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This project examines the mechanism of signal transduction by the estrogen receptor (ER), a hormone dependent transcriptional regulator involved in many human breast tumors. Our goal is to elucidate these mechanisms through the identification and characterization of proteins involved in the ER signal transduction pathway using genetic strategies. Our aims are to identify proteins that functionally interact with the estrogen receptor via dosage suppression screens in yeast and to isolate proteins that physically interact with the estrogen receptor using the yeast two hybrid system. Using dosage suppression analysis, we have isolated several candidate genes that when overexpressed restore the hormone-dependent activity to receptors defective in either ligand binding or receptor phosphorylation. We have also identified three proteins that physically interact with the amino-terminal transcriptional activation domain of ER using the yeast two-hybrid system. Future studies will include characterization of the gene products and analysis of their participation in transcription and receptor signaling. Since nearly half of all human breast cancers depend upon estrogen for growth, understanding how these factors function in steroid signaling may provide valuable targets for inhibiting ER action and stopping cancer growth.

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5) Introduction

Estrogen is a steroid hormone responsible for the proper function of multiple physiological processes. Its role in the female reproductive system has long been established: while estrogen suppresses Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) production from the anterior pituitary, it stimulates prolactin synthesis and production[1, 2]. In addition, estrogen can induce uterine hyperplasia and is essential for development of the mammary glands. Estrogen's role in breast cancer is currently a focus of intensive study, as length of exposure to estrogen correlates with risk of developing the disease, a correlation that has been strengthened by laboratory studies[3-5]. In fact, it was known as early as 1896 that ovariectomy resulted in a regression of metastatic breast cancer[6]. Studies of breast cancer cell lines have suggested that this is a direct effect: estrogen has been shown to accelerate the proliferation of certain mammary epithelial cells, while antagonists of estrogen have been shown to impede growth.

The full spectrum of estrogen's role in mammalian physiology outside of the reproductive system, however, has only recently begun to be fully understood. Estrogen has been recognized to have effects within the cardiovascular system. It increases left ventricular contractility, reduces aortic stiffness, and is protective against coronary artery disease[7]. It can also, however, cause anemia through the inhibition of erythropoesis[8]. Estrogen is also important in the proper functioning of the skeletal system, where it has been shown to be essential for the proper maintenance of bone density: decreased estrogen levels, as seen in menopause, are thought to be the cause of post menopausal osteoporosis[9]. The immune system is also suspected to be regulated by estrogen, as females have more vigorous cellular and humoral immune responses than males[10]. This difference in immune responses is believed to be responsible for the higher susceptibility of females to autoimmune diseases. The recent identifications of the estrogen receptor (see below) in cultured endothelial cells, vascular smooth muscle cells, mature osteoclasts, as well as the cells of the immune system (macrophages[10], thymocytes[11], and lymphocytes[12]) suggest that these estrogen associated affects are direct. The recent production of ER knockout mice has already proven the importance of ER in the development of the female reproductive system and proper maintenance of bone density, and will surely prove invaluable for the further study of all ER dependent phenomena[13, 14]. Thus, it is the ongoing realization of the hormone's wide ranging physiological effects on human physiology that fuels the drive to understand how estrogen hormone signaling is perceived and acted upon at the cellular level.

The mediator of estrogen signaling is the estrogen receptor (ER), a protein found in all estrogen responsive tissues. A member of the nuclear steroid receptor superfamily, the

ER is a ligand-induced transcription factor, believed to function in a manner analogous to other nuclear steroid receptors. The estrogen pathway is thought to proceed in a manner as depicted in Figure 1. In the absence of estrogen, the ER is found predominantly in the nucleus, bound in monomer form to a number of proteins including heat shock proteins hsp 90, hsp 70, and hsp 56. The resulting "aporeceptor complex " is believed to keep the ER in a conformation that both 1) allows it to bind ligand, and 2) inhibits any transcriptional activation[15, 16]. Upon binding estrogen, the ER dissociates from the aporeceptor complex and binds to another ER monomer. The ER dimer is now capable of recognizing specific DNA sequences within promoter regions of estrogen responsive genes called estrogen response elements (ERE)[17]. Once bound to an ERE, the ER dimer is believed to modulate transcription of the downstream estrogen responsive gene via recruitment of transcriptional machinery. Though predominantly a transcriptional activator, ER is also capable of repressing transcription through direct inhibitory interactions with other transcription factors: inhibition of GATA-1 transcriptional activity by ER in hematopoesis interferes with erythroid differentiation, resulting in anemia[8].

The ER gene spans over 140kB and is broken into eight introns which code for a 67 kD protein[18]. ER shares the structural compartmentalization exhibited throughout the nuclear steroid receptor family. The ER protein is functionally divided into six different domains (A-F), based upon its homology with the other nuclear steroid receptors (Figure 2). The A/B region, the most variant of these domains, consists of the amino terminal 185 amino acids. The least understood region, it contains an intrinsic transcriptional activation domain (referred to as TAF-1) that is able to activate transcription when fused to a heterologous DNA binding domain[19]. Studies using A/B fragment chimeras suggest that the regions responsible for this transcriptional activation are localized to amino acids 51-93 and 102-149. This transcriptional activity of the A/B domain is dependent upon both promoter context and cell type: it has been demonstrated that, between cell types, the requirement of TAF-1 function varies, even for an identical promoter[20-22]. The A/B domain also contains many potential phosphorylation sites believed to be integral to the control of ER (see below).

The following 70 residues comprise the DNA binding region (region C), and are more highly conserved between steroid receptors. Crystal structures of the estrogen receptor bound to DNA show that two important subdomains exist: the zinc finger motifs, and the P box[23]. The two zinc fingers each contain four cysteine residues that tetrahedrally coordinate a zinc ion between them. The first zinc finger helix is responsible for specifically binding to the ERE sequence (AGGTCAnnnTGACCT), while the second is thought to stabilize this interaction[24]. The P box, the three residues located within the

alpha helix immediately following the first zinc finger, is crucial to ER's ability to recognize the ERE sequence[25]. Mutating these residues or altering the spacing between the half palindromic sites in the ERE abolishes ER-DNA binding.

The D, E, and F domains make up the remainder of the protein, the ligand binding domain. The D domain is a non-conserved sequence among steroid receptors, and is therefore thought to be a hinge region not highly important to the function of ER[22]. Conversely, the E/F domain is responsible for several important functions: upon binding ligand, this domain undergoes conformational change[26] which 1) allows functional dimer formation through exposure of the receptor dimerization domain, 2) exposes the DNA binding domain, allowing the receptor to bind the ERE, and 3) activates a distinct transcriptional activating domain, TAF-2[22, 27, 28]. These distinct functions map to distinct regions within the domain, and can be functionally separated by certain estrogen antagonists and ER mutations[29, 30]. In addition, this is the only region which has been shown to interact with potential coactivators. Grip1[31], TIF2[32], and RIP 140[33] have all been found to interact with the TAF-2 region in a ligand inducible manner, thereby increasing its transactivating potential.

