

# A Genetic Analysis of Glucocorticoid Receptor Signaling: Identification and Characterization of Ligand-Effect Modulators in *Saccharomyces cerevisiae*

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## ABSTRACT

To find novel components in the glucocorticoid signal transduction pathway, we performed a yeast genetic screen to identify ligand-effect modulators (LEMs), proteins that modulate the cellular response to hormone. We isolated several mutants that conferred increased glucocorticoid receptor (GR) activity in response to dexamethasone and analyzed two of them in detail. These studies identify two genes, *LEM3* and *LEM4*, which correspond to *YNL323w* and *ERG6*, respectively. *LEM3* is a putative transmembrane protein of unknown function, and *ERG6* is a methyltransferase in the ergosterol biosynthetic pathway. Analysis of null mutants indicates that *LEM3* and *ERG6* act at different steps in the GR signal transduction pathway.

THE glucocorticoid receptor (GR) is a ligand-dependent transcriptional regulator that mediates a panoply of developmental, physiological, and behavioral processes (TRONCHE *et al.* 1998). GR integrates, coordinates, and responds to numerous cellular signals to accomplish its diverse functions. Regulation of glucocorticoid-responsive genes is a multistep process (BEATO *et al.* 1996; YAMAMOTO 1997). In the absence of hormone, the inactive apo-GR resides in the cytoplasm in association with molecular chaperones that maintain the apo-receptor in a high-affinity hormone-binding state. Hormone binding provokes dissociation of the chaperone machinery and nuclear translocation of the hormone-receptor complex. Once in the nucleus, GR binds selectively to specific genomic sites [glucocorticoid response elements (GREs)] and positively or negatively regulates transcription from nearby promoters (YAMAMOTO 1985). GR is composed of three modular domains that harbor distinct, separable functions (see Figure 3A). The N terminus contains sequences responsible for transcriptional activation, repression, and synergy control. The central domain mediates Zn<sup>2+</sup> interaction, dimerization, DNA binding, and nuclear localization. The C terminus interacts with molecular chaperones, coactivators, and hormone, and contains nuclear import and export sequences (PICARD and YAMAMOTO 1987; WRIGHT *et al.* 1993; IÑIGUEZ-LLUHÍ *et al.* 1997).

GR is not the sole determinant of the cellular response

to ligands; other cellular factors are known to modulate GR action. For example, hormone transporters (KRALLI *et al.* 1995; MAHÉ *et al.* 1996), molecular chaperones (PICARD *et al.* 1990; BOHEN and YAMAMOTO 1994), chromatin remodeling factors (YOSHINAGA *et al.* 1992; FRYER and ARCHER 1998), coactivators (GLASS *et al.* 1997), as well as other transcription factors such as AP-1 (MINER *et al.* 1991) interact with GR and affect its function. The fact that hormone-dependent transcriptional activation by mammalian GR can be recapitulated in yeast (SCHENA and YAMAMOTO 1988; GARABEDIAN and YAMAMOTO 1992), an organism that lacks the entire intracellular receptor gene superfamily, suggests strongly that the GR signal transduction pathway evolved from pre-existing highly conserved components. Thus, given the ease of genetic manipulation and cloning in *Saccharomyces cerevisiae*, this heterologous species has proved advantageous for investigations of various factors in the GR signal transduction pathway (BOHEN and YAMAMOTO 1993; KRALLI *et al.* 1995; IMHOF and McDONNELL 1996; HONG *et al.* 1997; IÑIGUEZ-LLUHÍ *et al.* 1997; KRSTIC *et al.* 1997).

Whereas most prior genetic studies of the glucocorticoid receptor signaling pathway have analyzed candidate genes, here we carried out an unbiased genetic screen to identify factors that can potentially act at any step in the GR signal transduction pathway. For this, we have taken advantage of the weak potency of the synthetic glucocorticoid dexamethasone (dex) in yeast to screen for factors that modulate hormone responses. We anticipated that some of these modulators could be functionally homologous to factors that impart the highly distinct, context-dependent GR responses ob-

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served in mammalian cells (CASOLINI *et al.* 1993; KAVELAARS *et al.* 1995; BAMBERGER *et al.* 1996; LIM-TIO *et al.* 1997; MCCORMICK *et al.* 1998). In an earlier study (KRALLI *et al.* 1995), we identified PDR5/LEM1, an ABC transporter that selectively exports ligands such as dex out of cells. Although the potency of dex in a *pdr5Δ* strain is ~10-fold greater than in wild-type cells, it is still weak when compared to other glucocorticoid agonists in yeast and far below its potency in animal cells (A. KRALLI, unpublished results). These observations suggest that factors in addition to PDR5 determine hormone responsiveness in yeast. To identify these factors, we extended our initial screen with two modifications: first, we devised a selection scheme and second, we isolated *lem* mutants in a *pdr5Δ* strain to preclude obtaining mutants affecting PDR5 function.

## MATERIALS AND METHODS

**Yeast strains:** Yeast strains used in this study are isogenic derivatives of YPH499/500 (*ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*). YNK420 was derived from YPH499 in two steps. First, the *PDR5* locus was disrupted with the *LEU2* gene using the DNA insert of plasmid pTCA/*lem1::LEU2* (KRALLI *et al.* 1995). Next, cells were transformed with pRS314-N795 and the *KpnI* fragment from p314-3TAT-C-His3. Trp<sup>+</sup> transformants that had integrated the *GRE-HIS3* reporter displayed hormone-dependent growth on SD -histidine. YNK425 is a Leu<sup>+</sup>, hormone-dependent His<sup>+</sup> segregant from the cross of YNK420 to YPH500. To create the integrated *GRE-CAN1* strain (YNK508), YNK410 cells were transformed with pRS314 and the *EcoRI-PstI* fragment from ptGT3c-Can1. Trp<sup>+</sup> transformants were replica plated onto SD -arg + 50 μg/ml canavanine medium to select for canavanine-resistant (Can<sup>R</sup>) colonies. Twelve Can<sup>R</sup> isolates were further characterized. Upon introduction of GR expression plasmids, all were Can<sup>R</sup> in the absence of hormone and canavanine sensitive at high concentrations of agonist ligand. YRS350 and YNK558 are Lem<sup>-</sup> Leu<sup>-</sup> Can<sup>R</sup> segregants from crosses of YRS301 and YRS401 to YNK508, respectively. The Lem<sup>-</sup> phenotype was determined by assaying GR activity at 1 and 10 μM dex. Canavanine resistance was determined by checking for growth in the presence of 50 μg/ml canavanine.

