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Dual Activators of the Sterol Biosynthetic Pathway of *Saccharomyces cerevisiae*: Similar Activation/Regulatory Domains but Different Response Mechanisms

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Genes encoding biosynthetic enzymes that make ergosterol, the major fungal membrane sterol, are regulated, in part, at the transcriptional level. Two transcription factors, Upc2p and Ecm22p, bind to the promoters of most ergosterol biosynthetic (*ERG*) genes, including *ERG2* and *ERG3*, and activate these genes upon sterol depletion. We have identified the transcriptional activation domains of Upc2p and Ecm22p and found that *UPC2-1*, a mutation that allows cells to take up sterols aerobically, increased the potency of the activation domain. The equivalent mutation in *ECM22* also greatly enhanced transcriptional activation. The C-terminal regions of Upc2p and Ecm22p, which contained activation domains, also conferred regulation in response to sterol levels. Hence, the activation and regulatory domains of these proteins overlapped. However, the two proteins differed markedly in how they respond to an increased need for sterols. Upon inducing conditions, Upc2p levels increased, and chromatin immunoprecipitation experiments revealed more Upc2p at promoters even when the activation/regulatory domains were tethered to a different DNA-binding domain. However, induction resulted in decreased Ecm22p levels and a corresponding decrease in the amount of Ecm22p bound to promoters. Thus, these two activators differ in their contributions to the regulation of their targets.

Because of its medical importance, the regulation of cholesterol levels in mammalian cells is well understood. Part of the regulatory response involves controlling the transcription of genes that encode enzymes of the cholesterol biosynthetic pathway and proteins that control cholesterol processing and uptake. The major regulators of these genes are a pair of transcription factors known as sterol regulatory element binding proteins 1 and 2 (SREBP1 and -2). When cholesterol levels are adequate, SREBPs are tethered to the membrane of the endoplasmic reticulum, where they are spatially restricted from activating their target genes. When cholesterol levels are low, two sequential proteolytic cleavages free a portion of these proteins from their transmembrane tether, whereupon the liberated fragments bind *s*terol *r*egulatory *e*lements (SREs) and activate genes involved in both cholesterol biosynthesis and LDL uptake (15).

Regulation of ergosterol biosynthesis, the fungal equivalent of cholesterol biosynthesis (41), is not as extensively studied. Although the regulation of ergosterol biosynthesis in *Schizosaccharomyces pombe* appears to share features with mammalian cholesterol regulation (18), other fungal species, including *Saccharomyces cerevisiae*, lack protein homologs to mammalian cholesterol regulators and thus appear to have evolved a distinct regulatory approach. Because the ergosterol biosynthetic pathway contains the major targets for antifungal therapy, it is both interesting and important to understand its regulation in detail. Like the genes involved in cholesterol biosynthesis, the genes encoding enzymes for ergosterol biosynthesis are transcriptionally regulated in response to the need for ergosterol (5, 10, 23, 36), but in ways that differ markedly from regulation in mammals (10).

In the yeast *Saccharomyces cerevisiae*, two Zn(II)2Cys6 binuclear cluster transcription factors (39), Upc2p and Ecm22p, have been implicated in ergosterol regulation (40). When sterol levels are reduced, the expression of ergosterol biosynthesis (*ERG*) genes encoding the enzymes that catalyze late steps in ergosterol biosynthesis, such as *ERG2* and *ERG3*, is substantially increased (5, 36, 37, 40). This induction can be mediated by either Upc2p or Ecm22p; mutants defective in either protein can induce these genes in response to sterol depletion, but the deletion of both genes eliminates induction (40). Both Upc2p and Ecm22p bind to the same regulatory element upstream of *ERG2* and *ERG3*. This binding is necessary for transcriptional activation by Upc2p or Ecm22p. Both in vivo promoter deletions and in vitro binding studies have identified a short sequence motif that is both necessary and sufficient to bind either Upc2p or Ecm22p (40). One or more copies of this motif occur upstream of most other *ERG* genes, although few have been tested directly for function.

In addition to binding the promoters of *ERG* genes, Upc2p binds a similar regulatory element upstream of the anaerobically induced mannoprotein-encoding genes known as the *DAN/TIR* genes (1, 2). Indeed, Upc2p may be responsible for the activation of up to one-third of all anaerobically induced genes (25). Ecm22p's role in anaerobiosis is not as thoroughly explored. Ecm22p plays a role in the expression of *DAN2* and *DAN3* but not in the expression of any other *DAN/TIR* genes (1).