Along with the binding of ligand, phosphorylation is also believed to play an important role in ER function. The estrogen receptor contains several serine and tyrosine residues which are believed to be phosphorylated in both a constitutive and hormone-inducible manner (Figure 2)[34-37]. Residues phosphorylated in the presence of hormone are thought to be the means by which both estrogen and other signaling pathways exert control over ER function: estrogen binding, EGF stimulation, and cAMP pathway activators are all able to effect changes in ER phosphorylation status and transcriptional activity[36, 38]. The serine residues thought to be phosphorylated after ligand binding are contained within the A/B region of the receptor, although the exact residues modified appear to differ with cell type. ER transiently transfected into COS-1 cells is phosphorylated on S104, S106, and S118[34], while S167 seemed to be the major target in ER isolated from MCF-7 cells[39]. Phosphorylation of other sites is also believed to occur, but has not been fully characterized.

A single tyrosine residue, Y537, has also been shown to be phosphorylated, although in a manner that is independent of estrogen treatment[40, 41]. Evidence suggests that this modification may be a crucial event for control of ER function since only tyrosine phosphorylated ER was found to be 1) nuclear, 2) hyperphosphorylated at serine residues, and 3) capable of binding DNA[40]. These observations, however, conflict with *in vitro* studies which show that ER unphosphorylated at Y537 is still capable of binding DNA.

As mentioned above, phosphorylation is a possible mechanism by which other signaling pathways may affect ER function. The best characterized interaction is that between ER and the EGF/ras/MAPK signaling pathway[42]. There are currently three proposed steps at which this interaction takes place. It is believed that MAPK is able to increase transcription by the ligand bound ER through phosphorylation of the S118 residue located in the amino terminal[34, 43]. In addition, it has been shown that this same phosphorylation event can lead, in part, to activation of unliganded ER[44]: this provides a possible explanation as to how EGF is able to mimic many of estrogen's physiological effects in uterine tissue[45]. Evidence also exists, however, that ER can, in turn, activate the EGF pathway through activation of src kinase[46]. Additional interaction is believed to exist between ligand bound ER and the AP-1 proteins fos and jun. This interaction is complex in that it can be both synergistic or competitive, and that evidence exists for both DNA dependent and direct protein-protein interaction[47-50].

Similar patterns are seen in the interaction of the ER and PKC signaling pathways. Activators of PKC lead to increased transcriptional activation by the ligand-bound estrogen receptor, including activation of mutant ERs that are unable to induce transcription in response to hormone alone[38, 51, 52]. Unlike MAPK, however, PKC is believed to act through a domain other than the amino terminal[36]. There is also evidence that ER may be able to activate PKC: administration of estrogen hormone leads to the induction of PI turnover, leading to DAG production[53]. Just as in the EGF pathway, there is also the interaction between ER and the AP-1 proteins.

There remains the possibility that ER function is regulated not only through alteration of the protein itself, but also through regulation of other proteins with which ER must associate to activate transcription. The search for such cofactors has led to a number of possible candidate proteins which bind to specific ER domains in an estrogen dependent manner, but whose relevance to ER function remains to be fully characterized[54, 55].

It appears that evidence in recent years points to the possibility that the traditional view of ER function may be somewhat limited, and therefore in need of revision and expansion in order to fully understand the many roles ER plays in the various cell types in which it is found. The complexity of the protein, with its multiple structural domains, along with the many pathways it appears to interact with, seems fitting for a protein that affects so many different physiological responses in different tissues. Crucial to the elucidation of this complexity is the further identification of proteins which interact either physically or functionally with the ER.

6) Body

Yeast Dosage Suppression Screens

The genetic capabilities of yeast make it a powerful system in which to study ER function. It has been well established that the ER signal transduction pathway is faithfully conserved in yeast. Our overall goal is to use genetic approaches to identify proteins that affect ER function within this system. We expect that the characterization of these proteins will lead to a greater understanding of the ER signal transduction pathway, and ultimately, to the identification of mammalian counterparts involved in ER function.

Factors that interact functionally with ER can be identified in yeast through dosage suppression analysis. Mutant ER proteins that display altered ability to activate transcription can be used as substrates in a dosage suppression screen to isolate yeast genes that are capable of overcoming this discrepancy in activity. Overexpression of factors important for ER function can in principle overcome the mutational block by favoring the interactions that facilitate ER function. The advantage of this procedure is that it results in the direct cloning of genes of interest.

Although the yeast *S. cerevisiae* does not contain endogenous nuclear steroid hormone receptors, the mechanics of eukaryotic gene transcription appear to be sufficiently conserved so that these proteins are able to function in yeast in a manner analogous to that in mammalian cells[29, 56]. It has been established that human ER expressed in yeast can bind DNA in response to ligand, subsequently activating transcription from EREs located in promoters upstream of reporter genes. The two transcriptional activation regions of ER, TAF-1 and TAF-2, have both been shown to retain their ability to activate transcription, and the residues important for TAF1 function in yeast have been shown to overlap with the residues important for its function in mammalian cells[19]. Indeed, the yeast system has already been used to identify proteins that seem to be important for maintenance of the aporeceptor complex, transactivation by the TAF-2 region, and DNA binding[15, 57, 58].

Although the study of ER function in yeast is unlikely to lead to the understanding of the varied estrogen-induced effects seen in mammalian cells, it is a useful model for trying to determine the basic mechanisms by which ER activates transcription-- knowledge essential to understanding the more complicated aspects of ER function. To identify proteins involved in these basic mechanisms, we will exploit the phenotypes of mutant ERs which are defective in their ability to fully activate transcription in response to hormone binding. These phenotypes will be used as the basis for dosage suppressor screens using a high copy yeast genomic library that will allow for the identification of yeast proteins important for the proper functioning of the ER pathway.

A strong argument can be made for the isolation of these proteins through the exploitation of the genetic capabilities of yeast. The smaller yeast genome allows for the screening of only thousands of colonies versus the millions that are necessary to screen a mammalian library. The recently completed sequencing of the yeast genome makes possible the rapid identification of the entire isolated genomic fragment sequence by merely matching the flanking sequences to the yeast genome database. Characterization of any isolated protein's normal function in yeast is greatly facilitated by the relatively simple construction of yeast strains that lack the genes of interest.

We have evaluated the function of two mutant ERs in the yeast system in order to determine if their effects were consistent with their observed phenotypes in the mammalian cells. The two different mutations, a replacement of serine with alanine at residue 118 (S118A) and a replacement of glycine with valine at position 400 (G400V), have been shown to affect different aspects of ER function in mammalian cells, giving rise to distinct phenotypes that can be exploited in dosage suppression screens.

The first mutation, S118A, results in the removal of a phosphorylation site from the amino terminus of ER, an event that has been shown to impair the full activation of ER in mammalian cells. We hypothesize that this decreased transcriptional activation by the S118A mutant reflects a reduced ability of ER to interact with endogenous proteins important in transcriptional activation. This protein-protein interaction might normally be regulated through either direct interaction with the phosphorylated residue, or by conformational changes dependent upon phosphorylation of S118. In the first case, overexpression of the interacting protein would be one way in which to increase the rate of a protein interaction that has become energetically unfavorable by removal of the phosphorylated serine (a la Le Chatelier's principle). Similarly, if the interaction were dependent on intramolecular conformational changes brought about by phosphorylation of S118, one could again expect that overexpression of the interacting protein would result in increased formation of the activating complex as the ER shifted between its varying conformations. It should be noted, however, that overexpression of any yeast protein important to the activation of ER could conceivably result in increased activity of the S118A ER mutant and may also be identified in such a screen. Although these proteins would not be directly dependent upon the state of ER phosphorylation per se, they will still be informative as to the functioning of the ER signaling pathway, and will therefore also be further characterized.