Disruptions of *LEM3* and *ERG6* were generated in diploid strains using PCR-mediated gene targeting (WACH *et al.* 1994). For the *LEM3* disruption (YNK760), oligos *lem3-5UT* (GTG TCCTTTTAGAAATACGAGAGGGTGGTAAAAATATTGAAGA GGTTCGACGGTACCCC) and *lem3-3UT* (AAATTTTACAGGG TTAATAAATAAAGAAAACCATCACTCATCGATGAATTCGA GCTCG) were used to amplify a DNA fragment containing the *kanMX* resistance marker flanked by 42 nucleotides (nt) of 5' and 41 nt of 3' untranslated (UT) *LEM3* DNA sequence. Following transformation with the *lem3::kanMX* DNA fragment, integration events were selected by isolating kanamycin-resistant (Kan<sup>R</sup>) colonies [growth in the presence of 200 g/ml Geneticin (GIBCO)] and confirmed by PCR analysis of genomic DNA. For the disruption of *ERG6* (YNK597), oligonucleotides *erg6-1s* (TATATAGTTTCGGGTGTTTTCTCCTACC TCTGCTGCTCTCGAATTCCTGGGATCCCG) and *erg6-2a* (GCATCGGACAGTCTGTTTGTAAGGCCTGCTAGCAATGA ACCAAGCTAGCTTGCTGCAG) were used to amplify the *HIS3* gene flanked by *ERG6* sequence from the YDp-H plasmid (BERBEN *et al.* 1991). Following transformation with the *erg6::HIS3* fragment, integration events were selected by

growth in the absence of histidine and confirmed by PCR analysis of genomic DNA. YNK598 is a His<sup>+</sup> Kan<sup>R</sup> meiotic segregant generated from a cross of YNK761 to YNK597.

**Plasmids:** pRS314-N795, pRS314-N525, pL2G-407C, pG1-F620S, and pΔS26X were all described previously (SCHENA and YAMAMOTO 1988; GARABEDIAN and YAMAMOTO 1992). The p314-3TAT-C-His3 integration construct (gift of J. Iñiguez-Lluhi) contains a 5' *KpnI-EcoRI HIS3* fragment followed by three glucocorticoid response elements from the tyrosine amino-transferase 3 gene (*TAT3*) and the entire *HIS3*-coding sequence. The ptGT3C-Can1 integration construct contains the following integration cassette: 833 bp of *CAN1* 5' UT sequence followed by the yeast phosphoglycerate kinase terminator, three glucocorticoid response elements from *TAT3* (SCHENA and YAMAMOTO 1988), the *CYC1* promoter, and the first 822 nt of the *CAN1*-coding sequence. pRS316-LEM3 contains a *PmlI-XbaI* LEM3-containing fragment cloned into *HindIII-XbaI* of pRS316. pRS306-ERG6 and pRS316-ERG6 were made by subcloning an *ERG6*-containing *NheI-EcoRI* fragment into the *XbaI-EcoRI* sites of pRS306 and pRS316, respectively.

**Yeast methods:** With the exception of *lem4/erg6* cells, yeast transformations were carried out using lithium-acetate protocols (GIETZ *et al.* 1995; AGATEP *et al.* 1998). Mutant *erg6* cells were transformed by electroporation (AUSUBEL *et al.* 1994). Briefly, cells were grown and harvested as described in the lithium-acetate protocol. Following a 1-hr incubation in 100 mM Tris, pH 7.5, 1 mM EDTA, pH 8, 10 mM dithiothreitol, and 100 mM LiOAc, cells were washed in ddH<sub>2</sub>O, then in 1 M sorbitol, and resuspended in 1 ml ice-cold sorbitol to a cell density of 10<sup>10</sup> cells/ml. A total of 50 μl of competent cells were added to 200 ng of transforming DNA and pulsed in a 0.2 cm-gap electroporation cuvette at 1.5 kV, 25 μF, and 200 Ω. Cells were allowed to recover for 1 hr in 1 ml of YEPD + 1 M sorbitol, resuspended in 1 M sorbitol, and spread onto SD medium + 1 M sorbitol lacking the appropriate amino acids.

Plate β-galactosidase (β-gal) assays were carried out by replica-plating yeast colonies or patches onto the appropriate SD medium in the absence or presence of hormone and growing overnight at 30°. Cells were overlaid with 0.5% agarose, 0.5 M NaPO<sub>4</sub> (pH 7), 0.1% SDS, 2% *N,N*-dimethylformamide, and 0.05% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and incubated at room temperature for color development. Liquid β-gal assays were carried out in 96-well microtiter plates as described previously (IÑIGUEZ-LLUHÍ *et al.* 1997). Briefly, cells were grown to saturation at 30°, diluted 1:25 in the absence or presence of dex, and grown for 12–14 hr until cells were at OD<sub>650</sub> 0.2–0.4. A total of 10 μl of cells were permeabilized for 5 min at 25° with 10 μl of 2× Z buffer (120 mM NaPO<sub>4</sub>, pH 7.0, 10 mM KCl, 1 mM MgSO<sub>4</sub>, and 20 mM β-mercaptoethanol) containing 5% CHAPS, and reactions were initiated by adding 180 μl of 0.5 mM chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) in 1× Z buffer. Activity units were derived from the following equation:  $A = (\Delta OD_{550-650} \times cv) / (OD_{650} \times sv)$ .

**Mutagenesis, selection, and isolation of *lem* mutants:** YNK420 was transformed with pRS314-N795 and pΔS26X. For UV mutagenesis, cells were exposed to UV light for 20 or 25 sec, resulting in 43 and 28% cell survival, respectively. Mutants were then plated at a density of 4.35 × 10<sup>4</sup> on SD -his -trp -ura plates containing 25 μg/ml 3-amino-triazole (3-AT) to suppress leaky *HIS* expression. For EMS mutagenesis, cells were treated with 0.03% EMS for 10 or 20 min, resulting in 50 and 25% survival, respectively. Approximately 10<sup>4</sup> survivors were plated on selective medium. UV- and EMS-induced mutants that grew over a period of 1–3 days were picked, restreaked to isolate single colonies, and assayed for *GRE-lacZ* activity by plate as well as liquid culture assays. RE and RU refer to EMS- and UV-induced mutations, respectively.

**Genetic analyses:** *lem* mutants (RE10, RE21, RE41, RU41, RU48, RU68, RU91, and RU97) were crossed to YNK425 and *GRE-lacZ* activity was assayed in the heterozygous diploid. The RE10/WT diploid displayed increased GR activity, comparable to that of the RE10 haploid (data not shown). The remaining *lem*/WT diploids all had wild-type phenotypes. We sporulated RE41, RU48, RU68, and RU97 diploids and performed tetrad dissections and analyses. Wild-type and mutant phenotypes segregated 2:2 in four to eight complete tetrads. From these tetrads we isolated *lem* mutants of opposite mating types to generate crosses for complementation studies. As RE41/RU48, RE41/RU97, RU48/RU97, RU68/RE41, RU68/RU48, and RU68/RU97 heterozygous diploids all displayed wild-type GR activity at 1  $\mu$ M dex, we concluded that they comprise four complementation groups. We backcrossed RU97/*lem3* and RU48/*lem4/erg6* to YNK425 once more and then crossed to YNK508. After sporulation and tetrad analysis, we isolated Lem<sup>-</sup> Leu<sup>-</sup> Can<sup>S</sup> tetrads to proceed with the cloning of the wild-type alleles.

