in Fig.1 along with the depicted expression vectors. The extent of the deletions is indicated at left. HER241G deletes amino acids 250-303; HE12 deletes amino acids 270-300. B) Analysis of the contribution of the NLS sequences to activation at AP-1 sites in the background of an ER missing the ligand binding domain. Various ER deletions with the C-terminal amino acid indicated were transfected into HeLa cells as in Fig. 1B and tested for activation of AP-1 and ERE reporters. The location of the NLS sequences is indicated at left.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+63
ERE
-60
+63
AF1
AF2
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mAF2
S118A
HE0
HER
L547A,
M548A
HER
S118A
No Hormone
ICI
Tamoxifen
Estrogen
0
5
10
15
20
Light Units
0
10
20
30
40
50
60
Cat Activity
B)
None
SG5
HE15
HE15
S118A
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10
20
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40
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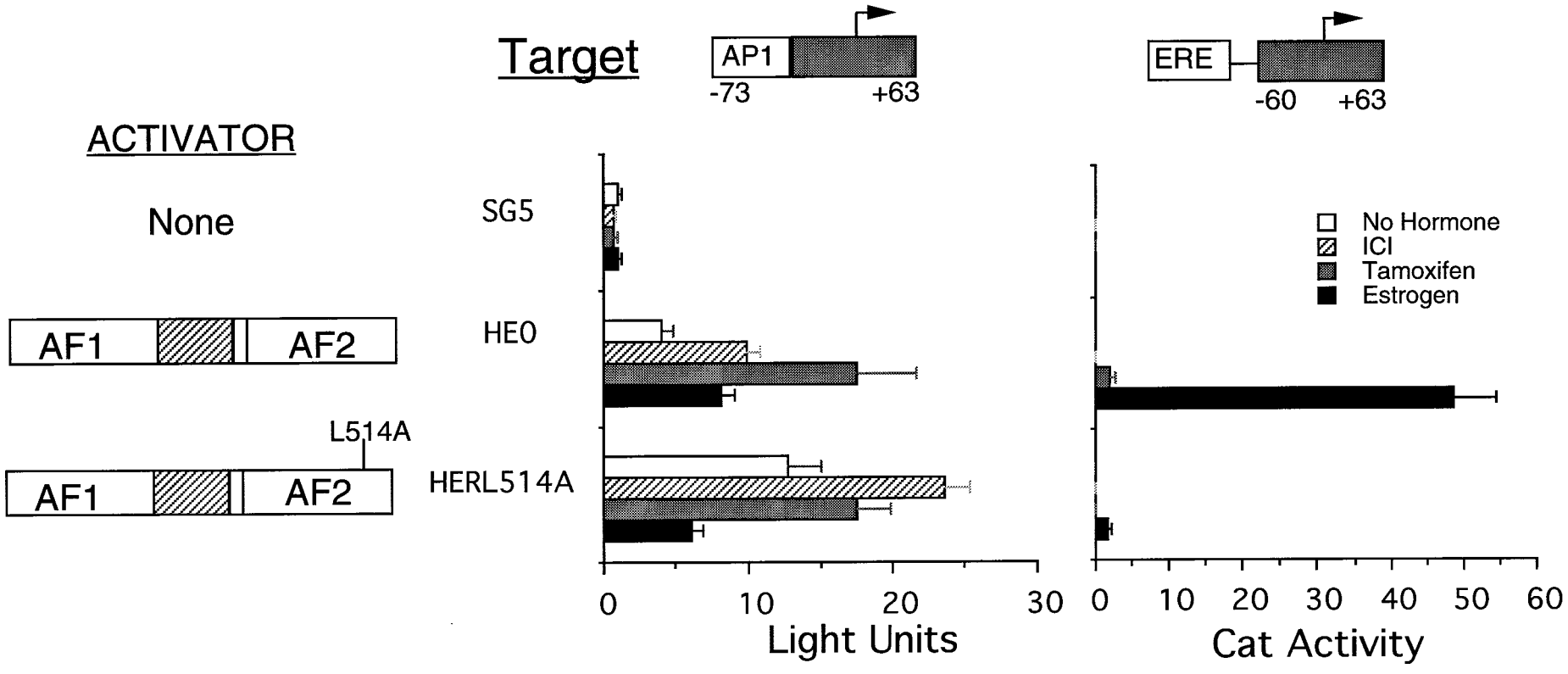
Light Units
0
1
2
3
4
5
6
Cat Activity