The S118 residue is believed to be phosphorylated by MAPK, an event that may serve as one of the mechanisms by which the EGF and IGF pathways exert control over both the ligand bound and unbound forms of the ER. In mammalian COS-1 cells, the

S118A mutation was shown to result in a decreased maximal level of ER transcriptional activation[36]. In yeast, we found the S118A mutant to behave in a manner analogous to that seen in mammalian cells. If one examines transcriptional activation by the WT and S118A ER proteins as a function of hormone concentration, one sees that although S118A ER becomes transcriptionally active at the same concentrations as the WT ER, the ability of S118A ER to activate transcription never reaches the same maximal activity at saturating ligand concentrations (Figure 3). Interestingly, this difference was most striking when cells were grown in conditions that result in only low levels of ER protein expression. We believe this finding strengthens our hypothesis that S118A plays a role in cofactor signaling, for the overexpression of ER results in suppression of the mutant phenotype. We therefore expect to see similar suppression when the interacting protein is overexpressed. Though we have not yet shown that S118 is phosphorylated in yeast, three lines of evidence suggest to us that it is: 1) the S118A mutation results in the identical phenotype in yeast and mammalian cells, 2) yeast have MAPK homologues, which could potentially phosphorylate S118, and 3) the phosphorylation of other steroid receptors has been found to be conserved in yeast[59].

The G400V mutation has previously been demonstrated in mammalian cells to affect ER's ability to bind ligand, and therefore also affects ER's ability to activate transcription at certain levels of estrogen treatment[60]. As might be expected, this glycine residue lies within the hormone binding domain, a region that has been additionally implicated in the control of ER dimerization and TAF-2 function. When this ER mutation was expressed in yeast, we found that, when measuring transcriptional activity as a function of hormone concentration, G400V exhibits a decreased affinity for ligand when compared with WT ER. G400V requires a 100 fold increase in ligand concentration before it begins to show transcriptional activation, as compared to WT ER (Figure 4). Unlike S118A, however, G400V is able to reach the same maximal activity as WT ER at saturating concentrations of ligand. This suggests that once the hindrance to ligand binding is overcome, the receptor is able to act as efficiently as the WT receptor in entering the various interactions, both protein-DNA and protein-protein, that are necessary for transcriptional activation. Thus, the distinctive dose response curves seen for the two different mutants, S118A and G400V, would suggest that two discrete aspects of the ER pathway are being affected. Identification of proteins important at either step will prove useful in the ongoing attempt to better understand how ER functions in mammalian cells.

After having ascertained characteristic dose response curves for each mutant ER, we were able to use these data to establish conditions for dosage suppression screens. Yeast plates were made with media containing the particular carbon source and level of

ligand that resulted in the largest discrepancy between the mutant ER and WT ER function. S118A ER yeast were grown on X-gal plates containing 2% raffinose as the sole source of carbon, along with 10 nM 17B-estradiol, while G400V ER yeast were grown on 2% galactose, 1% raffinose X-gal plates with 17B-estradiol at a concentration of 1nM. When grown under these conditions, the decreased activity of the mutant ERs versus the wt ER becomes readily discernible through the differential induction of an ERE dependent B-galactosidase reporter gene: yeast colonies containing WT ER are dark blue, while mutant ERs are pale blue(S118A) or white(G400V). Yeast strains containing these mutant ER proteins will be transformed with a high copy yeast genomic library, and then screened via expression of B-galactosidase. Dark blue colonies will be suppressor candidates that may have overcome the effects of receptor mutation through overexpression of a factor that normally interacts with the receptor.

In an effort to find suppressors of the S118A ER mutation, we screened approximately 5,000 colonies, which represents about half the genome. After transformation with the high copy genomic library, colonies were allowed to grow on glucose containing plates that selected for the -leu marker present on the library plasmids. Colonies were then replica plated onto X-gal indicator plates containing 2% raffinose and 10nM 17B-estradiol. The darkest colonies were selected and patched onto a single -leu glucose plate. This allowed for the side by side comparison of the candidate suppressors with each other and WT ER when this plate was replica plated onto the raffinose X-gal plates. In this manner, eight colonies were chosen for further analysis. After isolating the library plasmids and reintroducing them into S118A ER yeast, one of the eight library plasmids seemed to suppress to a level greater than the remaining seven. In an effort to learn more about this suppressor, we sequenced approximately 150 bp from both ends and then matched them to the yeast genomic sequence database. The suppressor was found to contain a genomic fragment of 6980 bp from yeast chromosome IV. Three open reading frames were identified in this region, though only one was present in its entirety (Figure 5). The two truncated genes located at either end of the fragment, SRP101 and "D9189.5", are likely to produce partial, non-functional proteins incapable of enacting suppression, although this remains to be determined. In addition, their known functions do not suggest a direct mechanism by which they might enact suppression: SRP101 is homologous to the signal recognition particle receptor, while D9189.5 shares homology with glutamate decarboxylase.

The ssd1 gene, on the other hand, is present in its entirety. This yeast protein has been isolated in several other suppression screens. It is a suppressor of 1) heat shock sensitivity characteristic of a cAMP phosphodiesterase mutant, 2) the *ins1* mutation, which

blocks G1-S phase transition, 3) the lethality resulting from a mutation of SIT4 phosphatase (a protein implicated in the G1-S phase transition)[61], and 4) yeast RNA polymerase I,II, and III mutations[62]. Recently, ssd1 has been shown to be involved in RNA processing-- its role in such a general cellular process may explain how it might suppress mutations affecting such disparate processes within the cell. In addition, it is important to note that the yeast strain used for our assay, w303, contains an ssd1 allele (ssd1-d2) whose mRNA is expressed at only half the level of other ssd1 alleles[61]. Thus, on the basis of its proposed function in RNA processing, we do not consider ssd1 to be a specific candidate suppressor of the S118A mutation in ER.

In an attempt to find suppressors of the G400V ER mutation, a total of 5622 transformed colonies were screened, representing approximately half the yeast genome. Of the 13 chosen for further study, only 5 were found to be linked to the library plasmid. After liquid B-galactosidase analysis, one library plasmid greatly exceeded the level of suppression the others exhibited, and this suppressor became the focus of our analysis. The clone contains a large genomic fragment with several open reading frames (Figure 6). At the 3' end is a truncated ORF encoding a putative protein kinase. Along with a tRNA gene, there are two complete open reading frames that code for unknown proteins: YKL520 and YKL518. There also exists, however, two complete ORFs which have homology to known proteins: the YKL522 gene is a mitochondrial ADP/ATP carrier protein homologue, and the YKL525 gene encodes a protein which shows limited homology to TUP1. TUP1 is a yeast protein which has been shown to be a universal repressor of transcription when complexed with another yeast protein, SSN6[63, 64]. Devoid of any inherent DNA binding ability, these two proteins have been found to be recruited by other DNA binding repressor proteins to effect efficient transcriptional repression, possibly through interactions with histones. The TUP1 homologue might behave in an analogous fashion, though resulting in increased, rather than decreased, transcriptional activation. It is also quite possible, however, that any of the other ORFs might be the gene of interest. Further characterization will require the systematic study of smaller pieces of this genomic fragment in order to locate the ORF which is specifically responsible for the suppression of the G400V phenotype.