**Western blot analysis:** *lem* mutant and wild-type cells were grown to OD<sub>600</sub> 0.6–0.8 in 10-ml cultures. Cells were harvested, washed with H<sub>2</sub>O and 1 ml of high salt extraction buffer (400 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1  $\mu$ g/ml aprotinin, leupeptin, and pepstatin A), and resuspended in 200  $\mu$ l of high salt extraction buffer. An equal volume of glass beads was added and samples were shaken for 20 min at 4°. Extracts were collected and centrifuged at 14,000  $\times$  g for 10 min to separate insoluble material. Supernatants were transferred to a new tube and protein concentrations (8–15  $\mu$ g/ $\mu$ l) were determined using the Bio-Rad protein assay. Samples were used immediately or frozen in liquid nitrogen and stored at –80° until use. Forty nanograms of total protein was run on a 7.5% SDS-PAGE gel and transferred onto nitrocellulose using a semidry gel blotter with transfer buffer (0.3% Tris, 1.12% glycine, 0.035% SDS, 20% methanol). Next, the membrane was incubated with 5% nonfat dry milk overnight at 4° in TBST (150 mM NaCl, 10 mM Tris (pH 7.5), and 0.05% Tween-20) and then with a 1:100 dilution of the primary GR antibody BuGR2 (GAMETCHU and HARRISON 1984) in TBST for 1 hr at 25°. After three 10-min washes in TBST at room temperature, the membrane was incubated with a 1:4000 dilution of secondary antibody (goat anti-mouse IgG alkaline phosphatase conjugate) in TBST for 1 hr. The membrane was washed three times for 10 min with TBST and rinsed briefly with ddH<sub>2</sub>O. Signals were detected using Supersignal chemiluminescent substrate (Pierce Chemical, Rockford, IL).

**In vivo hormone-binding assay:** The GR variant F620S displays increased hormone binding in yeast and is therefore more amenable for such studies (GARABEDIAN and YAMAMOTO 1992). *lem3-1* and *erg6-101* have similar effects on wild-type GR and F620S GR in yeast (data not shown). Overnight cultures of yeast cells expressing pGI-F620S were grown to saturation, diluted 1:5, and grown until OD<sub>600</sub> = 0.4–0.5 (at least two doublings). <sup>3</sup>H-dex (1–2  $\times$  10<sup>6</sup> cpm/ml) minus or plus 150-fold excess of cold hormone was added to cells and cultures were incubated at 30° for 2 hr. Samples were put on ice, centrifuged at 14,000  $\times$  g for 5 min at 4°, washed three times with 1 ml of ice-cold PBS/2% glucose (w/v), and resuspended in 50  $\mu$ l of PBS/2% glucose. The amount of bound <sup>3</sup>H-dex was quantitated by liquid scintillation.

**Canavanine-negative selection cloning strategy:** YRS350 (*can1::GRE3-CAN1 lem3-1 pdr5::GRE-lacZ*) was transformed with the CEN-ARS, *LEU2*-marked yeast genomic library, p366 (American Type Culture Collection, Rockville, MD), and 10<sup>5</sup> transformants were plated on SD -arg -trp -ura, 300  $\mu$ M dex, and 100  $\mu$ g/ml canavanine. A total of 545 canavanine-resistant (Can<sup>R</sup>) cells grew after 3 days at 30°. Since cells could evade

hormone-induced toxicity by silencing GR activity or losing receptor expression (*e.g.*, by acquiring *TRP1* from the library at the expense of the *TRP1*-marked GR-expression plasmid), we checked that Can<sup>R</sup> cells were still able to respond to deoxycorticosterone (DOC), a potent GR ligand in yeast. Library DNAs from 26 colonies that contained active receptor and displayed wild-type phenotypes (Can<sup>R</sup> and low *GRE-lacZ* activity) were isolated. Southern blot analysis revealed that five clones corresponded to *PDR5* and 21 clones contained common, overlapping, or identical sequences (data not shown). An *XbaI-PmlI* fragment containing only the YNL323w ORF present in the 21 clones was both necessary and sufficient to confer plasmid-dependent complementation (Figure 4C).

YNK558 was transformed with the *URA*-marked, CEN-ARS yeast genomic library Ycplac33 (gift of M. Hall) using electroporation, plated on SD -arg -trp -ura, 3  $\mu$ M dex, 100  $\mu$ g/ml canavanine, and 1 M sorbitol, and allowed to grow for 4–6 days at 30°. A total of 336 Can<sup>R</sup> colonies were picked from 7.5  $\times$  10<sup>4</sup> Ura<sup>+</sup> transformants. A total of 87 colonies displayed *lacZ* activity in the presence of 20  $\mu$ M dex (indicating the presence of active GR). To select for loss of the *URA* marker, cells were grown in the presence of 1  $\mu$ g/ml 5-fluoroorotic acid (5-FOA). Two of these colonies exhibited plasmid-dependent, wild-type GR activity at 1  $\mu$ M dex. Plasmids were isolated from these colonies and reintroduced into *lem4* cells to confirm their ability to decrease response to dex.

## RESULTS

**Isolation of mutants with increased GR responses to ligand:** To identify proteins that modulate the response of GR to ligand, we looked for mutants with increased ligand responses. Our starting strain, YNK420, contained a GR expression vector and two reporter genes, *GRE-HIS3* and *GRE-lacZ*. YNK420 is able to grow in the absence of histidine and exhibits robust  $\beta$ -galactosidase activity when grown in the presence of 1  $\mu$ M DOC, a potent GR ligand in yeast. In contrast, YNK420 is unable to grow in the presence of 300 nM dex on minimal medium lacking histidine and forms white colonies on 5-bromo-4-chloro-1-indolyl  $\beta$ -D-galactosidase (X-gal) plates (Figure 1A). YNK420 cells were mutagenized by exposure to UV light or EMS to a survival of 25–50%. Mutants that grew in 300 nM dex in the absence of histidine were obtained at a frequency of 3  $\times$  10<sup>-4</sup>.

