Estradiol-Inducible Squelching and Cell Growth Arrest by a Chimeric VP16-Estrogen Receptor Expressed in *Saccharomyces cerevisiae*: Suppression by an Allele of *PDR1*

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Received 3 August 1992/Accepted 9 October 1992

We have constructed and characterized a flexible system for analyzing the phenomenon of squelching and estrogen receptor function in the yeast *Saccharomyces cerevisiae*. The A/B region of the human estrogen receptor was replaced with the transcriptional activating domain of VP16 and expressed in yeast cells from high-copy-number plasmids. Addition of hormone resulted in an immediate inhibition of expression (squelching) of a chromosomally integrated GAL1::lacZ reporter gene and the eventual arrest of cell growth (toxicity). In order to determine whether a relationship exists between toxicity and squelching, mutations were made in this chimeric receptor (VEO) and their effects on transcriptional activation, squelching, and toxicity were compared. A direct correlation was found between mutations in VEO that reduced VP16 transactivation ability in yeast cells and those that reduced both squelching and toxicity. Surprisingly, mutations in the DNA binding domain (DBD) of VEO dramatically reduced squelching and completely relieved toxicity, suggesting a role for the DBD in squelching and strengthening the correlation between squelching and toxicity. To demonstrate the utility of this system for carrying out genetic selection, a plasmid-based yeast genomic bank was screened for genes that can relieve the toxicity of VEO by means of an elevated copy number, resulting in the repeated cloning of an allele of the *PDR1* (pleiotropic drug resistance) gene. We present evidence that mutations in *PDR1* can modulate the intracellular availability of estradiol by the same mechanism that leads to multiple drug resistance in yeast cells. Taken together, our results provide evidence that cell growth arrest occurs when squelching exceeds a certain threshold and that strong squelching requires both a DBD and a transcriptional activating domain. Furthermore, we show that growth arrest can provide a useful phenotype for carrying out the genetic analysis of both squelching and estrogen receptor function in yeast cells.

Transcriptional activators can often interfere with the expression of genes lacking binding sites for these factors, both in vivo (15, 33, 41, 44) and in vitro (5, 21). This transcriptional interference, or squelching, is presumed to be due to competition between activating factors for a productive interaction with limiting transcription factors (reference 25 and references therein). In fact, the efficiency with which a particular activator can squelch has been shown to be related to its ability to activate transcription (15) and has in some cases been shown to be independent of the presence of a DNA binding domain (DBD) (15, 41). The identity of the limiting transcription factor(s) is not known; however, in some cases squelching has been shown to specifically affect activated and not basal-level transcription (5) or to be limited to certain combinations of activating factors (31, 41). The specificity of these effects has led to the proposal of a novel class of transcriptional intermediary factors (TIFs) (coactivators, mediators, or adaptors) that interact with specific transcriptional activation domains, independently of DNA binding, and ultimately contact the basic transcription apparatus to stimulate transcription (reference 25 and references therein).

Direct evidence for the existence of TIFs has come from in vitro studies in which a yeast nuclear preparation (21) and a HeLa whole-cell extract fraction (49), capable of relieving squelching by GAL4VP16 (a transactivator consisting of the DBD of GAL4 and the acidic activating domain [AAD] of VP16), have been separated from the components required for basal-level transcription. Additional evidence for the existence of TIFs has been provided by the observation that recombinant TFIIH can be used to reconstitute basal-level, but not activated, transcription in vitro (20, 36, 39). HeLa cell partially purified TFIIH fractions, which do support activated transcription in vitro, actually consist of several additional proteins (10), at least some of which are required for activated, but not basal-level, transcription. Whether or not these TFIIH-associated proteins are the same limiting proteins that are titrated by squelching remains to be demonstrated.

An alternative to the biochemical purification of these putative TIFs is to try to clone the genes encoding them directly, taking advantage of yeast genetics. It has been observed that the overexpression of certain activating factors in *Saccharomyces cerevisiae* can cause a severe reduction in growth rate (6, 30, 46, 51). In particular, it is
extremely difficult to transform yeast strains with high-copy-number plasmids expressing GAL4VP16 (6, 17). Because of the potency of GAL4VP16 in squelching transcription in vitro and in vivo, we hypothesized that growth inhibition (toxicity) might be due to squelching of essential yeast transactivators by VP16. This hypothesis suggested an obvious approach to clone the limiting TIF by adding back the TIF gene from a plasmid-based yeast genomic bank, thereby increasing TIF concentration and relieving toxicity. One potential problem was that GAL4VP16 is a constitutively toxic protein, requiring one to rely on yeast transformation efficiency and colony size as a measure of GAL4VP16 toxicity. In addition, GAL4VP16 contains a natural yeast DBD; therefore, toxicity could be a result of the inappropriate expression of particular yeast genes. Since it has been shown that the estrogen receptor (ER) functions in S. cerevisiae (32) and that the ER hormone binding domain (HBD) can confer hormone dependence on the functions of linked protein domains (11, 12, 37), we replaced the ER A/B domain (which contains the transcriptional activator TAF-1) with the AAD of VP16. By doing this, we created a protein in which the VP16 activation function is inducible by estradiol.

In addition to providing a useful system for the analysis of squelching, we anticipated that an estrogen-dependent growth arrest phenotype could provide a means to select for mutations involved in the activation of the ER by estradiol. Since the utility of such selection systems depends on an understanding of the nature of the growth arrest phenotype, we undertook to determine whether the toxicity of VP16 in S. cerevisiae is related to squelching and whether it can provide a phenotype that is useful for genetic selection.

We show here that when expressed to high levels in S. cerevisiae, this VP16-ER chimeric protein (VEO) causes a gradual and complete arrest of cell growth. Growth arrest is entirely dependent on the addition of estradiol so that, in the absence of hormone, high levels of this protein can be maintained in the cell with no detectable effect on cell growth. We also show that VEO can rapidly squelch the expression of a GAL4-responsive promoter (GAL1::lacZ). Maximal squelching and growth arrest are both dependent on an intact VP16 AAD and the DBD of VEO, providing correlative evidence that toxicity results from strong squelching. An attempt to suppress toxicity by using a yeast genomic library led to the repeated cloning of an allele of PDR1 capable of conferring resistance of yeast strains to estradiol, demonstrating that the first step in ER activation, the entry of diffusible ligand into the cell, is regulated by the multidrug resistance pathway.