Yeast Two Hybrid Analysis

Our second strategy for finding proteins that interact with the ER uses the yeast two hybrid system. This technique can be used to find proteins that physically interact with a protein of interest, but it says nothing of the biological significance of that interaction. The protein (or fragment of a protein) of interest is fused to the DNA binding domain of the LEX protein (Figure 10). This fusion "bait" protein binds to a LEX operator which lies

upstream of two reporter genes: a leu 2 gene, encoding a protein involved in the leucine biosynthesis pathway, and a B-galactosidase gene, which codes for an enzyme which can cleave chromogenic substrates, providing a visual measure of interaction. The cDNA library to be screened is expressed as a "prey" protein, fused to a transcriptional activation domain. The two fusion proteins are functionally silent in the yeast unless the "bait" protein can physically interact with the library protein present in the "prey". In this event, the activator domain of the "prey" is brought to the promoter region of the reporter genes and activates transcription. This allows for selection of interacting library proteins by the yeast's ability to 1) grow in the absence of leucine, and 2) express B-galactosidase.

In our yeast two hybrid screen we used the amino terminal 1-185 residues of ER as the bait. This region corresponds to the A/B domain, a region which contains the TAF-1 activity of the ER and has been shown to be able to activate transcription when fused to a heterologous DNA binding domain. Despite the ability of the A/B region to activate transcription in some systems, we found that our 1-185 ER-Lex fusion protein was transcriptionally silent when expressed in yeast, as was demonstrated by both absence of growth on -leu plates, and lack of blue pigment observed when grown on X-gal plates. Western blot analysis showed a protein of expected size was produced. Other shorter truncations of the amino terminal did, however, transcriptionally activate in a constitutive manner: 1-115 ER and 1-121 ER- Lex fusion constructs activate transcription in the absence of any prey product. This suggests to us that the C-terminal of our 1-185 ER construct is somehow able to repress the inherent TAF-1 activity of this domain in yeast, thereby making the use of the 1-185 ER fragment possible. Using this bait, we screened a HeLa cell cDNA library for interacting proteins.

In a preliminary experiment, we isolated fifty positive clones. We proceeded to test the specificity of these interactions by testing these isolated "preys" against baits which we obtained from other labs; TAF 130, a nuclear protein found in the TFIID complex (from the Tanese Lab); DRG, a GTP-binding protein of unknown function (from the Sun Lab), and the A/B domains of both thyroid receptors alpha and beta (from the Samuels Lab). To rule out any interactions that were specific to the Lex DNA binding domain, we tested the library preys against the Lex DNA binding region alone. Based on these specificity tests, we ruled out the vast majority of our clones as nonspecific interactions: on the basis of these criteria-- specific interaction with ER and selective interaction with other baits-- we chose three clones for further study.

The first clone, D18, shows the highest specificity of all clones tested. It interacted strongly with the 1-185 ER bait, and failed to show any interaction with the TAF 130, DRG, thyroid hormone receptor baits, or Lex DNA binding region alone. Partial sequence

analysis of D18 and subsequent database searches showed it to be a novel protein, though matches were found to sequences in the expressed sequence tagged database (EST). These matches will be used to corroborate our sequencing of the cDNA (see below). Restriction analysis showed the insert length of the two hybrid clone to be approximately 1.2kb long. Using this fragment, we were able to carry out Northern analysis on polyA+ mRNA from HeLa, SOAS2, U20S, and MCF-7 cells. A mRNA of approximately 1.6 kb was observed in all four cell lines, with all cell lines expressing a similar abundance of D18 in relation to GPDH control. A cDNA of this approximate length was isolated from a human teratoma lambda phage cDNA library and cloned into the pBSK+ vector. Efforts to fully sequence this message are ongoing.

In addition to the isolation of the full length transcript, we have used the two hybrid system in an effort to better map D18's interaction with ER. We attempted to ascertain whether the interaction would still occur if the D18 fragment were switched from the prey to the bait construct. Surprisingly, when the D18 clone was expressed as a fusion protein with the Lex DNA binding domain, it caused a decreased growth rate of the yeast when grown in galactose. This phenotype was not evident when the yeast were grown in glucose, despite the fact that the protein is expressed under both conditions. We hypothesize that D18 may be interfering with, or sequestering, factors specific to the galactose pathway, perhaps by direct interaction. Whatever the mechanism by which D18 is suppressing growth in yeast, this phenotype prevents us from assaying D18 interaction with constructs which must necessarily be expressed as fusion "prey" proteins due to their ability to activate transcription when expressed within the "bait" context. For example, the shorter amino terminal ER fragments (1-82, 1-115, 1-121), the GR amino terminal 106-414 (TAF-1), as well as the full length ER protein (Figure 10), all activate transcription when fused to the Lex DNA binding domain.

We are interested in our second clone, D7, because its specificity pattern included not only our ER construct, but TAF 130 as well. Thus, the ability of D7 to interact with two proteins implicated in transcriptional activation makes it inherently interesting. After sequencing the ends of D7, a database search found it to be a novel protein with matches in the EST database. We again carried out a Northern analysis of polyA+ mRNA from HeLa, SOAS2, U20S, and MCF-7 cells, and found the message length to be approximately 1kb, and equally expressed in all four cell lines. We were able to isolate a cDNA of this approximate length from a human teratoma lambda phage library, and have cloned this cDNA into the pBSK+ vector. We are currently sequencing this cDNA along with D18.

Unlike D18, however, D7 does not express any unusual phenotypes when switched from the prey construct to the bait protein, thereby allowing for the further

mapping of the D7-ER interaction (Figure 7). We found that the D7 bait is able to interact with our 1-185 ER fragment in the prey context, though it did not interact with any of our shorter ER constructs (1-82, 1-115, and 1-121). The D7 bait, however, was able to bind to the full-length ER construct in the presence of ligand. This suggests that the crucial piece of ER required for D7 binding lies within the residues 121-185, and that this region may be hidden in the unliganded ER protein. Binding of ligand and truncation of the protein to form 1-185 ER may both serve to release the relevant residues from a conformational repression. Finally, in addition to being able to bind the ER amino terminal, D7 also demonstrated some ability to interact with residues 104-414 of the amino terminal of the glucocorticoid receptor, a region which encompasses the TAF-1 activity of GR.

Sequencing of the last clone, D6, revealed it to be the carboxy terminal domain of the Grb-2 protein, which encodes the complete C terminal SH3 domain. Though D6 bound strongly to our ER bait, we found it also interacted with TAF 130, DRG, and the GR amino terminal, though no interaction was seen with the Lex DNA binding region alone. By switching the D6 fragment to the bait protein, we were able to test its ability to associate with three ER truncation preys (1-82, 1-115, 1-121) as well as the full length ER protein (Figure 8). We found that D6 bound specifically to ER truncation 1-115, but did not bind to the slightly longer 1-121 truncation. In addition, we found that the D6 interaction with the full length ER was greatly enhanced in the presence of hormone, suggesting that the interacting domain of ER only becomes accessible when bound to ligand. These two pieces of data suggest that the residues 83-115 were important for Grb-2 binding, and that in both the full length and 1-121 truncated ER, these residues were sufficiently masked to prevent this interaction. Note, however, that the 1-185 ER is able to interact with Grb-2, suggesting that the relevant residues would also be exposed in this truncation.