His<sup>+</sup> isolates were restreaked to single colonies, assayed for expression of the *GRE-lacZ* reporter in response to hormone, and grouped into phenotypic classes (MATERIALS AND METHODS). Of 156 mutants, 16 formed white colonies in the presence of dex and X-gal, suggesting that their His<sup>+</sup> phenotypes were independent of GR. Forty mutants showed high GR activity both in the absence and in the presence of ligand; many of these likely reflect lesions in the GR gene that truncate the C-terminal hormone-binding/signaling domain, yielding fragments with constitutive activity (GODOWSKI *et al.* 1987; SCHENA and YAMAMOTO 1988). The remaining 100 mutants exhibited increased ligand-dependent  $\beta$ -gal activity and thus met both criteria for increased GR activity in response to ligand. To determine whether the mutations were chromosomal or plasmid

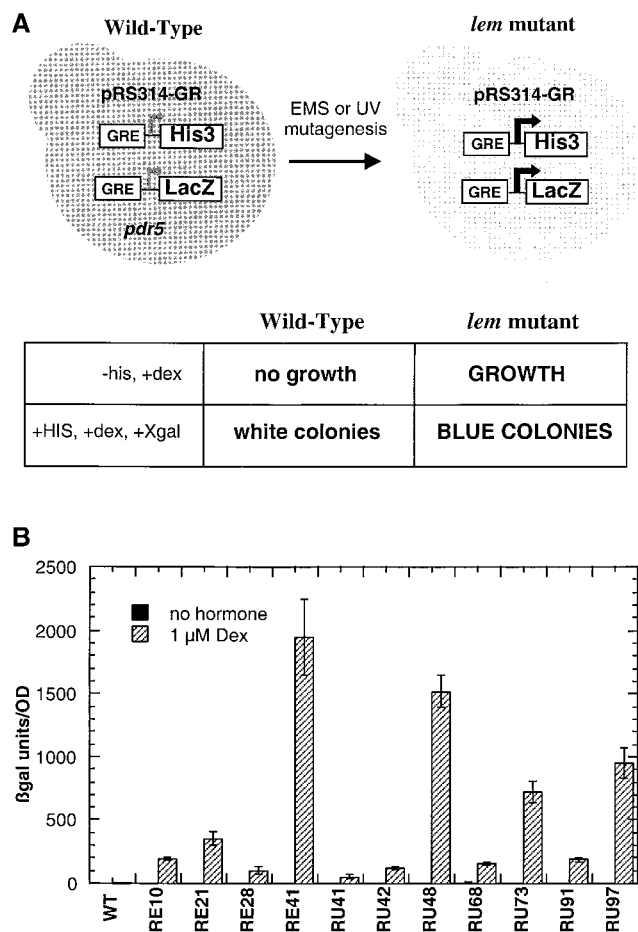


FIGURE 1.—Screen in yeast for ligand-effect modulators (LEMs). (A) Schematic of *lem* screen. YNK420 cells containing the *GRE-lacZ* reporter (pΔS26X) and pRS314-N795 were UV- or EMS-mutagenized and plated on selective media (-trp, -ura, -his, +300 nM dex, +100 μg/ml 3-AT). Under these conditions, only mutants with increased GR activity were able to grow. GR activity was quantitated by assaying *GRE-lacZ* reporter gene response. (B) Phenotypes of representative *lem* mutants. Liquid β-gal assays were performed on lysates from wild-type and mutant cells carrying full-length GR and *GRE-lacZ*, and grown in the absence or presence of 300 nM dex.

borne, we selected isolates that had lost the plasmids and retransformed with wild-type GR and reporter plasmids; 95% retained their ligand-dependent His<sup>+</sup> LacZ<sup>+</sup> phenotype. The phenotypes of 11 representative *lem* mutants are shown in Figure 1B.

**Genetic analysis and characterization of *lem* mutants:** Eight *lem* mutants were backcrossed to wild-type strains, and GR activity was assayed in the heterozygous diploids to determine whether the mutations were dominant or recessive. One mutant (RE10) was dominant; the others (RE21, RE41, RU41, RU48, RU68, RU91, and RU97) were recessive (MATERIALS AND METHODS). We sporulated *lem* × wild-type diploids and analyzed tetrads from RE41, RU48, RU68, and RU97 crosses. In all cases, the mutant phenotypes segregated in a 2:2 ratio, indicating single-gene alterations. Complementation studies re-

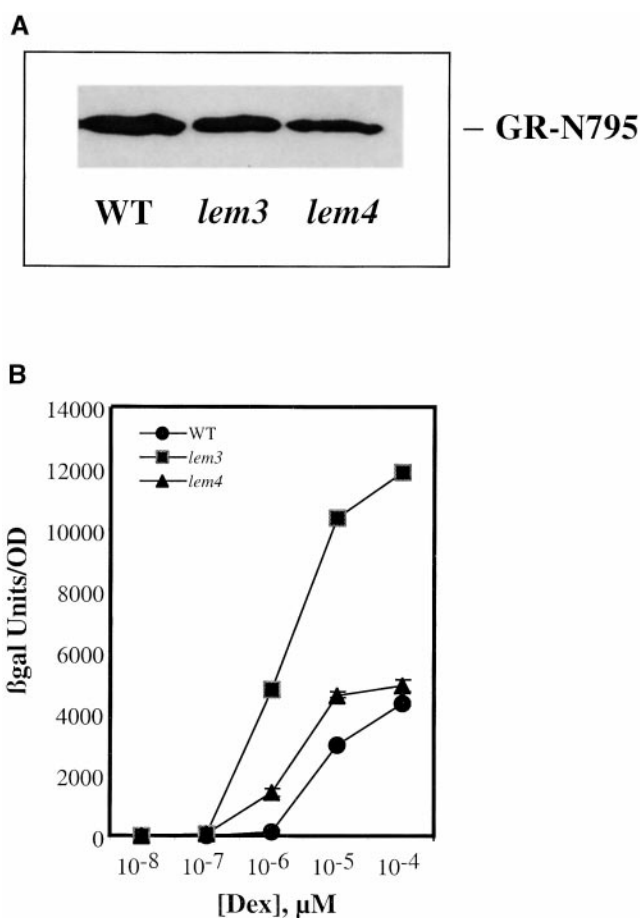


FIGURE 2.—Phenotype of *lem3* and *lem4* mutants. (A) Western blot of whole-cell extract (40 ng of protein) from wild-type, *lem3*, and *lem4* strains, detecting GR using the BuGR2 monoclonal antibody. (B) Dex dose-response curve: *lem3* affects both GR potency and efficacy while *lem4* affects potency.

vealed that these mutants define four complementation groups (see MATERIALS AND METHODS).

We chose for further characterization two mutants with relatively pronounced phenotypes, RU97 and RU48. The mutants, renamed *lem3-1* and *lem4-101*, respectively, also exhibited moderate growth defects. Western blots of whole-cell extracts using the GR-specific monoclonal antibody BuGR2 revealed similar levels of accumulated GR in the wild-type and mutant backgrounds (Figure 2A). Thus, *lem3-1* and *lem4-101* appear to affect the GR signaling pathway downstream of receptor expression.