MATERIALS AND METHODS

Yeast strains, transformations, and media. S. cerevisiae TGY14.1 (MATa leu2-3,112 ura3 pep4-3 gal2) was obtained from Transgene. US50-18C (MATa ura3 his3 pdr1-3) was a gift from E. Balzi (3). SC334 (MATa leu2-3,112 gal1 regl-sol1 ura3-52 lys2 cyh2 gal1) was a gift from A. Sachani. The construction of strain PL3 (ura3-Δ his3-Δ leu2-3 trpl1::SER-413 [SER-4 indicates three estrogen-responsive elements]) has been described elsewhere (38). Yeast transformations were carried out either by electroporation or by polyethylene glycol-assisted protoplast transformation. Transformants were selected on YNB (0.7% yeast nitrogen base [YNB], 2% glucose) supplemented (per liter) with 30 mg of uracil, 5 g of Casamino Acids, or 20 to 60 mg of individual amino acids as appropriate. Colonies were established under defective LEU2 (dLEU2) selective pressure at about 1% of the frequency of colonies established under URA3 selective pressure, using the protoplast transformation procedure (electroporation was not successful in giving rise to dLEU2-selected transformants). TGY14.1/pDGY3 strains were made by protoplast transformation of TGY14.1 with pDGY3, selecting directly for dLEU2. Transformants grow slowly under these conditions (see Fig. 3) and also display the aseptate morphology typical of cells carrying high copy numbers of 2 μm plasmids (35); we found this morphology to be a convenient screen for cell lines carrying high copy numbers of pDGY3. Reversion (to estradiol-resistant cells) rates between individual transformants in medium containing 100 μM estradiol varied between 10^-5 and 10^-7. Passaging of TGY14.1/pDGY3 for several weeks resulted in a gradual increase in the frequency of reversion, and storage at 4°C in YNB resulted in rapid (within a few weeks) death of the cell line. Reversion was accompanied by a loss of the septate morphology and the inability to rescue pDGY3 to Escherichia coli. Experiments using TGY14.1/pDGY3 were carried out with fresh transformants that had reversion frequencies of <10^-6.

G334B was constructed by electroporation of strain SC334 with the integrating plasmid pRY131Leu2B (described below), after linearization with XhoI, selecting for leucine prototrophy. PDR1 disruption of PL3 to make strain GPL3A was done by electroporating PL3 with p19-32Xmal/SacII Trp1a (described below) after digi-mentation with ApaI, XmaI, and SnaBI (to destroy 2 μm se-quences), selecting for tryptophan prototrophy. Colonies were then screened for sensitivity to cycloheximide. Disruption was confirmed by Southern blot analysis of DNA prepared from those colonies demonstrating increased sensitivity to cycloheximide.

Plasmid constructions. pTG848 (26), pAB6.5 (3), pRS313 (38), and pFL1 (also known as pYEP24) (8) have been described elsewhere, and pYE90 is a HIS3-selectable derivative of pTG848 (38). Receptor VEO was constructed by linking the EcoRI-KpnI fragment from VP16(N)(41) to the KpnI-EcoRI fragment of HE80 (23) and cloned into the EcoRI site of pSG5 (16). For expression in yeast strains, the various constructs (Fig. 1) were excised as EcoRI fragments and either were cloned into the EcoRI site of pYE90 (for transformation of PL3) or were first cleaved into the EcoRI site of an EMBL3-derived plasmid (pD18 in which the polylinker was exchanged for a polylinker containing the constitutive restriction sites BamHI-EcoRI-BamHI). Receptors cloned into the EcoRI site of pD6 were excised with BamHI and cloned into the BglII sites of pTG848 (for transformation of TGY14.1 or G334B), pDGY3 was made by excising the URA3 gene of pTG848 (by digestion with HindIII and recircularization), resulting in pTG848AURA, and then cloning VEO as a BamHI fragment into the BglII sites of pTG848ΔURA. Receptor mutants VE1 and VE11 were made by in vitro mutagenesis of VEO in pD6. VE4 was made by exchanging the SacI-XmaI fragment of VEO with the same fragment encoding an alanine at position Phe-442 of VP16 (a gift of C. Brou). VE5 and VE6 were made by deleting the DNA between the XhoI and SacI sites representing VP16 amino acids 411 to 422 of VEO or VE4. VE7 and VE8 were made by deleting the DNA between the XmaI and KpnI sites representing VP16 amino acids 454 to 490 of VEO or VE4. VE9 and VE10 were made by introducing both VP16 deletions into VEO and VE4. VE12 was made by deleting the DNA between the two XhoI sites representing ER amino acids 287 to 552 of VEO. The integrating plasmid
FIG. 1. Schematic diagram of receptors. The 595-amino-acid hER can be divided into six domains (A to F) as shown. The activation domain of VP16 is contained in amino acids 411 to 490 of VP16. VEO is a chimera consisting of the VP16 activation domain (residues 411 to 490) linked in frame to CDEF (175 to 595) of hER, and VE1 through VE12 are derivatives of this construct containing various mutations or deletions as indicated. VE1 contains two Cys→Gly mutations at amino acid positions 202 and 205 in the first Zn$^{2+}$ finger of the hER C domain. These amino acids have been shown to be essential for DNA binding by the ER (23). VE2 contains a deletion of hER amino acids 176 to 230 containing the DBD (C). Deletions of the N-terminal (411 to 423) or C-terminal (453 to 490) amino acids of the VP16 domain were introduced in VE5 to VE10. The entire VP16 domain was deleted in VE11. VE4, VE6, VE8, and VE10 contain a Phe→Ala mutation at position 442 of the VP16 domain. VE12 contains a deletion of hER amino acids 286 to 555 containing the HBD (E) (see Materials and Methods for details of constructions). All proteins contain the same short N-terminal sequence of amino acids containing a Kozak sequence and the nuclear localization signal of simian virus 40 T antigen (19), described by Tasset et al. (41). VE3 (not shown) is a construct not included in this study. VE3 is identical to VE2 except that the hER D domain was also deleted. This protein was not localized to the nucleus, indicating that the simian virus 40 T-antigen nuclear localization signal does not function in S. cerevisiae and that the D domain of the ER is required for nuclear localization of these receptors in yeast cells (14).

pRY131Leu2B was made by deleting the DNA between XhoI and NsiI sites of pRY131 (48) and replacing it with a Sall genomic fragment containing the LEU2 gene with the direction of transcription of LEU2 opposite that of GAL1: lacZ. We found that this orientation consistently gave 50% greater expression of GAL1: lacZ than did the opposite orientation when integrated into the LEU2 chromosomal locus. The plasmid used for PDR1 disruption was constructed by first deleting the DNA between the XmaI and SacII sites of the PDR1-containing genomic library plasmid p19-3 to give p19-3ΔXmaI/SacII. The TRP1 gene was then cloned into the XhoI site within the coding sequences of PDR1 to produce plasmid p19-3ΔXmaI/SacII Trp1a.