We chose to pursue the Grb-2-ER interaction based upon its proven role as an adaptor protein in the tyrosine kinase receptor cascade leading to activation of Ras and MAPK[65]. The SH2 domains of Grb-2 bind to phosphotyrosine moieties of activated growth factor receptors, while its SH3 domains interact with other proteins via proline rich motifs (P-X-X-P) to form active signaling complexes. Although interaction of ER and Grb-2 at first glance seems unlikely since Grb-2 is located predominantly in the cytoplasm and ER in the nucleus, the established relationship between the EGF and ER signaling pathways suggests that there may indeed be some kind of interaction between the two proteins. The EGF pathway can activate ER in the absence of hormone through phosphorylation of ER by MAPK. Interestingly, removal of the phosphorylation site

(S118), does not completely abolish the EGF pathway's ability to activate ER, suggesting that there is another mechanism by which the EGF pathway can exert control over ER function. ER is also tyrosine phosphorylated in its ligand binding domain, an event that is seen by some to be of great importance to ER's activation and translocation into the nucleus. In addition, there is now precedent for a direct interaction between a transcription factor and the receptor tyrosine kinases. The human proto-oncogene *vav* , a protein which contains a helix-loop-helix domain, a zinc finger domain, and a leucine zipper domain, also contains an SH2 motif and has been coimmunoprecipitated with activated EGF receptors[66]. Thus, it is this circumstantial evidence for an interaction between ER and Grb-2 that has convoked us to further pursue the possibility of a functional interaction between these two proteins.

7) Conclusion

The two yeast screens, the dosage suppression and two hybrid screens, have thus far yielded four possible proteins that interact either physically or functionally with the ER. Due to the different criteria used in the separate types of assays, the methods by which the candidate proteins will be further characterized is largely dependent upon the method in which they were isolated. The yeast dosage suppressor clone was selected on the basis that it could affect ER function within yeast. It remains to be shown whether it has relevance within the mammalian system. The two hybrid clones were isolated from a mammalian cDNA library based on their ability to physically interact with our ER bait. These interactions must now be proven to have functional significance. Future studies will include characterization of the gene products and analysis of their participation in transcription and receptor signaling.

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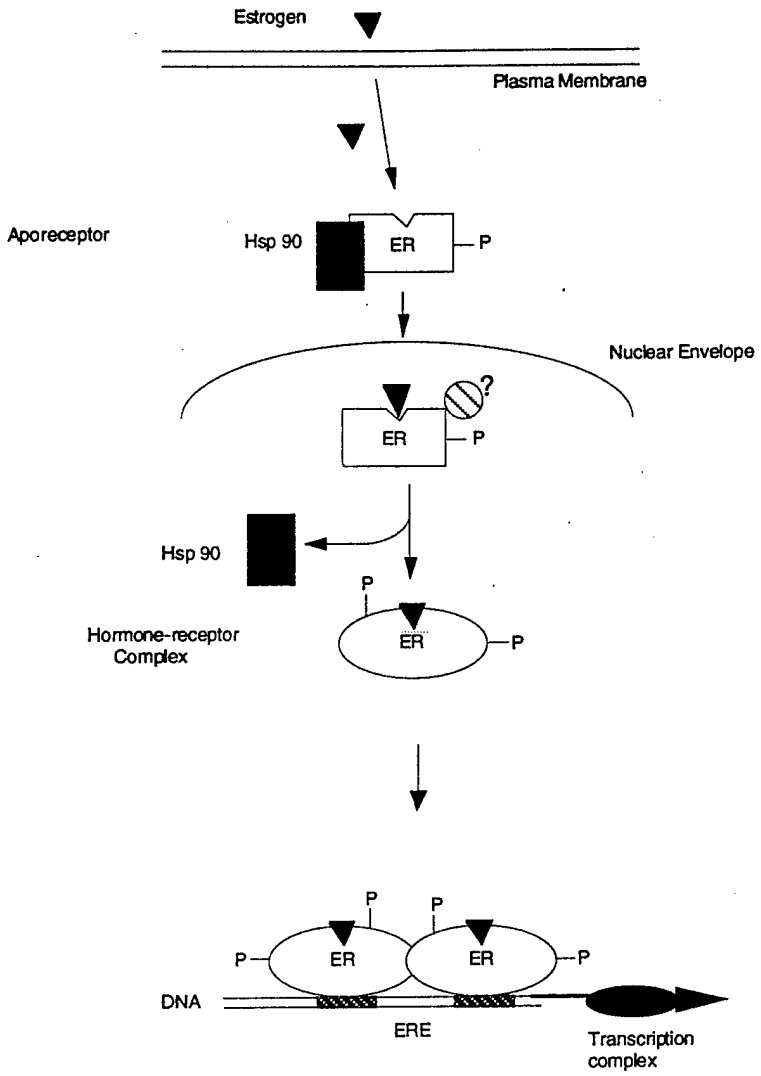


Transcriptional Enhancement

Figure 1. Signal transduction by the estrogen receptor. The current model for estrogen-dependent transcriptional activation is diagrammed. ER is believed to be primarily localized to the nucleus. Following stimulation with its cognate ligand, 17β-estradiol, ER is activated; it forms homodimers and binds to specific DNA response elements (EREs). ER is a phosphoprotein whose phosphorylation status is altered in response to ligand treatment and receptor activation. The addition of phosphate moieties to this transcriptional enhancer is likely to play a key role in its regulation.

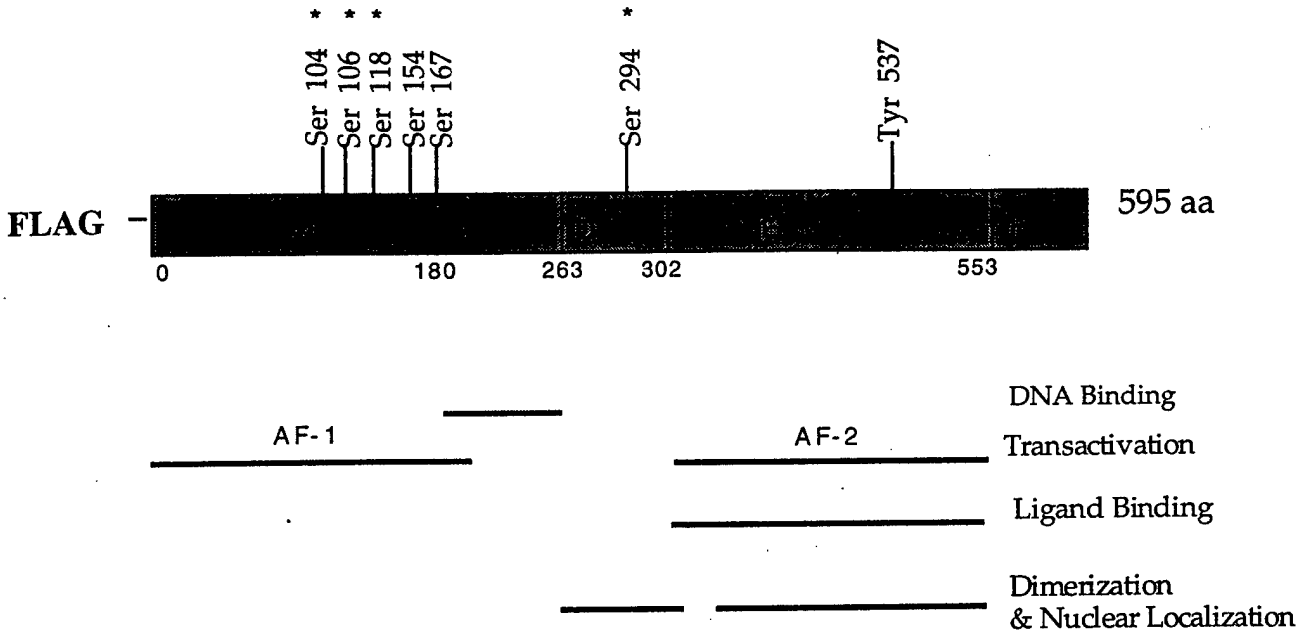


Figure 2. Domain structure of the human estrogen receptor. Six characterized domains of the estrogen receptor, designated A through F, are diagrammed. The estrogen receptor contains several putative phosphorylation sites, indicated by *, which match the consensus sequence (x-ser/thr-x-pro) targeted by a family of ser/thr-pro-directed protein kinases to which cyclin-dependent kinases and MAPK belong. These sites are believed to be important for receptor regulation and function.