We next performed hormone dose-response assays on *lem3-1* and *lem4-101* cultures. The results revealed that *lem4-101* displayed increased ligand potency (responding to lower concentrations of dex) whereas receptor efficacy (maximal activation levels at saturating hormone conditions) was similar to that of the wild type (Figure 2B). In contrast, *lem3-1* displayed marked increases in both potency and efficacy.

***lem3* and *lem4* affect different steps in the GR signal**

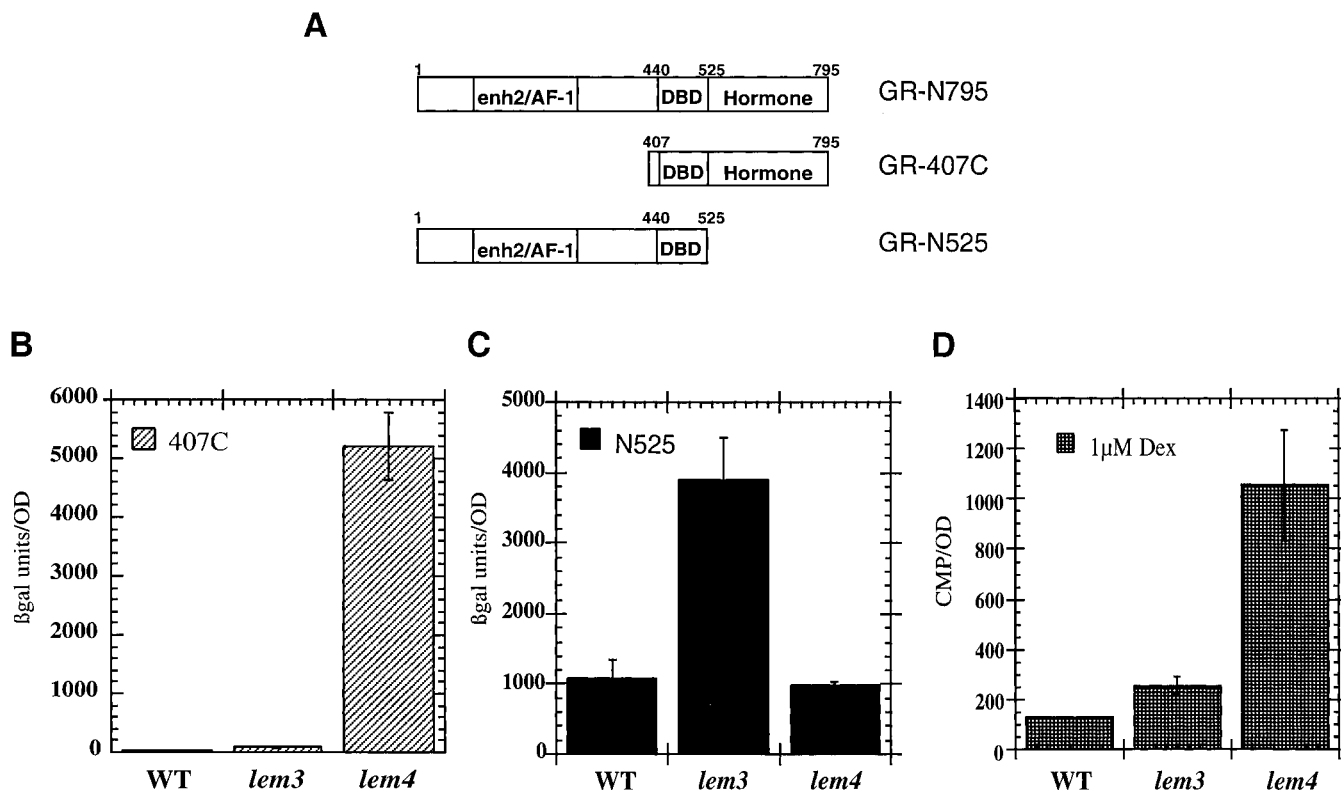


FIGURE 3.—Phenotypes of *lem3* and *lem4* mutants involve different GR domains. (A) Schematic of glucocorticoid receptor functional domains. Enh2/AF1 is the N-terminal regulatory domain that harbors activation, repression, and synergy functions. The ZFR contains zinc-binding, dimerization, DNA-binding, and nuclear localization sequences. The signaling domain is responsible for hormone binding, chaperone interaction, nuclear translocation, and transcriptional activation. (B) The activity of pG1-407C in response to 1  $\mu$ M dex. (C) *lem4* but not *lem3* requires the GR-LBD. pRS314-N525 activity is increased in *lem3* but remains unchanged in *lem4* compared to wild type. (D) *In vivo* hormone-binding assay. Cells expressing pG1-F620S were grown in the absence or presence of  $^3$ H-dex and bound hormone was quantitated by liquid scintillation. Significantly increased hormone occupancy by GR is seen in the *lem4* but not the *lem3* mutant.

**transduction pathway:** The dose-response assays implied that *lem3* and *lem4* affect different GR functions. Consistent with this view, we found that *lem3* and *lem4* require different segments of GR for their increased ligand responses. Thus, activity of the GR derivative 407C, which lacks the N-terminal domain, remained significantly increased in *lem4* cells but was only weakly increased in *lem3* cells (Figure 3, A and B). In contrast, the *lem4* phenotype was lost in the absence of the GR-ligand-binding domain (LBD), whereas the effect of the *lem3* mutation was retained on this truncated GR derivative GR-N525 (Figure 3, A and C).

The effect of *lem4* on hormone potency, as well as the requirement of the GR-LBD for the *lem4* phenotype, suggested that the *lem4* mutant might increase hormone availability or binding to GR. We tested this by measuring intracellular hormone accumulation *in vivo*, which reflects GR occupancy by hormone. Indeed, binding of  $^3$ H-dex was increased 8- to 10-fold in *lem4* relative to wild type. In contrast, *lem3* displayed only a twofold increase in hormone binding in the presence of 1  $\mu$ M dex (Figure 3D), which is clearly insufficient to account for the large increase in the transcriptional activity of

full-length GR at the same hormone concentration (Figure 2B). Together, these results indicate that *lem3* and *lem4* affect distinct GR domains and likely different steps in GR signaling: the *lem3-1* mutation appears to operate on the N-terminus/DNA-binding domain, whereas *lem4-101* affects GR functions housed in the C terminus, such as ligand binding.