Enzymatic, toxicity, and drug sensitivity assays. Orotidine 5'-monophosphate decarboxylase activity was measured according to the method Wolcott and Ross (50), with crude extracts obtained as previously described (27). Specific activity was expressed in nanomoles of substrate transformed per minute per milligram of protein. Protein content was assayed by the method of Bradford (7). β-Galactosidase assays were done as described by Miller (34) except that the calculation of activity was determined by using the optical density at 600 nm (OD$_{600}$) of the actual permeabilized cell preparation used to carry out the reaction. Drop tests (Fig. 2) were done by placing a standardized concentration of cells in a 10-μl aliquot onto agar medium and scoring the resultant growth after 2 to 4 days. Viability assays were carried out by culturing cells to exponential phase in YNBD containing Casamino Acids (selection for uracil prototrophy) and then plating serial dilutions of each culture to YNBD plates supplemented with either Casamino Acids, Casamino Acids plus 100 nM estradiol, or 100 nM estradiol alone or to YNBD plates with no supplements. Colonies were counted after 2 days on media containing Casamino Acids or after 4 days on plates with no amino acid supplements. The number of CFU on YNBD plus Casamino Acids was taken to be 100% viability, and the viability under all other conditions was calculated relative to this number. Viability assays were done three to nine times, depending on the actual number of colonies scored (hence, the degree of confidence). Drug sensitivities were determined and expressed by the procedure of Balzi et al. (3). Briefly, equal quantities of cells taken
from a fresh plate were drop tested in the appropriate medium containing increasing concentrations of cycloheximide, oligomycin, or estradiol and scored for growth after 4 days on YNB, YNB, or YPG or after 3 days on YPD. Cycloheximide sensitivity was determined on YPD (1\% yeast extract, 2\% Bacto Peptone [YP], 2\% glucose) agar with 0.0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 \( \mu \text{g} \) of cycloheximide per ml, except for strains containing plasmids, which were tested on YNB with the appropriate supplements and 0.0, 0.025, 0.05, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 3.0 \( \mu \text{g} \) of cycloheximide per ml. Oligomycin sensitivity was tested on YPG (YP plus 2\% glycerol) with 0.0, 0.02, 0.1, 0.5, 2.5, and 10.0 \( \mu \text{g} \) of oligomycin per ml or on YNB (YNB plus 2\% glycerol) with 0.0, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 \( \mu \text{g} \) of oligomycin per ml for strains containing plasmids. The maximum noninhibitory concentration (maximum concentration of drug at which normal growth was observed; MNIC) of estradiol was determined on YNB with 0.0, 1.0, 2.0, 4.0, 6.0, 10.0, 20.0, 30.0, 40.0, and 50.0 nM estradiol, and the maximum nonstimulatory concentration (maximum concentration at which cells are still auxotrophic for uracil; MNSC) of estradiol was determined on YNB with 0.0, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 nM estradiol.

**Immunodetection and yeast immunofluorescence.** Western immunoblotting was carried out on yeast extracts by using a monoclonal antibody raised against the F domain of the ER (1). In situ detection of receptors by immunofluorescence was accomplished by the procedure of Kilmartin and Adams (22), using the anti-F-domain antibody and Texas red-conjugated goat anti-mouse immunoglobulin G.

**Library screening.** The yeast genomic library used in these studies was a gift from F. Lacroute and is estimated to contain at least three complete genomes. The library was constructed by cloning 5- to 7-kb fragments of yeast genomic DNA partially digested with *Sac*3A into the *Bam*HI site of pFL1 (YPep24). A via! containing 1 ml of this bank frozen at an early passage was diluted into 1 liter of rich medium, and ampicillin was added after 3 h of growth. At the time of preparation of the plasmid DNA (by alkaline lysis), bacteria were plated for individual colonies, and 9 of 12 separate colonies tested contained inserts of the appropriate size into the pFL1 vector. TGY14.1/pDGY3 cells were grown to exponential phase in selective medium (YNBD) supplemented with uracil and then subsequently grown for several more generations on rich medium (YPD) before spermidoplasts were made, to facilitate efficient regeneration and transformation. Protoplasts were then transformed with a pFL1 yeast genomic bank or the vector pFL1 alone and overlaid on YNB. After 24 h, a second overlay of agar containing 100 nM estradiol (to achieve a final estradiol concentration of \( -33 \text{nM} \) was applied, and colonies were picked and restreaked to YNB containing 30 nM estradiol as they arose between 4 days and 2 weeks after transformation. Of over 600 colonies that were picked from plates of cells transformed with the library, 43 continued to grow in the presence of estradiol; no colonies grew on the control plates of cells transformed with the pFL1 vector. DNA from the 43 clones was rescued to *E. coli* by the glass bead lysis method. Southern blotting was performed with the total plasmid DNA isolated from pools of transformed *E. coli*, and the blots were hybridized to an oligonucleotide representing 50 bases of the *LEU2* promoter to detect clones that had received the wild-type *LEU2* gene from the library or to a DNA fragment containing the coding sequences of VEO to detect loss or rearrangement of VEO. pDGY3 isolated from the three *PDR1*-containing clones was found to be unarrested by restriction mapping and capable of conferring estradiol-inducible toxicity upon retransformation of TGY14.1 cells. VEO protein was demonstrated to be present at normal levels by Western blotting and to be localized to the nucleus by immunofluorescence in cells containing any one of the three library plasmids both in the presence and in the absence of estradiol.

The lower-copy-number (*URA3*-selected) library plasmids from the three clones containing intact pDGY3-containing colonies were selectively rescued by transformation into a strain of *E. coli* containing a mutation at the pyrF locus, which can be complemented on minimal medium by the yeast *URA3* gene. Purified library plasmids (designated p9-7, p19-3, and p20-8) were restriction mapped, and an overlapping *XhoI* fragment was hybridized back to genomic yeast DNA by Southern blotting, confirming that it is a unique contiguous fragment (not shown). Fragments of p19-3 were subcloned, retransformed into TGY14.1/pDGY3, and tested for the ability to suppress estradiol-inducible toxicity by determining transformation efficiency in the presence of estradiol. The levels of VEO protein and the nuclear localization of VEO were verified in TGY14.1 cells retransformed with each of the three separately isolated library inserts and grown either in the presence or in the absence of estradiol.