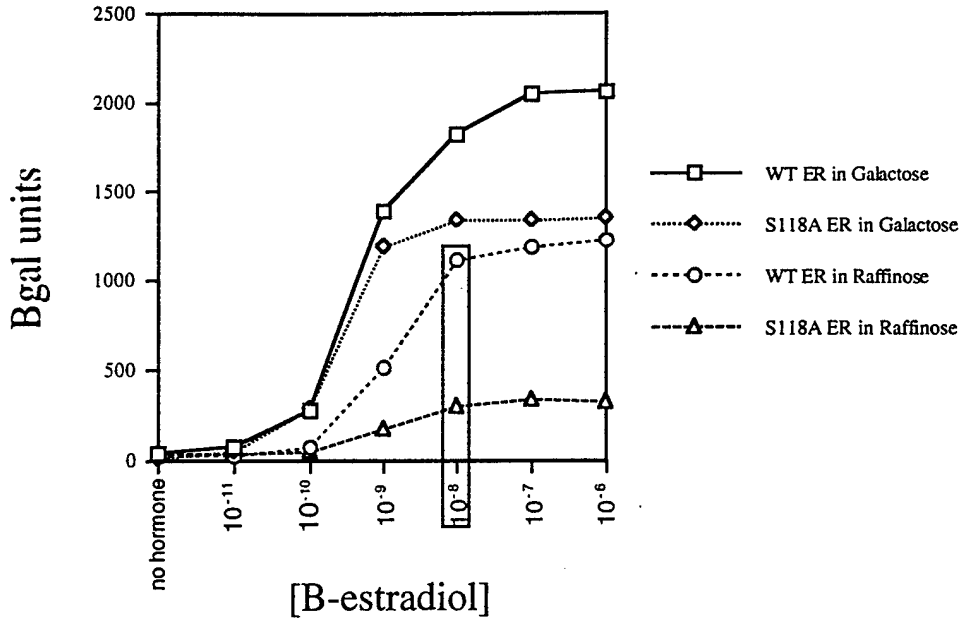


Figure 3: The above graph depicts the decreased level of transcriptional activation exhibited by the S118A ER mutation within yeast. The yeast strain w303a was transformed with 1) a galactose inducible expression vector containing either the wild type or S118A ER and 2) a reporter plasmid containing the B-galactosidase gene under the control of an ERE. These strains were then used to carry out liquid B-galactosidase assays. Different levels of ER expression were achieved through differential induction of the GAL 1-10 promoter: incubating the yeast strains in galactose-containing media resulted in high expression of the ER proteins, while incubation in raffinose resulted in lower levels of expression. The data points indicated by the box reflect the ligand concentration and carbon source which were selected for the dosage suppression screen: screen plates contained 10nM B-estradiol and 2% raffinose.

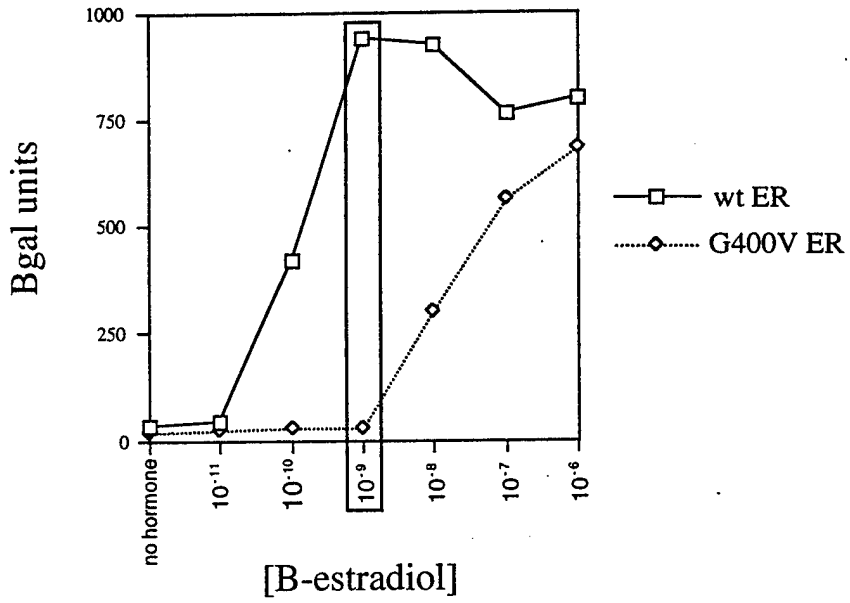


Figure 4: The above graph illustrates the decreased affinity of the G400V ER mutant for ligand, resulting in a corresponding decrease in transcriptional activation. The w303a yeast strain was transformed with 1) a galactose inducible expression vector containing either the wild type or G400V ER and 2) a reporter plasmid containing the B-galactosidase gene under the control of an ERE. These strains were then used to carry out a liquid B-galactosidase assay in media containing galactose, which results in high expression levels of the ER proteins. A 100-fold increase in ligand concentration is needed in order for G400V ER to activate transcription at wild type ER levels. The data points indicated by the box show the ligand concentration which was selected for the dosage suppression screen: the screen plates contained 1nM B-estradiol with galactose as carbon source.