**Cloning wild-type LEM genes:** To isolate the wild-type LEM alleles, we devised a negative selection scheme based on hormone-dependent expression of a toxic gene (Figure 4A). For this, the *lem3* and *lem4* mutations were introduced into a yeast strain that harbors two integrated GR-responsive reporters: *GRE-lacZ* and *GRE-CAN1*. CAN1, the yeast arginine permease, allows entry of the toxic arginine analog canavanine into yeast (BOLLER *et al.* 1975). The *lem3* and *lem4* mutants (YRS350 and YNK558) thus fail to grow in the presence of dex and canavanine, in contrast to wild-type cells, which are canavanine resistant (Figure 4A).

To isolate LEM3, YRS350 was transformed with a low-copy LEU2 yeast genomic library and plated on medium lacking arginine and containing 300 nM dex and 100  $\mu$ g/ml canavanine. Of  $10^5$  Leu<sup>+</sup> transformants, 545 iso-

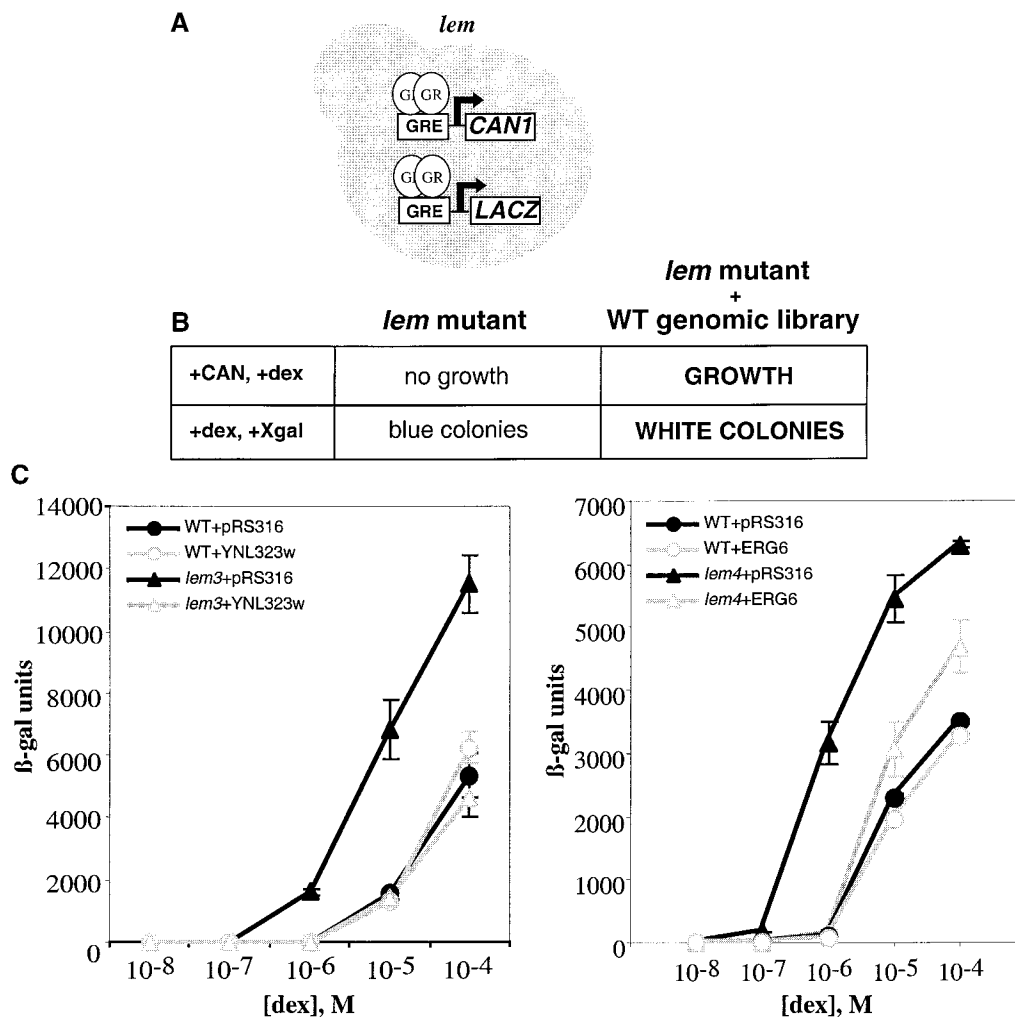


FIGURE 4.—Cloning wild-type Lem alleles. (A) Schematic of canavanine-negative selection cloning strategy. *GRE-lacZ* and *GRE-CAN1* reporter genes are integrated into the yeast genome. Cells were transformed with pRS314-GRN795 and the wild-type genomic yeast library p366. (B) Growth of wild-type and mutant cells in the absence and presence of canavanine and dex. The growth of *lem3* is greatly compromised and *lem4* completely fails to grow in the presence of 100  $\mu\text{g/ml}$  canavanine and 3  $\mu\text{M}$  dex. (C) Complementation of *lem3* and *lem4* by YNL323W and ERG6, respectively.

lates were canavanine resistant. These were assayed for hormone-responsive *lacZ* expression in the presence of 1  $\mu\text{M}$  dex and 10  $\mu\text{M}$  DOC (see MATERIALS AND METHODS). Twenty-six isolates responded to the high concentration of DOC and displayed little response to the low concentration of dex, as expected for complementation of the *lem3* defect to produce wild-type receptor activity. Library DNAs were purified and reintroduced into *lem3* cells to confirm plasmid-dependent complementation (Figure 4B). As our *lem3* strain lacks *PDR5* (Table 1), we expected to recover both the *PDR5* and *LEM3* genes with our cloning strategy. Indeed, Southern blot analysis revealed that 5 clones contained *PDR5*; the remaining 21 clones contained inserts overlapping with each other but not with *PDR5* (data not shown). Deletion analysis and sequencing revealed that YNL323w was both necessary and sufficient to complement *lem3* (see MATERIALS AND METHODS).

To isolate *LEM4*, YNK558 was transformed with a low-copy *URA3* yeast genomic library. Of 75,000 *Ura*<sup>+</sup> transformants, 336 were canavanine resistant. Eighty-seven retained active GR (*lacZ* expression in the presence of

20  $\mu\text{M}$  dex) and displayed wild-type transcriptional responses to dex (Figure 4B). After selection for loss of the *URA3*-marked library plasmids on 5-FOA plates, two isolates reverted to the Lem<sup>4</sup> phenotype, indicating that complementation was plasmid dependent in those cells. Sequencing revealed that one plasmid carried the *PDR5* gene; the other contained two fragments from two different chromosomes that coded for five complete open reading frames (ORFs). Deletion analysis and sub-cloning (see MATERIALS AND METHODS) showed the complementing ORF to be YML008c (*ERG6*).