**RESULTS**

**VEO imparts estradiol-dependent cell growth arrest.** VEO (Fig. 1) was constructed by linking the VP16 transcriptional AAD (amino acids 411 to 490) in frame to a truncated derivative of the human ER (hER) (amino acids 175 to 595) containing the DBD and ligand binding domain. The plasmids expressing VEO and all constructs reported in this study were derived from pTG848, a 2.0 \( \mu \text{m} \) shuttle plasmid containing the promoter and terminator from the PGK gene designed for high-level expression of cDNAs cloned into the *BglII* site of this plasmid (26). pTG848 contains as selectable markers the *URA3* gene and the *LEU2* structural gene with a defective promoter (*dLEU2*). Selection for uracil prototrophy yields transformants in which the plasmid is maintained at normal levels (approximately 20 copies per cell [8]); however, selection for leucine prototrophs requires maintenance of *dLEU2*-containing plasmids at an abnormally high level (approximately 100 copies per cell [13]). Thus the level of expression of cDNAs cloned into these plasmids can be controlled by choice of selection marker (28).

To determine whether VEO is toxic in yeast cells, we transformed a *ura3 leu2 yeast strain* (TGY14.1 with pTG848 or derivative plasmids expressing either VEO or the complete hER (HEO), selecting for either *URA3* or *dLEU2*. Several independent clones from each transformation were then tested for the ability to grow on media containing estradiol. An example of each is shown in Fig. 2A. When VEO is expressed under selection for *URA3*, the presence of estradiol inhibits cell growth; however, growth arrest is not complete, and at least some of the population of VEO-expressing cells continue to grow. On the other hand, expression of VEO under selection for *dLEU2* leads to a complete arrest of cell growth upon the addition of estradiol. Under these experimental conditions, the parental expression vector or the vector expressing ER (HEO) did not detectably alter cell growth (Fig. 2A and data not shown), indicating that the strong toxic effect of VEO is due to the presence of the VP16 AAD.

To determine how rapidly estradiol affects cell growth and
cell death, even after 24 h in the presence of estradiol (not shown). For this reason, we refer to the estradiol-induced phenotype as growth arrest.

**Estradiol addition rapidly inhibits GAL4-stimulated gene expression in cells expressing VEO.** To determine whether estradiol-dependent growth arrest in cells expressing VEO is related to squelching of endogenous gene expression, a reporter gene expressing β-galactosidase under the control of the yeast GAL1 promoter was integrated into the LEU2 locus of a reg1-502 gal1 yeast strain (designated G334B). With this yeast strain, galactose can be added as a gratuitous inducer to healthy cultures of cells growing in medium containing glucose (18). The GAL1 promoter is activated by the yeast transactivator GAL4, which contains an AAD that can be squelched by the AAD of VP16 (41). G334B cells were transformed with pTGG84 expressing VEO (selecting for URA3), and a single transformant was divided into nine identical exponentially growing cultures. Estradiol was added at various times before and after the addition of galactose, and both OD600 and the induction of β-galactosidase activity were measured (Fig. 3). Whether estradiol was added before or coincident with the addition of galactose, induction of the GAL1 promoter was inhibited to the same extent, indicating that activation of the VEO receptor rapidly squelches this promoter. Addition of estradiol at various times after the onset of GAL1 promoter stimulation led to an abrupt inhibition of β-galactosidase induction. In each case, the kinetics of induction of β-galactosidase were similar after the addition of estradiol, demonstrating that squelching by VEO can effectively inhibit transcription of an actively transcribed gene.

Since VEO has such a strong effect on cell growth under dLEU2 selection, the experiments described above were carried out under selection for URA3, in which case the inhibition of cell growth is much less severe (Fig. 3B versus Fig. 2B). We therefore assume that our results underestimate the squelching potential of VEO that is achieved under selection for the dLEU2 gene.

**Maximal squelching and toxicity by VEO require both a transcriptionally potent VP16 AAD and an intact DBD.** The results described above indicate that the addition of estradiol to cultures expressing VEO leads to an immediate squelching of GAL4-stimulated gene expression and a gradual inhibition of cell growth. To determine whether there is a correlation between the squelching and growth arrest effects of VEO, the receptor was mutated at a number of potentially important locations and the resulting mutant receptors were assayed for the ability to (i) activate transcription from a reporter gene containing an estrogen-responsive promoter, (ii) squelch the expression of the GAL1: lacZ reporter gene described above, and (iii) arrest cell growth. All of the receptors described in this report are represented in Fig. 1. VE1 contains point mutations in two cysteine residues (ER amino acids 202 and 205) known to be required for binding of the ER to DNA (23). VE2 contains a deletion in the ER DBD (ER amino acids 176 to 250). Receptor mutants VE4 to VE10 contain either a mutation in the Phe-442 residue of VP16, previously shown to be important for transcriptional activation in mammalian cells (9), deletions in VP16, or a combination of deletions and the Phe-442 mutation (a plasmid encoding VP16 with an alanine at this position was kindly provided by Christel Brou). VE11 contains an exact deletion of all VP16 amino acids. VE12 contains a deletion of the ER HBD. Since the HBD also contains TAF-2 (43), a hormone-dependent transcriptional activation domain that also functions in yeast cells (38), VE11 and VE12 allow a determina-
Each of these receptors was tested for its ability to activate transcription by using the yeast reporter strain PL3. This strain contains a single URA3 gene, integrated at the TRP1 locus, in which sequences required for both basal and activated transcription were replaced by three EREs in tandem, and has been shown to be a sensitive indicator of the activating potential of ERs (38). Since the reporter gene is URA3, each receptor was first cloned into the PKG expression cassette of a pTG848-derived vector (pYE90) in which the URA3 and dLEU2 selection markers have been exchanged for HIS3. The results of this analysis are presented in Table 1. As expected, mutation or deletion of the DBD abolished the ability of the receptor to activate transcription of the ERE-containing promoter. A small deletion in the N-terminal part of the VP16 AAD (deleting VP16 residues 411 to 422) had little or no effect, while a larger deletion in the more acidic C-terminal part of the VP16 AAD (deleting VP16 residues 454 to 490) reduced its activity approximately 10-fold. Substitution of an alanine at Phe-442 of VP16 had no striking effect on activation in the context of the entire VP16 activation domain or the N-terminally truncated VP16 domain; however, this same mutation decreased three- to fivefold the remaining 10% activity of receptors containing the C-terminally truncated VP16 domain. Deletion of the HBD resulted in transcriptional activity independent of the addition of estradiol as expected (24) and to a level comparable to that of the induced VEO. TAF-2 alone (VE11) activated transcription to a level only 1% of that of the VP16 AAD alone (VE12). Thus, virtually all of the transcriptional activity of VEO is due to the VP16 AAD.