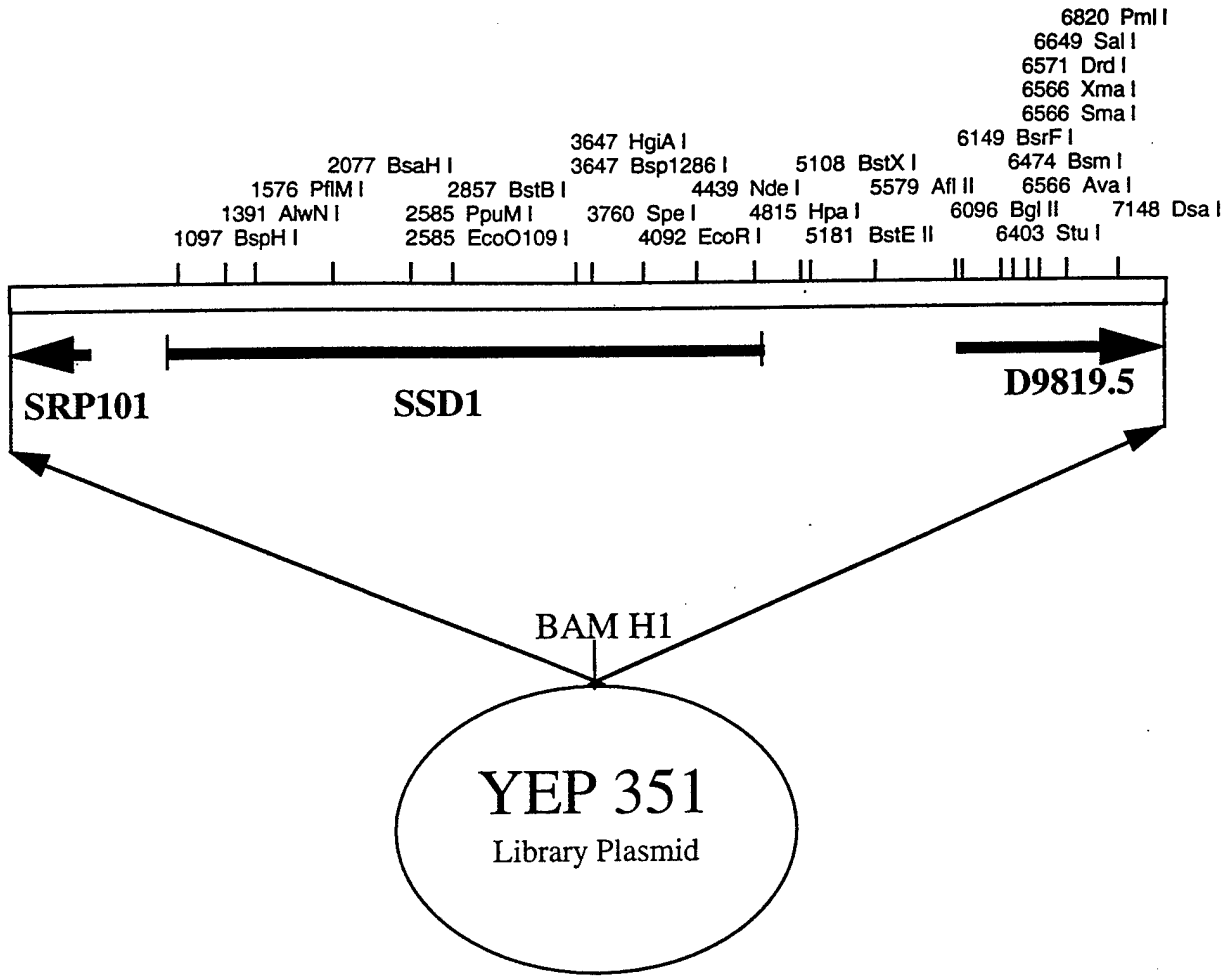


Figure 5: A restriction map of the full genomic fragment contained within the candidate S118A suppressor is shown. The corresponding positions of the genes within this fragment are indicated. Ssd1 is the only gene present in its entirety, while the SRP101 and D9819.5 genes are truncated at the 5' and 3' ends, respectively.

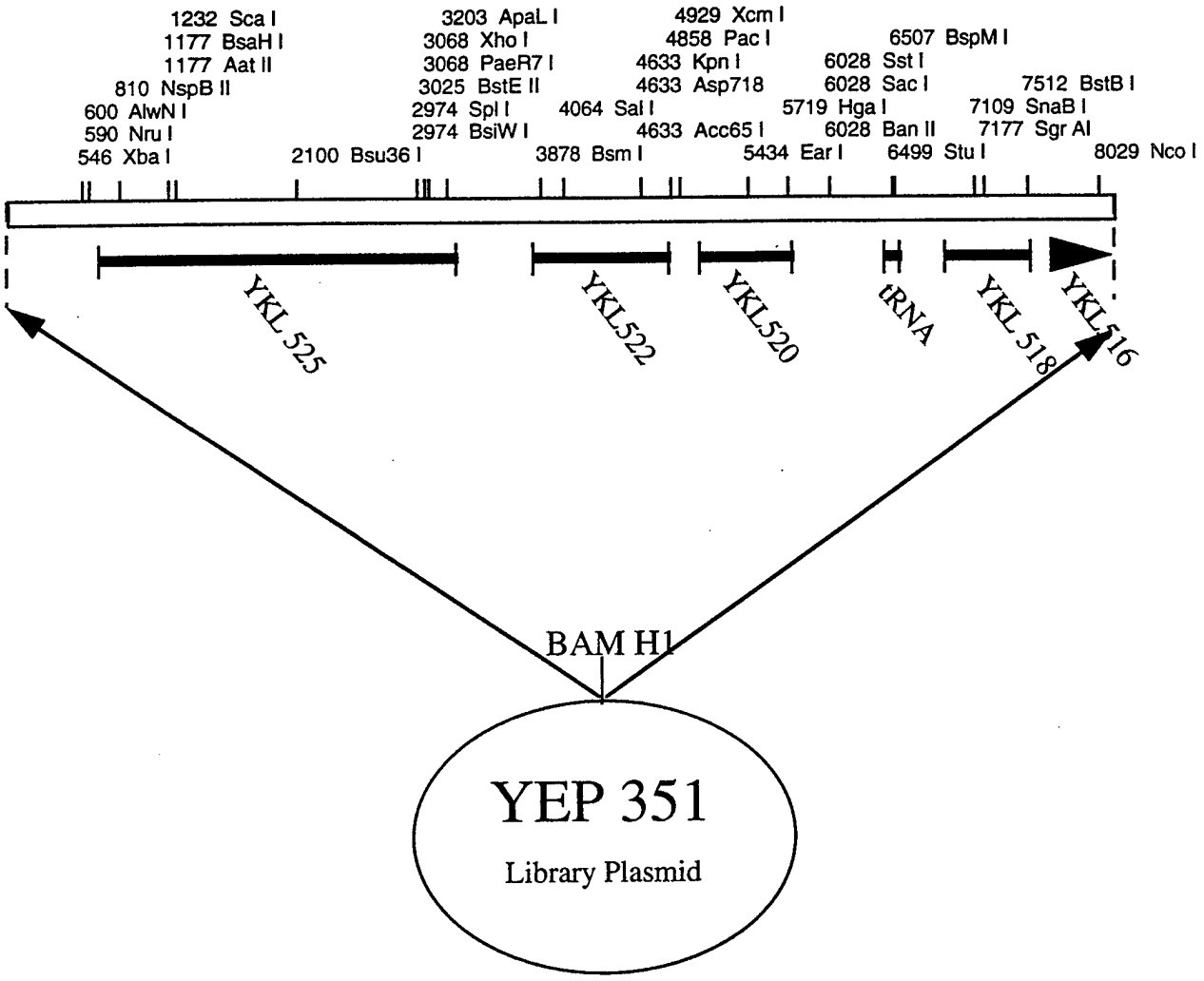


Figure 6: A restriction map of the full genomic fragment contained within the candidate G400V suppressor is shown. The corresponding positions of the six genes are indicated. Four of the genes code for complete proteins (YKL 518, YKL 520, YKL 522, YKL 525), while the YKL 516 gene is truncated at the 3' end.

D7 Interactions

PEG + PJG45	Gal/Raff Xgal	+BE2
1-185ER + D7	+	++
D7 +PJG45	-	-
D7 + 1-82ER	-	-
D7 + 1-115ER	-	-
D7 + 1-121ER	-	-
D7 + 1-185ER	+	+
D7 + 1-595ER	-	+
D7 + GR106-414	-	+

Figure 7: The results of our two hybrid analysis of the ER- D7 interaction are shown. The filled box in the D7 + 1-185ER row is meant to indicate sequences which seem to be important for the interaction of the two proteins. Reflected in the data is a general increase in Bgalactosidase staining observed on plates containing B-estradiol.