Figure 1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+63
ERE
-60
+63
ACTIVATOR
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AF2
L514A
AF1
AF2
SG5
HEO
HERL514A
0
10
20
30
Light Units
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10
20
30
40
50
60
Cat Activity
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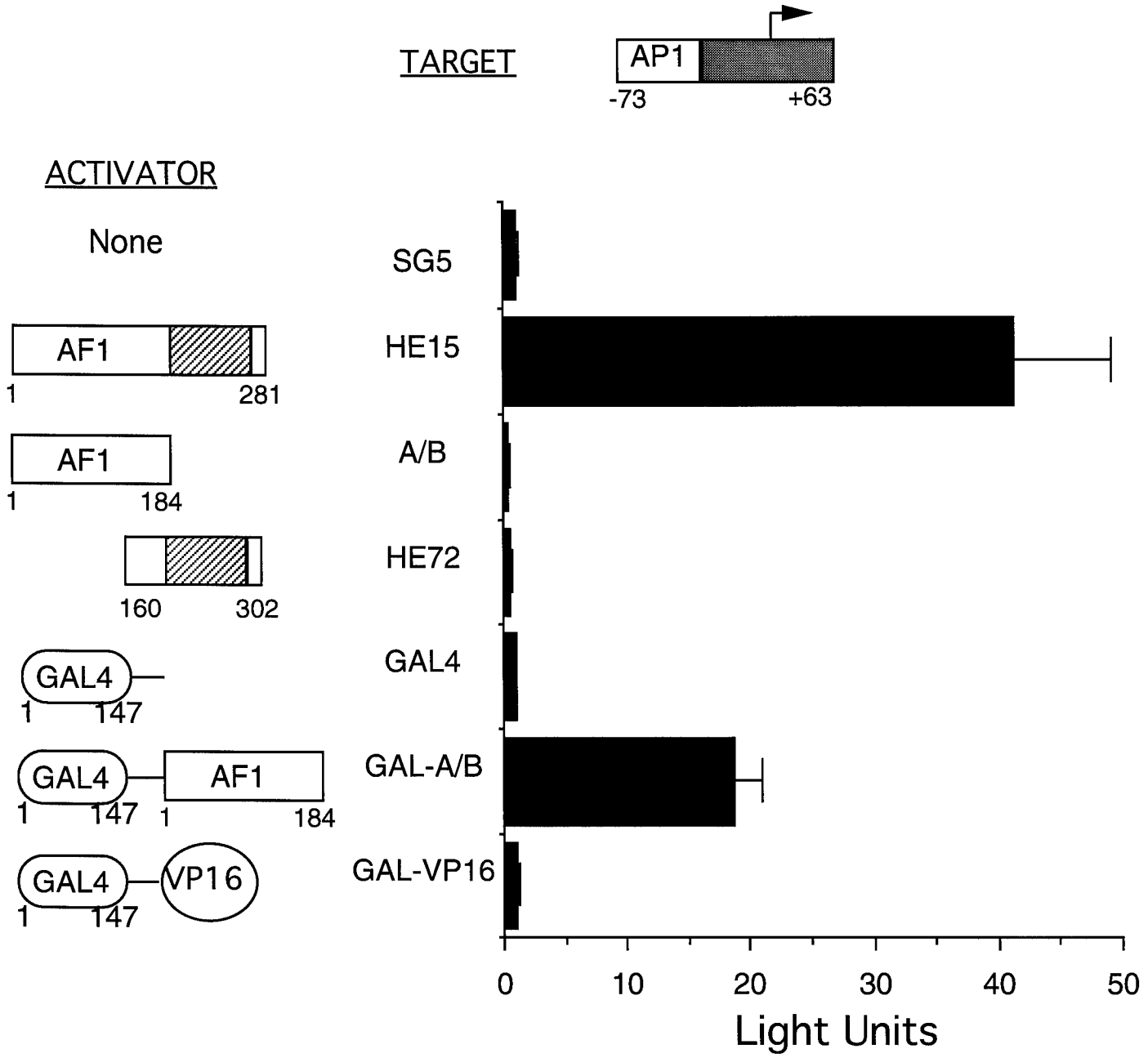
ICI
Tamoxifen
Estrogen