Squelching has been proposed to result from the titration of transcription factors due to the presence of competing transcriptional activating domains (see the introduction). Hence, interfering with the ability of an activating domain to activate transcription should also interfere with its ability to squelch the transcription of another gene. To determine whether a correlation exists between the ability of various VEO mutations to activate transcription and their ability to squelch the AAD of GAL4, each receptor was expressed from pTG848 (under selection for URA3) in yeast strain G234B, and β-galactosidase activity was assayed as described above. Individual transformants were cultured for 36 h in the continued presence of galactose (and estradiol where indicated) to bring all β-galactosidase expression levels to steady state for a particular clonal derivative (Table 1). Consistent with Fig. 3A, VEO had a strong estradiol-dependent squelching effect on the expression of the GAL1: lacZ reporter gene. The N-terminal truncation of the VP16 AAD did not affect the magnitude of squelching by VEO; however, the C-terminal truncation of VP16, which severely reduced the transcriptional activating potential of VEO, dramatically reduced its ability to squelch GAL4-stimulated activation. The Phe–442 mutation in the VP16 AAD (residues 411 to 490) caused a slight reduction in squelching ability, and a combination of the VP16 C-terminal deletion (residues 411 to 453) and Phe–442 mutation reduced squelching to the level achieved by molecules completely lacking the VP16 AAD. These results demonstrate that mutations affecting the ability of the VP16 AAD to activate transcription also affect its ability to squelch GAL4-stimulated expression from the yeast GAL1 promoter. In addition, mutations in the DD (VE1 and VE2), in the presence of the complete VP16 AAD, reduced the squelching activity of VEO as effectively as did a deletion of the VP16 AAD.

To determine whether there was a correlation between the squelching potential of VEO and its ability to arrest cell growth, we have quantitated the relative contributions of the VP16 AAD and TAF-2 to the various effects of the VEO receptor. All mutations were made internal to the receptor, retaining the integrity of the N terminus to facilitate similar translational efficiencies of all receptors and retaining the C-terminal F domain to allow the detection of full-length protein products. Western blotting indicated that the steady-state levels of all protein products were similar when expressed in yeast cells from pTG848 (data not shown). HEO, VEO, VE1 to VE6, and VE11 proteins were localized to the yeast nucleus, as determined by immunofluorescence of yeast cells (as described in Materials and Methods) both in the presence and in the absence of estradiol (data not shown).
growth, the toxicity of each of these receptors was also examined (Table 1). To provide a quantitative measure of the toxicity of each receptor, a viability assay was used. TGY14.1 cells were transformed with pTG848 plasmids containing each receptor, selecting for URA3, and several individual clones were tested for viability under URA3 or dLEU2 selection and in the presence or absence of estradiol. A strong correlation was found between the toxicity of the VEO receptor for cell growth and the strength of the acidic region of VP16 in transcriptional activation and squelching. In addition, mutations in the DBD that dramatically reduced the squelching potential of VEO completely abolished toxicity.

**An allele of PDR1 that relieves the estrogen-induced effects of VEO.** The results presented above suggest that cell growth arrest is related to squelching by VEO. If squelching was due to the titration of a limiting transcription factor, we reasoned that increasing the number of copies of the gene encoding the titrated factor, prior to the addition of estradiol, should relieve the growth arrest phenotype. To test this hypothesis, TGY14.1 cells were transformed with pDGY3 (a pTG848-derived plasmid expressing VEO in which the URA3 gene has been deleted to allow subsequent transformation with a genomic bank carrying URA3 as a selectable marker), selecting directly for the dLEU2 marker. Twelve colonies were tested for their frequency of reversion to estradiol resistance, and an individual transformant (designated TGY14.1/pDGY3) with a reversion frequency of $5 \times 10^{-7}$ was subsequently transformed with a complete yeast genomic library cloned in the UR13-selectable 2-μm plasmid pFL1 (see Materials and Methods). These cells were plated to selective medium for both dLEU2 and URA3 and incubated for 24 h to facilitate the expression and accumulation of the putative limiting TF both before the addition of an overlay of agar containing estradiol. Of approximately $6 \times 10^4$ TGY14.1/pDGY3 cells that were transformed by the genomic library (as estimated from the number of colonies on control plates receiving no estradiol), only 43 transformants survived estradiol challenge. Hybridization of plasmid DNA rescued from the 43 clones with a fragment of DNA encoding VEO showed that only three retained pDGY3 at high copy number. The pDGY3 plasmids rescued from these clones exhibited no obvious rearrangements and retained estradiol-inducible toxicity when retransformed into TGY14.1, demonstrating that they were intact. The remainder of the clones either had lost pDGY3 entirely (nine clones having received the complete LEU2 gene from the library, thus relieving the selection for pDGY3) or contained rearranged derivatives of this plasmid.