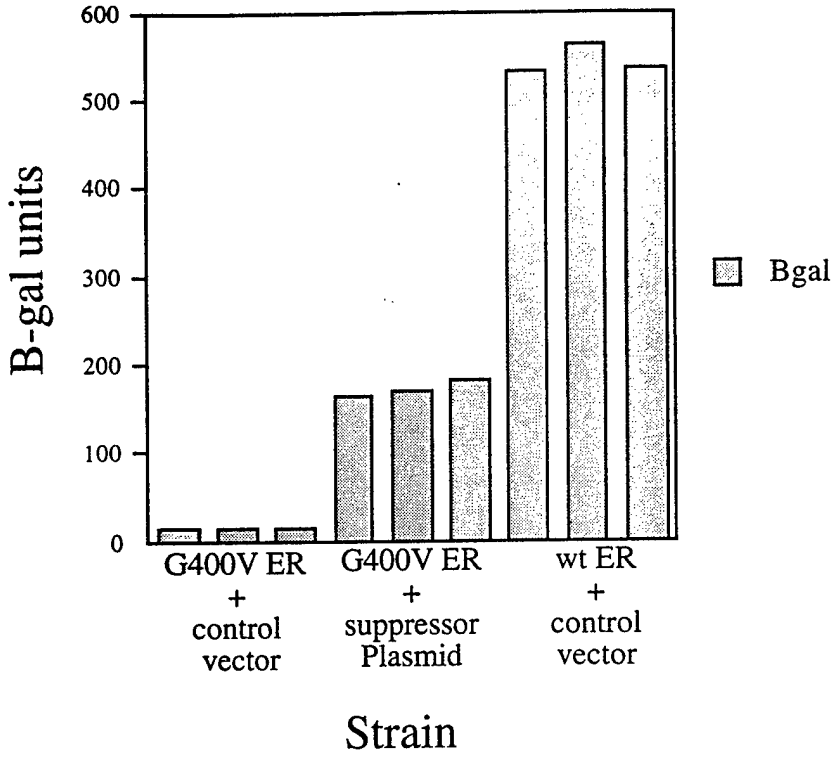
D6 Interactions

Grb-2 SH3 domain

PEG + PJG45	Gal/Raff Xgal	+BE2
1-185ER+D6	+	++
D6 + PJG45	-	-/+
D6 + 1-82ER	-	-/+
D6 + 1-115ER	+	++
D6 + 1-121ER	-	-
D6 + 1-185ER	+/-	+
D6 + 1-595ER	+	+++
D6 + GR106-414	++	+++

Figure 8: The results of our two hybrid analysis of the Grb-2/ER interaction are shown. The filled box in the D6 + 1-115ER row is meant to indicate sequences which seem to be important for the interaction of the two proteins. Reflected in the data is a general increase in Bgalactosidase staining observed on plates containing B-estradiol.

A)



B)

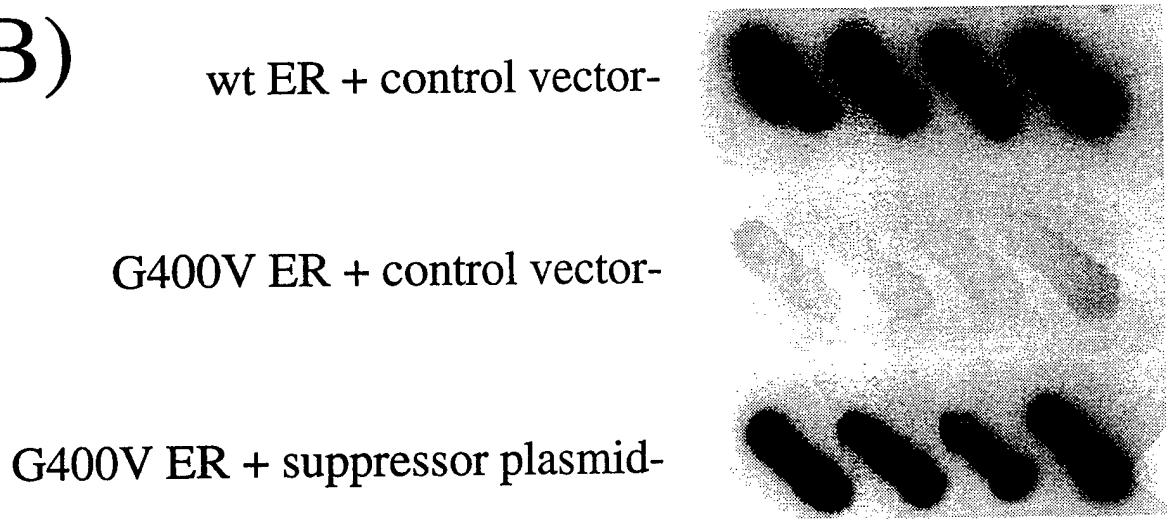
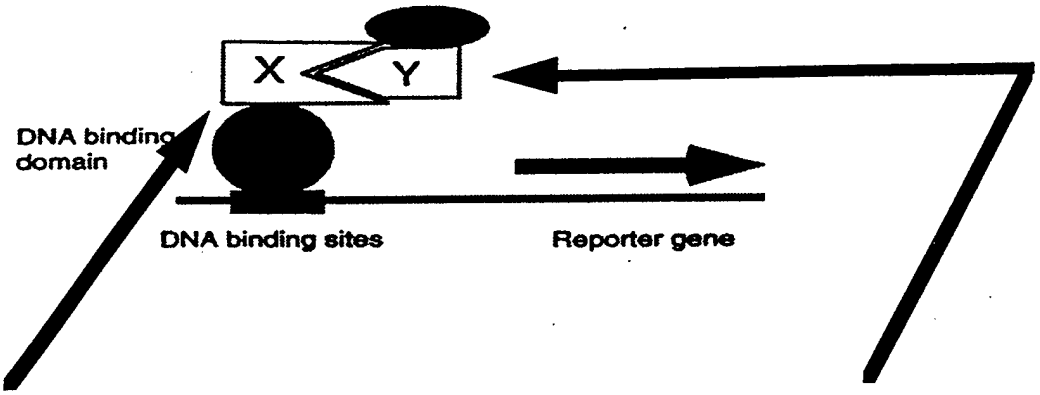


Figure 9: Suppression of G400V ER phenotype by the candidate suppressor is shown. A) liquid B-galactosidase assays carried out in media containing galactose as carbon source, with B-estradiol at a concentration of 10^{-9}, shows the suppressor is able to bring G400V ER activity to approximately 33% of wt ER activity. B) G400V ER phenotype suppression as seen on plate assay: indicator plates contain galactose as carbon source with B-estradiol at 10^{-9} final concentration.

Two Hybrid System



Baits

ER 1-185
DRG
TAF 130
T3 (A,B)
DNA Binding Domain alone

Preys

ER 1-82
ER 1-115
ER 1-121
ER 1-185
ER 1-595
GR 106-414
Activation Domain alone

Figure 10: The two hybrid strategy is illustrated above. The "bait" is a Lex DNA Binding Domain fused to the protein of interest "X"(e.g. ER 1-185). The library cDNAs ("Y") are expressed within the "prey", which contains an transcriptional activation domain. Induction of reporter gene transcription occurs when the bait and prey proteins are capable of interation, thereby bringing the activation domain to the promoter of the reporter gene. Below the schematic is listed the various baits and preys which have been used in our two hybrid analysis of isolated clones.