Figure 2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AF1
1
147
1
184
GAL4
VP16
1
147
SG5
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30
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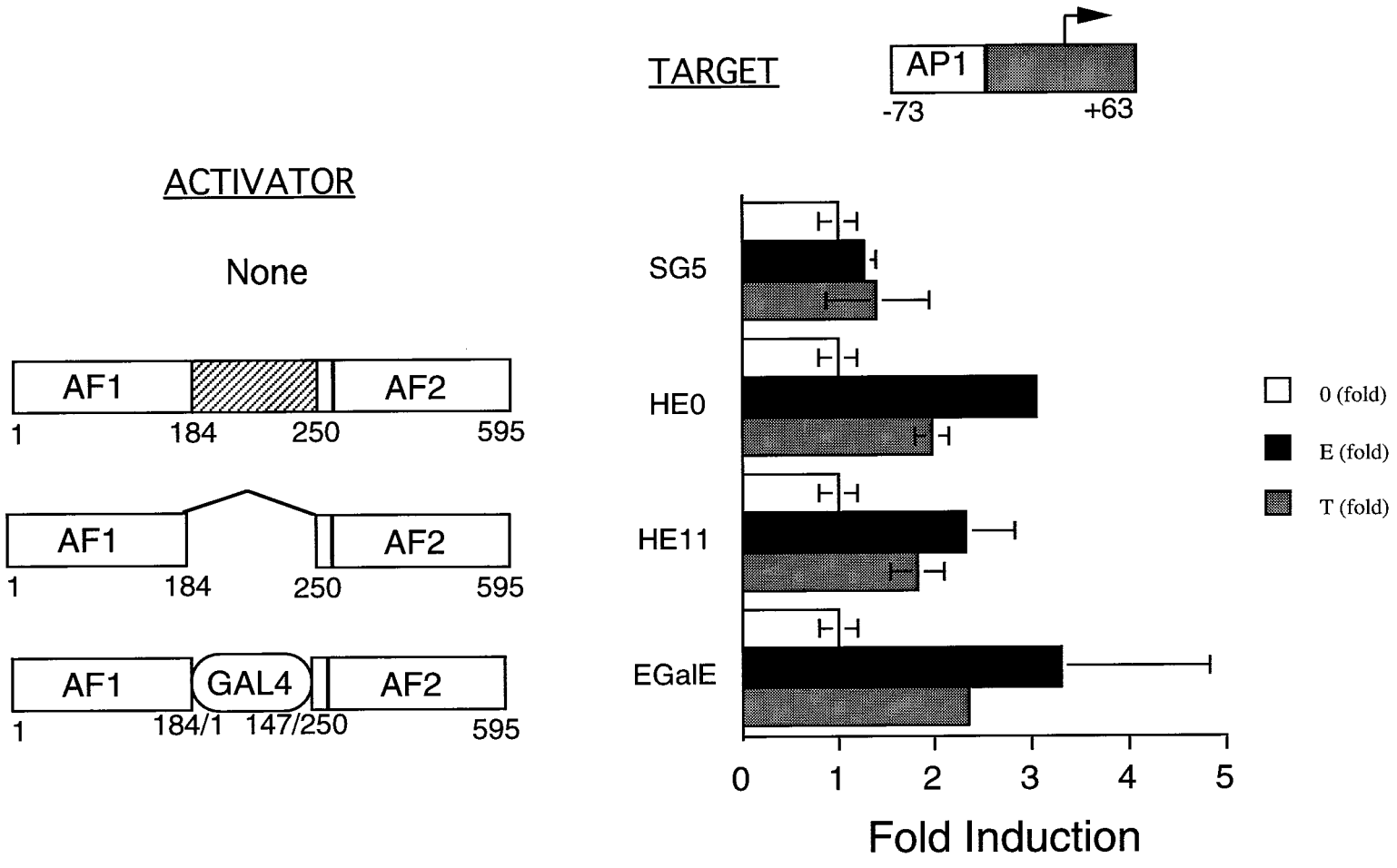
Light Units