Restriction mapping of the inserts contained in the library plasmids from these three yeast clones revealed that they shared a common sequence: two containing the same genomic fragment, and the third containing a larger overlapping fragment. Partial sequence analysis and screening of sequence data bases revealed that the library inserts contained the PDR1 gene and that the DNA encompasses the entire genomic inserts of two of our clones had previously been sequenced (+). TGY14.1/pDGY3 was retransformed with plasmids containing deletions of the previously identified (++) open reading frames flanking either the 5' or 3' border of the PDR1 gene. In each case, relief of toxicity was observed, verifying that the PDR1 gene, and not other linked

<table>
<thead>
<tr>
<th>Receptor</th>
<th>OMPdecase activity* (nmol of substrate transformed: (nmol/min/mg)</th>
<th>β-Galactosidase activity* (Miller units/mg)</th>
<th>% Survival*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-E2</td>
<td>+E2</td>
<td>-E2</td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>301</td>
</tr>
<tr>
<td>HEO</td>
<td>&lt;0.01</td>
<td>30.00</td>
<td>288</td>
</tr>
<tr>
<td>VEO</td>
<td>3.53</td>
<td>192.50</td>
<td>298</td>
</tr>
<tr>
<td>VE1</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>313</td>
</tr>
<tr>
<td>VE2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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</tr>
<tr>
<td>VE4</td>
<td>2.50</td>
<td>222.80</td>
<td>309</td>
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<td>1.10</td>
<td>225.50</td>
<td>298</td>
</tr>
<tr>
<td>VE7</td>
<td>0.11</td>
<td>24.63</td>
<td>322</td>
</tr>
<tr>
<td>VE8</td>
<td>0.05</td>
<td>4.82</td>
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<td>VE9</td>
<td>0.08</td>
<td>15.53</td>
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<tr>
<td>VE10</td>
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</tr>
<tr>
<td>VE11</td>
<td>0.10</td>
<td>1.76</td>
<td>301</td>
</tr>
<tr>
<td>VE12</td>
<td>176.80</td>
<td>NAf</td>
<td>94</td>
</tr>
</tbody>
</table>

* For determination of transactivation, the yeast reporter strain PL3 was transformed with pYES9 or expressing HEO or the YE series. Activation of the 3ERE:URA3 reporter gene was determined by measuring the specific activity of the URA3 gene product crotoxin 5'-monophosphate deacylase (OMPdecase). Transformants were cultured on selective media containing uracil in the presence or absence of 100 mM estradiol (E2) for five generations.

b For determination of squelching of GAL4-stimulated gene expression, yeast strain G3348 was transformed with pTG848 without insert or expressing each of the VE constructs. Single transformants were assayed for β-galactosidase activity after 36 h of growth in the presence of 2% galactose and in the presence or absence of estradiol, as indicated. Results represent average activities obtained from six independently isolated transformants.

c For determination of the toxicity of receptor mutants, TGY14.1 cells were transformed with each of the receptor mutants expressed from pTG848, selecting on YNBD supplemented with Casamino Acids (selection for URA3). Cells were grown in liquid medium (YNBD plus Casamino Acids) to exponential phase, and serial dilutions from these cultures were simultaneously plated on YNBD plus Casamino Acids, YNBD plus Casamino Acids supplemented with 100 mM estradiol (selection for URA3 plus estradiol), and YNBD supplemented with 100 mM estradiol (selection for dLEU2 plus estradiol). Shown is the relative number of CFU for each transformant, calculated as a percentage of the exact number of CFU on YNBD plus Casamino Acids (URA3 selection alone). Results represent averages for at least three independently isolated transformants.

d Colonies formed were noticeably smaller than normal.

e Colonies were obtained at variable frequencies below the value shown.

f NA, not applicable.
TABLE 2. Relief of estradiol-inducible toxicity by a mutated allele of PDR1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>MNIC</th>
<th>Cycloheximide (µg/ml)</th>
<th>Oligomycin (µg/ml)</th>
<th>Estradiol (nM)</th>
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</thead>
<tbody>
<tr>
<td>US50-18C</td>
<td>None</td>
<td>1.0</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pAB6.5</td>
<td>0.25</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p19.3</td>
<td>0.75</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGY14.1(pDGY3)</td>
<td>pFL1</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pAB6.5</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p19.3</td>
<td>40.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* TGY14.1(pDGY3) and the pleiotropic drug-resistant yeast strain US50-18C (ura3 his1 pdr1-3) were transformed with a plasmid containing the wild-type PDR1 gene (pAB6.5), the allele of PDR1 cloned in this study (p19.3), or the vector control of pFL1. The effects of these plasmids on the MNICs of estradiol [TGY14.1(pDGY3)] and of cycloheximide and oligomycin (US50-18C) were estimated by drop test assays on selective medium plates containing increasing concentrations of these drugs (see Materials and Methods).

open reading frames, was responsible for the suppression of growth arrest (data not shown).

PDR1 is a transcription factor controlling the expression of a variety of membrane proteins that export small molecules across the plasma membrane. Mutations in PDR1 can lead to the resistance of yeast strains to a range of toxic compounds by increasing the expression of these membrane efflux proteins (reference 4 and references therein). This finding suggested that the suppression of estradiol toxicity by PDR1 could be due to the increased export of estradiol by way of this pathway, most commonly known as multiple or pleiotropic drug resistance. However, it has been shown that PDR1 expressed from a multicycoplasmid does not lead to a multiple drug resistance phenotype; in fact, the presence of the wild-type PDR1 on a multicycoplasmid lowers the resistance of multiple drug-resistant [pdr1] yeast strains (3).

To determine whether we had fortuitously cloned a mutated allele of PDR1, we compared the ability of a cloned wild-type PDR1 allele (pAB6.5) and the PDR1 allele cloned in our laboratory (p19-3) to functionally complement an already characterized PDR1 mutation (pdr1-3) in yeast strain US50-18C (3) and to suppress the estradiol-sensitive phenotype of TGY14.1(pDGY3).

US50-18C cells were transformed with either pAB6.5 of p19-3, and the MNICs of cycloheximide and oligomycin were determined (Table 2). As has been previously reported (3), pAB6.5 significantly reduced the MNICs of both oligomycin and cycloheximide relative to the levels of resistance of untransformed US50-18C. By contrast, p19-3 slightly reduced the MNIC of cycloheximide and increased the MNIC of oligomycin for US50-18C. To determine whether the wild-type PDR1 gene is capable of suppressing the growth arrest phenotype caused by VEO, TGY14.1(pDGY3) cells were transformed with either pAB6.5, p19-3, or pFL1, and the MNIC of estradiol was determined (Table 2). While p19-3 conferred a 20-fold increase in the MNIC of estradiol, transformation with pAB6.5 had no effect on estradiol sensitivity.

Since PDR1 is a transcription factor, it was important to determine whether the suppression of growth arrest was indeed due to a decrease in the sensitivity to estradiol via the multiple drug resistance pathway and not specifically related to the squelching phenotype induced by VEO. To address this question, a disruption was made in the PDR1 locus of yeast strain PL3, and the effect of this disruption on the response of PL3 to estradiol was determined. Since PL3 carries a deletion at the endogenous URA4 locus, it is dependent on ER-induced expression of the 3ERE:URA4 reporter gene for growth on minimal medium lacking uracil (38). Therefore, when this strain is transformed with an ER, the MNIC can be used as a measurement of the sensitivity of this yeast strain to estradiol, comparable to the MNIC used to express sensitivity to growth-inhibiting drugs.