To confirm that defects in YNL323w and ERG6 gave rise to the *lem3* and *lem4* mutants, respectively, we disrupted these genes in our YNK410 strain background (see MATERIALS AND METHODS). The resultant *ynl323* $\Delta$  and *erg6* $\Delta$  strains showed increased responses to hormone, parallel to those seen in the original *lem3-1* and *lem4-101* mutants. Notably, GR activity in *lem3* $\Delta$  was higher than in our *lem3* mutant strain, suggesting that the null phenotype is more severe than that of the *lem3-1* allele. Mating of *ynl323w* $\Delta$  and *lem3-1* gave rise to a diploid with a Lem<sup>-</sup> phenotype, indicating that *ynl323w*

TABLE 1  
*S. cerevisiae* strains used in this study

Strain	Genotype	Reference
YPH499	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	SIKORSKI and HIETER (1989)
YPH500	<i>MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	SIKORSKI and HIETER (1989)
YNK410	<i>MATa pdr5::leu2::GRE-lacZ</i>	EGNER <i>et al.</i> (1998)
YNK420	<i>MATa his3::GRE-HIS3 pdr5::LEU2</i>	This study
YNK425	<i>MATα his3::GRE-HIS3 pdr5::LEU2</i>	This study
YNK508	<i>MATa can1::GRE-CAN1 pdr5::GRE-lacZ</i>	This study
YRS300	<i>MATa lem3-1 his3::GRE-HIS3 pdr5::LEU2</i>	This study
YRS301	<i>MATα lem3-1 his3::GRE-HIS3 pdr5::LEU2</i>	This study
YRS400	<i>MATa erg6-101 his3::GRE-HIS3 pdr5::LEU2</i>	This study
YRS401	<i>MATα erg6-101 his3::GRE-HIS3 pdr5::LEU2</i>	This study
YRS350	<i>MATa lem3-1 can1::GRE-CAN1 pdr5::GRE-lacZ</i>	This study
YNK760	<i>MATa lem3::kanMX pdr5::GRE-lacZ</i>	This study
YNK761	<i>MATα lem3::kanMX pdr5::GRE-lacZ</i>	This study
YNK558	<i>MATa erg6-101 can1::GRE-CAN1 pdr5::GRE-lacZ</i>	This study
YNK597	<i>MATa erg6::HIS3 pdr5::GRE-lacZ + pRS314-N795</i>	This study
YNK598	<i>MATa erg6::HIS3 lem3::kanMX pdr5::GRE-lacZ</i>	This study

and *lem3-1* affect the same complementation group. Furthermore, in eight tetrads derived from the *ynl323wΔ lem3-1* diploid, all segregants were Lem<sup>-</sup>. We concluded that *YNL323* and *LEM3* correspond to the same genetic locus. Thus, *YNL323* was renamed *LEM3*. Similarly, the cross of the *erg6Δ* strain to a *lem4-101* strain generated a diploid with a Lem4<sup>-</sup> phenotype (data not shown). Integration of an *ERG6*-containing DNA fragment from pRS303-ERG6 in a *lem4-101* strain at the *ERG6* locus restored wild-type GR activity. A cross of this strain to a wild-type strain yielded tetrads in which all segregants (95/95) exhibited wild-type response to hormone. Therefore, *LEM4* and *ERG6* are allelic. We shall refer to the *lem4-101* strain as *erg6-101*.

**Do *lem3* and *erg6* affect overlapping or independent regulatory pathways?** *LEM3* is predicted to encode a novel 414-amino-acid protein of unknown function, bearing two putative transmembrane domains. *ERG6* is a methyl transferase involved in ergosterol biosynthesis (XU and NES 1988). *lem3-1* and *erg6-101* appear to affect receptor activity by different mechanisms (Figure 3). To infer whether *lem3* and *erg6* affect independent or overlapping genetic pathways, we tested the phenotype of a *lem3Δ erg6Δ* double mutant (YNK598, Figure 5C). The phenotypes of *lem3Δ* and *erg6Δ* were additive in the *lem3Δ erg6Δ* strain, suggesting that they affect different pathways. Taken together, our data suggest that *LEM3* and *LEM4/ERG6* are components of separate, independent pathways that downregulate GR function.

## DISCUSSION

**Identification of *lems*:** A previous unbiased screen for mutants that modulate GR activity revealed the yeast ABC transporter PDR5/LEM1. PDR5 affects GR activity by actively exporting particular ligands from cells, chal-

lenging the notion that transit of steroids across the plasma membrane occurs solely by passive diffusion (KRALLI *et al.* 1995). To find additional ligand-effect modulators (LEMs), we devised a modified screen aimed at isolation of yeast mutants with increased response to dex. We performed the screen in a *pdr5* null background to preclude identifying proteins in the Pdr5 pathway that regulate hormone export. Analysis of two mutants, *lem3* and *lem4*, indicated that they negatively regulate GR activity in a Pdr5<sup>+</sup> cell. Genetic tests on four mutants revealed four complementation groups, indicating that there are many such modulators in yeast (and presumably in mammalian cells) and that this screen is far from saturated.

**Lem3 and Lem4/Erg6 affect different GR activities:** Characterization of two mutants, *lem3* and *lem4/erg6*, suggested that they affect GR activity at distinct steps, as analyses of various GR derivatives revealed that they operate primarily on different GR domains. Consistent with this view, the *erg6-101* but not the *lem3-1* mutation significantly increases hormone occupancy of GR *in vivo*. GR activity in both *lem3* and *lem4* mutant cells is increased in the presence of various ligands, such as dex, deoxycorticosterone, and corticosterone; moreover, *lem3* confers increased activity on other intracellular receptors, including the progesterone, estrogen, and mineralocorticoid receptors.

*LEM3* is predicted to encode a novel transmembrane protein of unknown function. It shares ~40% identity at the protein level with two predicted yeast membrane proteins of unknown function, CDC50 and YNR048w, as well as mammalian expressed sequence tags. We are currently determining whether GR activity is affected in CDC50 and YNR048w and whether the activity of other endogenous transcription factors is increased in the *lem3* mutant. It is unlikely that *LEM3* affects cellular