Figure 3a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+63
ACTIVATOR
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1
184
250
595
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595
AF1
GAL4
AF2
1
184/1
147/250
595
SG5
HE0
HE11
EGalE
0
1
2
3
4
5
Fold Induction
0 (fold)
E (fold)
T (fold)

Figure 3b

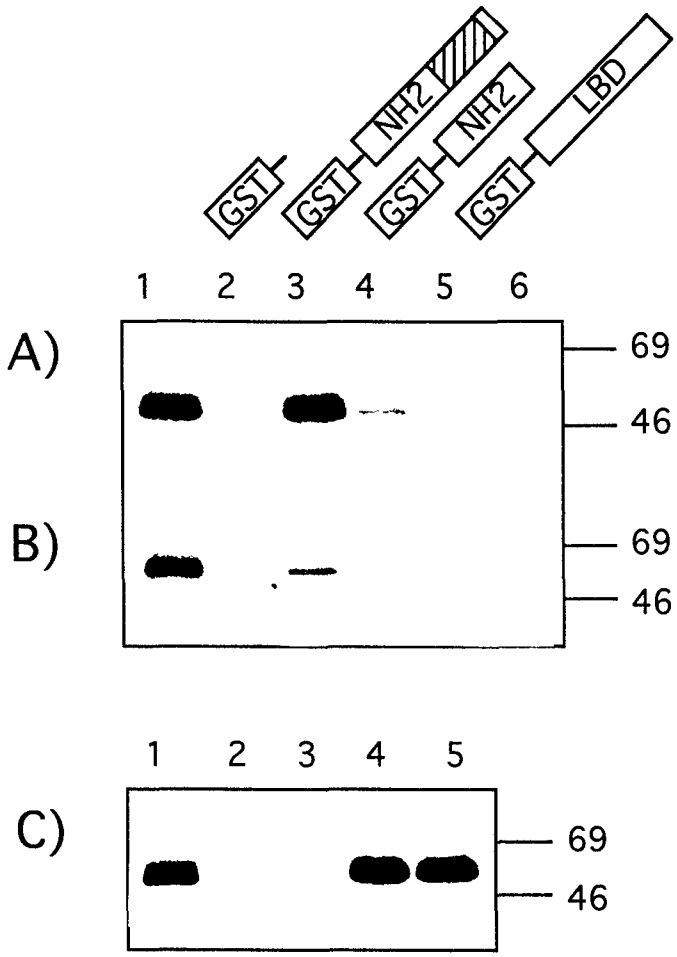

Figure 4

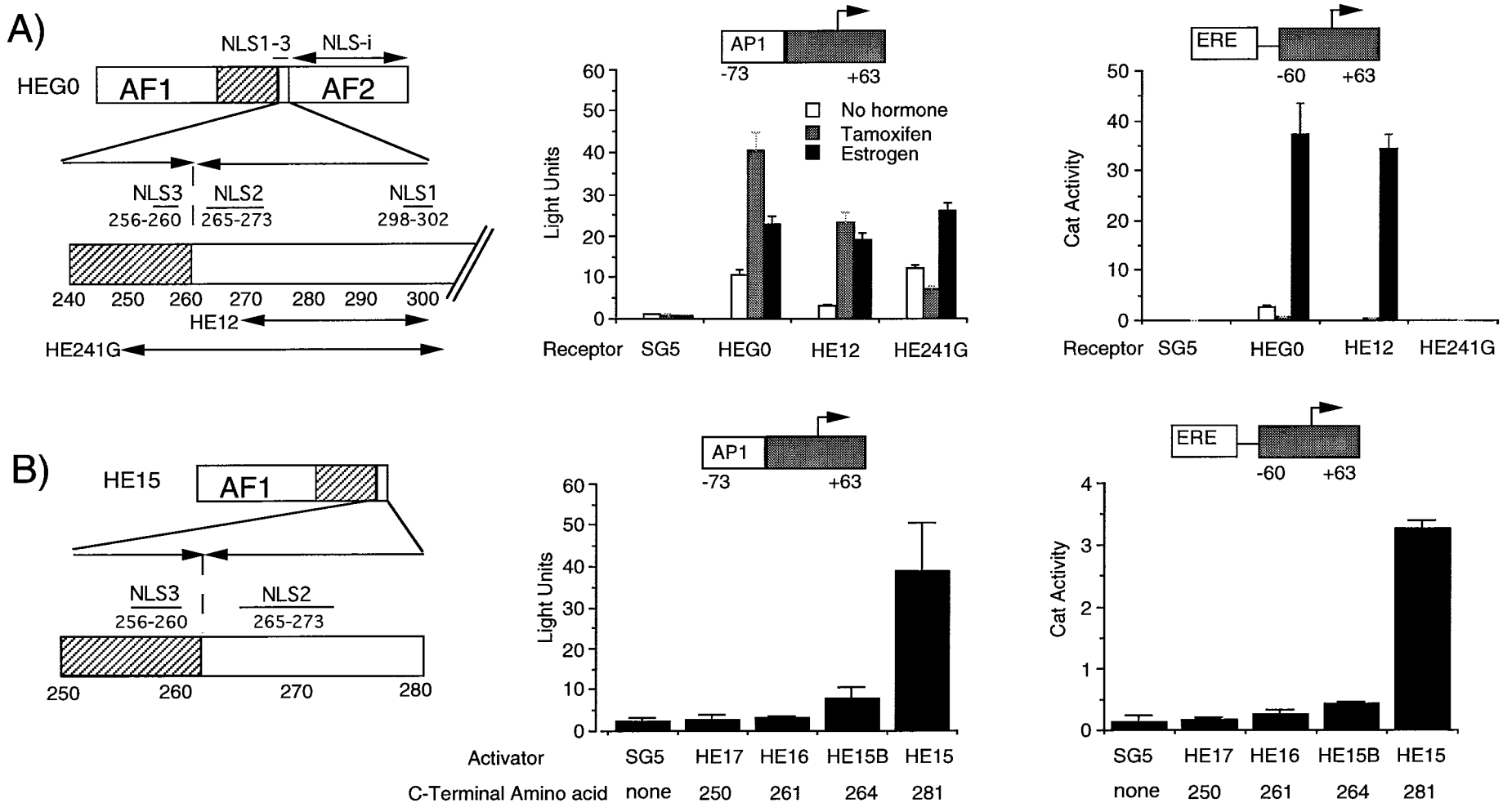
A)
NLS1-3
NLS-i
HEG0
AF1
AF2
NLS3
256-260
NLS2
265-273
NLS1
298-302
240 250 260 270 280 290 300
HE12
HE241G
AP1
-73
+63
No hormone
Tamoxifen
Estrogen
Light Units
Receptor SG5 HEG0 HE12 HE241G
ERE
-60
+63
Cat Activity
Receptor SG5 HEG0 HE12 HE241G
B)
HE15
AF1
NLS3
256-260
NLS2
265-273
250 260 270 280
AP1
-73
+63
Light Units
Activator SG5 HE17 HE16 HE15B HE15
C-Terminal Amino acid none 250 261 264 281
ERE
-60
+63
Cat Activity
SG5 HE17 HE16 HE15B HE15
none 250 261 264 281

Figure 5

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

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DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864
DAMD17-94-J-4267	ADB216187
DAMD17-94-J-4200	ADB216054
DAMD17-94-J-4185	ADB219284
DAMD17-94-J-4172	ADB224562
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DAMD17-94-J-4082	ADB215979
DAMD17-94-J-4053	ADB216052
DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

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PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management