Disruption of PDR1 has been shown to reduce the MNICs of cycloheximide and oligomycin in yeast cells (3). To verify the functional disruption of PDR1 in PL3, the MNICs of oligomycin and cycloheximide for PL3 were compared with that of the corresponding PDR1 disruptant, GPL3A (Table 3). As expected, disruption of PDR1 in PL3 reduced the MNICs of both drugs. To determine whether this same disruption also affected the sensitivity of PL3 to estradiol, both PL3 and GPL3A were transformed with a plasmid expressing the wild-type hER (pRS313HEGO [38]), and the MNIC was determined. The results (Table 3) demonstrate that a disruption of PDR1 in PL3 reduces the MNIC of estradiol from 1.0 nM to below 0.2 nM and provide evidence that multiple drug resistance is involved in modulating the response of yeast cells to estradiol in a context independent of the phenotype imposed by VEO. Taken together, these results suggest that the PDR1 allele cloned in this laboratory increases the expression of membrane proteins controlling the efflux of small molecules (including estradiol) from the cell, as has been suggested for previously characterized PDR1 mutations (4).

**DISCUSSION**

The results presented in this report describe an inducible system with which to study squelching in S. cerevisiae, which is amendable to genetic analysis. More specifically, replacement of the TAF-1 activation function of the ER with the potent AAD of VP16 produces an ER that rapidly squelches the expression of an endogenous yeast promoter and gradually causes the complete arrest of cell growth; both effects are entirely dependent on the addition of hormone. Maximal squelching and cell growth arrest are dependent on the integrity of the transcriptionally active acidic portion of VP16 and the DBD of the ER, providing correlative support that squelching above a threshold level leads to the eventual arrest of cell growth. The estradiol dependence of this system also allows for genetic analysis of ER function. We demonstrate the utility of this system for the cloning of
relevant genes by suppressing the growth arrest phenotype with a genetic bank, resulting in the demonstration that mutations in the yeast PDR1 gene that alter the sensitivity of yeast cells to a variety of drugs also affect the permeability of the cells to the steroid hormone estradiol.

**Squelching and the DBD.** Our results showing that an intact DBD significantly contributes to the level of squelching achieved in vivo differ from some previously reported results (15, 41) but are consistent with other results (6). Gill and Ptashne (15) have reported that a deletion of the DBD of GAL4 had no effect on the ability of this protein to squelch the expression of certain yeast promoters. Tasset et al. (41) have reported squelching by the isolated VP16 AAD in mammalian cells; however, it was necessary to introduce much more free VP16, compared with a DBD-linked VP16, to achieve the same amount of squelching in these experiments. During the preparation of this report, we learned that the inability to transform yeast strains with high-copy-number plasmids expressing GAL4VP16 can be relieved by mutations in the DBD of this protein, consistent with our results (6). The fact that the DBD of the hER is required for VEO toxicity indicates that the ability of the DBD to contribute to the strength of squelching in yeast cells is not restricted to the GAL4 DBD in particular, or any other yeast DBD, but can be fulfilled by a heterologous DBD.

Requirement for the activity of DNA binding in squelching could also provide an explanation for the relative amounts of toxicity observed by different activators with the same AAD. Deletion of the HBD of VEO resulted in a molecule that exhibited toxicity, but to a lesser degree than did VEO (Table 1). If squelching is enhanced by DNA binding per se, the diminished toxicity could be due to the fact that a deletion of the HBD eliminates the ability of receptors to dimerize, decreasing their affinity for DNA (23). GAL4VP16 is an extremely toxic protein for yeast cells; successful transformation has been reported only when the protein is expressed from single-copy plasmids (21). We have transformed yeast strains with either VE12 or GAL4VP16 expressed from the same 2 μm expression vector; and although VE12 contains the same AAD of VP16 as does GAL4VP16 (VP16 amino acids 411 to 490), VE12 can be readily transformed into yeast cells (under selection for normal 2 μM maintenance), while GAL4VP16 cannot (not shown). The primary difference between these molecules is the source of the DBD, providing further evidence for an involvement of the DBD in squelching and toxicity by the VP16 AAD. How the DBD of VEO could be involved in squelching is not known. It is possible that nonspecific DNA binding activity is necessary to localize VP16 to functionally important compartments of the nucleus. Alternatively, the DBD may interact directly with a limiting transcription factor and contribute to squelching either on its own or in combination with the activation domain. Experiments in vitro have shown that the DBD of GAL4VP16 is required for the inhibition of basal-level, but not activated, transcription (5). It is possible that we are observing a similar effect in vivo. It has also been shown that residues within the DBD of the activating factor ADR-1 are required for transcriptional activation (42). Furthermore, there is evidence that two components of EIA are required for squelching, one of which contains a Zn" finger (47), leading the investigators to propose an interaction between EIA and two limiting transcription factors. If the DBD does play a direct role in the observed in vivo squelching by VEO, it is clear from our data that the transcriptionally active component of VP16 is also necessary.

**Transcriptional activation and squelching by the VP16 AAD in S. cerevisiae.** Table 1 demonstrates that nearly all (>99%) of the activating potential of VEO is provided by the AAD of VP16 and that the transcriptional properties of VEO are a reflection of the properties of VP16 in yeast cells. Our analysis of transcriptional activation by various mutants of VP16 in S. cerevisiae yeast demonstrates that VP16 is a potent activator of transcription in yeast cells as it is in mammalian cells (40) and that its activity in yeast cells can be affected by changes in both net negative charge and specific amino acids (Phe-442), as has previously been demonstrated in mammalian cells (9). Cress and Triezenberg (9) have identified Phe-442 as a critical residue within the context of a truncated VP16 AAD (residues 427 to 451) in mammalian cells. We show that mutation of this same specific amino acid (Phe-442→Ala) can significantly affect the activity of a similarly truncated VP16 AAD (residues 423 to 454). However, we have found that this same mutation is innocuous in the context of the entire VP16 AAD (residues 411 to 490) or a truncated AAD consisting of amino acids 423 to 490. Furthermore, while VP16 amino acids 427 to 451 provide 50% of the activity of the complete VP16 AAD (residues 411 to 490) in mammalian cells (9), our data indicate that amino acids 454 to 490 supply most of the activity of the VP16 AAD in yeast.