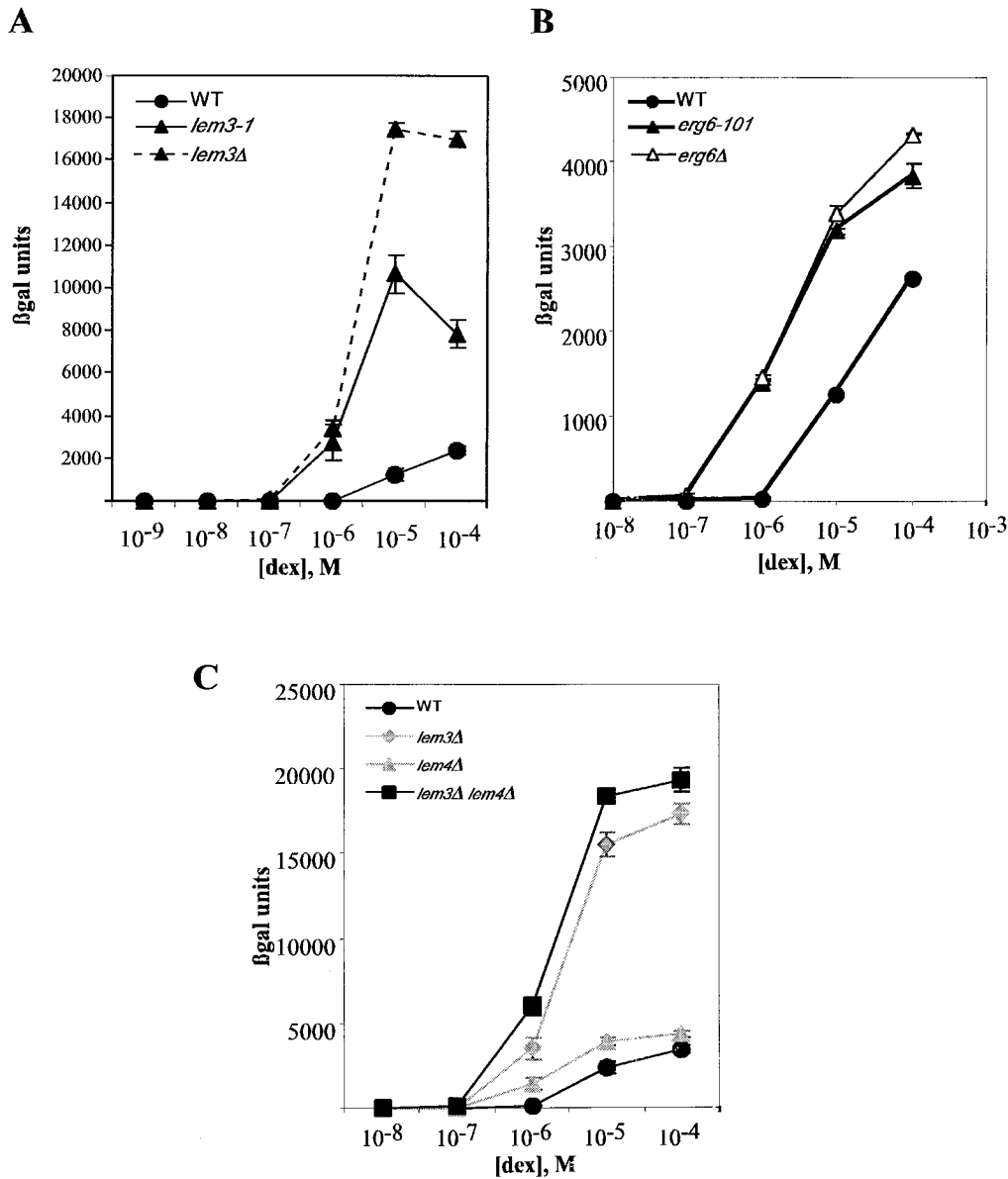


FIGURE 5.—Lem3 and Lem4/Erg6 are part of independent signaling pathways. (A) Phenotypes of *lem3-1* and *lem3Δ*. (B) Phenotypes of *erg6-101* and *erg6Δ*. (C) GR activity in *lem3Δ lem4Δ* double-mutant cells. *lem3Δ* and *lem4Δ* phenotypes are additive.

hormone levels or binding of hormone by GR given that *lem3* mutants affect the function of GR derivatives that lack the ligand binding domain. LEM3 is thus likely to affect a downstream step in the GR pathway, such as nuclear localization, DNA binding, or transcriptional activation.

*ERG6* encodes the *S*-adenosylmethionine  $\Delta$ -24-sterol-*C*-methyltransferase, which carries out side-chain methylation of zymosterol in the ergosterol biosynthesis pathway (GABER *et al.* 1989; HARDWICK and PELHAM 1994). Mutations in *ERG6* lead to altered membrane sterol composition and a pleiotropic sensitivity to numerous structurally diverse compounds, such as cycloheximide, Brefeldin A, and nalidixic acid (WELIHINDA *et al.* 1994; LEES *et al.* 1995; PRENDERGAST *et al.* 1995). How *ERG6* and/or sterol composition affect sensitivity to drugs is not clear.

Given the increase in hormone-bound GR in *erg6-101* cells, it seems plausible that the increased response to hormone results from higher intracellular hormone levels in these cells due to altered membrane composition. The identification of two genes, *PDR5* and *ERG6*, that likely modulate hormone transport across the membrane implies that establishment or maintenance of GR function in yeast may depend on hormone accessibility. Moreover, they demonstrate that there are multiple cellular pathways that can affect hormone access to the receptor. Previous studies on GR signaling have focused primarily on the endpoint of regulation, transcription, as a way to achieve tissue specificity of glucocorticoids. Mechanisms that influence the intracellular concentration of steroid hormones may be a distinct and novel way to account for selective actions of intracellular receptors.

**Implications for LEM functions:** Factors that modulate ligand responsiveness and/or ligand selectivity are likely to contribute to the context-specific actions of GR. For example, factors that catabolize ligand or sequester ligand partly explains why the mineralocorticoid receptor, which can bind with high affinity to both corticosterone and aldosterone, responds only to aldosterone in particular cells of the kidney (BENEDIKTSSON *et al.* 1995; FUNDER 1997). Furthermore, misregulation of a LEM could lead to aberrant responses of steroid receptors to their cognate ligands, such as hormone insensitivity or hypersensitivity, as is seen in several diseases and in therapeutic scenarios (BRÖNNEGÅRD *et al.* 1996). Glucocorticoid resistance and hypersensitivity can arise in individuals bearing wild-type glucocorticoid receptor (ADCOCK *et al.* 1995; BARNES and ADCOCK 1995); similarly, patients who respond well to hormone therapy can suddenly acquire resistance (KLUMPER *et al.* 1995; KAWA and THOMPSON 1996; LAMBERTS 1996).

**Evolution of GR modulators:** As eukaryotes evolved into multicellular organisms, they built upon highly conserved regulatory mechanisms to achieve more diverse and complex cellular functions. The emergence of intracellular receptors as transcriptional regulators and small hydrophobic molecules as signaling factors in metazoans reflects the use of pre-existing cellular factors to achieve context-specific activities. This is exemplified by the ability of the glucocorticoid receptor to confer heterologous, ligand-dependent transcriptional activation in yeast. Although it is quite likely that some GR modulators will be unique to mammalian cells, to date, many yeast factors that affect GR function appear to have functional homologues in mammalian cells (BOHEN and YAMAMOTO 1993; CAIRNS *et al.* 1996; IMHOF and McDONNELL 1996). Thus, the genetic approach we present here is likely to provide new insight into the regulation of intracellular receptor function.

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