The results presented in Fig. 3 indicate that the squelching effect of VEO is rapid and effective at any point during the induction of GAL1: lacZ by GAL4. The model for squelching, if correct, indicates that TIFs interacting with these two competing AADs are not committed to a particular complex once bound, but can freely dissociate from one AAD and interact with another. In fact, squelching may be a normal mechanism by which yeast cells can respond rapidly to changes in their environment. For example, a moderate decrease in cell growth rate was seen in control cultures upon the addition of galactose (Fig. 3B), which was then followed by a return to the normal growth rate, indicating that squelching may occur transiently upon activation of GAL4. Furthermore, our results indicate that cells can tolerate a certain amount of squelching with very little effect on cell growth. For example, the degree of squelching seen in Fig. 3A under selection for URAS3 (maintaining approximately 20 copies of expression vector per cell) produces only a moderate decrease in cell growth, as seen in Fig. 3B. Certain mutations in VEO that completely relieved toxicity retained some residual squelching ability (Table 1). Taken together, these results indicate that squelching must exceed a certain threshold level to become toxic and reveal a dynamic interplay between transcription factors in the cell that can occur rapidly whenever environmental conditions change. Normally, cells adjust rather quickly to these changes (Fig. 3B); however, under the exaggerated experimental conditions of strong AAD overexpression, cell growth is completely arrested.

**Is squelching due to the titration of a single limiting TIF?** In attempting to relieve growth arrest with a multicopy plasmid-based yeast genomic library, a number of assumptions were made. If there was a single limiting yeast TIF that was being titrated by the AAD of VP16 upon the addition of estradiol, then amplification of corresponding TIF gene, by way of its presence on a multicopy plasmid, should increase TIF concentration and relieve growth arrest. The inducible system that we developed to test these assumptions provided us with the ability to allow the TIF to accumulate intracellularly before inducing immediate and toxic levels of squelching by the addition of hormone. The low frequency of
spontaneous reversion (4 × 10⁻⁷) of the growth arrest phenotype by committed selection for stringent conditions, such as if the concentration of TIF had to fall within a certain range to overcome squelching. In fact, the LEU2 gene could overcome selection only in those cells that had a reduced VEO concentration at the time of estradiol addition, yet this gene was cloned in nine separate transformants. Cloning of the PDR1 gene required expression of PDR1 to levels dominant to the wild-type PDR1 in TGY14-1, an increase in the level of expression of the membrane proteins responsible for estradiol resistance, and functional integration of these proteins into the plasma membrane before estradiol addition (4). Also, PDR1 is a relatively large yeast gene (3), and the size of the library inserts carrying PDR1 was significantly larger than the size of an average library insert (Materials and Methods), making it likely that PDR1 was a relatively rare gene in our library. Therefore, we believe that the cloning of the PDR1 gene in three separate transformants makes it unlikely that any other single gene present in our library can relieve the toxicity of VEO.

There are a number of explanations for our inability to identify a single limiting factor. Assuming that growth arrest is due to squelching and that squelching is, in fact, the titration of limiting TIFs, it may be that squelching by VEO involves the simultaneous titration of more than one TIF, as has been proposed for E1A (47). This view is supported by our observations that squelching requires two components of VEO, the AAD of VP16 and the DBD. Alternatively, it may be that there is such a delicate balance between the levels of various transcription proteins (see above) that increasing the concentration of the TIF is itself inhibitory to cell growth and, hence, impossible in vivo. Finally, we should not disregard the possibility that the current model for squelching is incorrect. For example, what is observed as squelching of transcription in vivo may be an indirect effect of whatever mechanism leads to growth inhibition by these proteins. A direct demonstration that the titration of limiting concentrations of TIFs by VEO is the cause of squelching and eventual cell growth arrest awaits the identification of the gene products involved. We have demonstrated that direct library complementation may not be a viable method of identifying these genes. However, the fact that mutations in either the DBD or the AAD of VP16 domain can completely relieve toxicity suggests the involvement of specific macromolecular interactions. It may be possible, therefore, to mutate (rather than amplify) individual yeast genes whose products are involved in squelching and suppress the toxicity of VEO. Preliminary results indicate that UV mutagenesis can increase the frequency of reversion of the growth arrest phenotype, and studies using this approach are in progress (14).

Pleiotropic drug resistance in S. cerevisiae as a model for understanding hormone resistance in humans. The cloning of PDR1 demonstrates the utility of the system described in this report, but it also provides some interesting biological information. Our data suggest that we have cloned a mutated form of PDR1. It is likely that this mutation originated from the yeast strain used to make the genomic library (FL100), since it has been observed that FL100 is resistant to a number of drugs tested (29). Previous reports support a model by which PDR1 mutations increase the expression of a class of membrane proteins that export small toxic molecules across the plasma membrane, and that specific proteins in the PDR family of membrane proteins recognize and export certain classes of molecules (4). Our results place estrogens within the category of molecules that are recognized and exported by yeast PDR proteins. It has been suggested that multiple drug resistance in humans (also mediated by a membrane protein) may be responsible for the acquired tolerance of patients treated clinically with antihormones such as breast cancer patients (45). However, a direct link between multiple drug resistance and hormone tolerance has not, to our knowledge, been demonstrated. This provides the first example of the acquired resistance of yeast cells to a compound for which the mammalian multiple drug resistance gene (MDR) has been implicated and underscores the value of studies on the yeast PDR genes for the understanding of mammalian MDR.

ACKNOWLEDGMENTS

We thank S. Mader, L. Tora, D. Metzger, M. Leid, B. Pierrat, I. Kuhn, A. P. Sibler, E. Balzi, A. Goffeau, F. Lacroute, C. Brou, and H. Gronemeyer for helpful discussions and contribution of materials, C. Rosen, L. Guarente, and T. Kerpolla for critical reading of the manuscript, and J. Fuchs and coworkers for materials and technical assistance with yeast immunofluorescence. We also thank F. Ruffenach and A. Straub for synthesis of oligonucleotides, and we thank C. Werle, B. Boulay, and J. M. LaFontaine for help in preparing the manuscript.

D.M.G. and D.M.H. were supported by fellowships from EMBO. This work was supported by funds from the Association pour la Recherche sur le Cancer, the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the Fondation pour la Recherche Médicale.

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