Upc2p and Ecm22p may also play a role in the uptake of sterols under hypoxic conditions. Normally, yeast cells take up sterols from their environment only under anaerobic conditions (4). A mutant allele of *UPC2*, *UPC2-1*, allows the aerobic

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TABLE 1. Strains used in this study

Strain	Genotype	Source (reference)
PJ69-4a	*MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 ade2-201::GAL2-ADE2 met2::GAL7-lacZ*	James et al. (20)
W303-1a	*MAT**a** ade2-1 leu2-3,112 his3-1 ura3-52 trp1-100 can1-100*	R. Rothstein
JRY7179	W303-1a *upc2Δ::HIS3*	Vik and Rine (40)
JRY7180	W303-1a *ecm22Δ::TRP1*	Vik and Rine (40)
JRY7181	W303-1a *ecm22Δ::TRP1 upc2Δ::HIS3*	Vik and Rine (40)
JRY7520	W303-1a *bar1*	A. Kirchmaier
JRY7865	JRY7520 *UPC2-TAP*	This study
JRY7866	JRY7520 *ECM22-TAP*	This study
JRY7773	W303-1a *erg2Δ::TRP1*	This study
JRY7775	W303-1a *erg3Δ::TRP1*	This study
JRY7776	W303-1a *erg4Δ::TRP1*	This study
JRY7779	W303-1a *erg2Δ::TRP1 erg3Δ::TRP2* pJR2316	This study
JRY7939	W303-1a *erg3Δ::TRP1 upc2Δ::HIS3*	This study
JRY7940	W303-1a *erg3Δ::TRP1 ecm22Δ::TRP1*	This study
JRY7941	W303-1a *erg3Δ::TRP1 upc2Δ::HIS3 ecm22Δ::TRP1*	This study

uptake of sterols (26). This phenotype results from a single amino acid change in the C-terminal domain of Upc2p (8).

Upc2p and Ecm22p share a high level of sequence similarity in their DNA-binding domains. Both proteins also contain C-terminal regions of unknown function, which, although highly similar between the two proteins (33), have no sequence similarity to any other *Saccharomyces* proteins or any known nonfungal proteins. The highly similar DNA-binding domains and highly similar C-terminal regions are separated by highly dissimilar middle regions (see Fig. 1A).

In this work, we investigated the activity and regulation of Upc2p and Ecm22p. In particular, we tested which parts of the proteins contained the activation and regulatory domains and tested the mechanism by which the proteins respond to an increased need for sterols.

MATERIALS AND METHODS

Strains and media. All yeast strains were isogenic to W303-1a except those noted in Table 1. Gene deletions were made by a PCR-based gene disruption method (6), such that the entire open reading frame was replaced by the *TRP1* gene amplified from pRS304. Sequences encoding the tandem affinity purification (TAP) tag (32) were integrated in frame at the 3′ end of the open reading frames by use of homologous recombination and one-step gene integration of PCR-amplified modules.

All yeast strains were grown in complete synthetic medium (CSM; 0.67% Difco yeast nitrogen base without amino acids, complete supplemental mixture minus appropriate amino acids [Q-Biogene]) containing 2% glucose. A 25-mg/ml stock solution of lovastatin (a generous gift from James Bergstrom) was made as previously described (11). Lovastatin was added to liquid medium to final concentrations of 5 μg/ml (for PJ69-4a) or 30 μg/ml (for all other strains).

Plasmids. The plasmid constructions pJR2570 to -2595 made extensive use of pGBDU-C1 and pGBDU-C2 (20), both of which contain the coding sequence for the Gal4p DNA-binding domain.

pJR2570 to -2572 were made by subcloning the sequences encoding Upc2p amino acids (aa) 583 to 913, 390 to 913, and 1 to 913, respectively, into the multiple cloning site of pGBDU-C1, resulting in fusions of the DNA-binding domain of Gal4p and various portions of Upc2p.

The QuikChange protocol from Stratagene was used to make the deletions and point mutations described below, and a variation of the QuikChange protocol described by Geiser et al. (13) was used to make insertions.

pJR2573 and pJR2574 were made from pJR2572. Stop codons were introduced at sites corresponding to amino acid 106 (for pJR2573) and amino acid 583 (for pJR2574) of *UPC2* with primers BD50 and -51 (5′-GCAGACGGTAG CGTGT̲AGTCTGATTCATCGG-3′ and its reverse complement) for pJR2573 and with BD52 and -53 (5′-CGAAGCTGACT̲GAAGTTCTGTTTCTGCTG G-3′ and its reverse complement) for pJR2574.

The primers BD54 and 55 (5′-GTATCGCCGGAATTCCCCGGGGTGGAG TCTGATTCATCGGTAG-3′ and its reverse complement) were used to delete the coding sequence for Upc2p amino acids 1 to 104 in pJR2574 and pJR2572, resulting in pJR2575 and pJR2576, respectively.

pJR2585 to -2587 were made by subcloning the sequence encoding Ecm22p amino acids 479 to 814, 277 to 814, and 1 to 814, respectively, into the multiple cloning site of pGBDU-C1 (pJR2585 and 2586) or pGBDU-C2 (pJR2587), resulting in fusions of the DNA-binding domain of Gal4p and various portions of Ecm22p.

pJR2588 was created using the method of Geiser et al. (13) to insert the sequence encoding amino acids 448 to 478 of Ecm22p into pJR2585, resulting in a plasmid coding for a fusion of the Gal4p DNA-binding domain and amino acids 448 to 814 of Ecm22p.

pJR2589 and pJR2590 were made from pJR2587. The primers BD44 and -45 (5′-GATAGGGTAGGCAAGT̲AAAATCTGAGTGAC-3′and its reverse complement) and BD46 and -47 (5′-CAATCTTCTCCCCATT̲AAAACGCTTCAT C-3′ and its reverse complement), respectively, were used to create stop codons at sites corresponding to amino acid 100 (pJR2589) and amino acid 480 (pJR2590) of Ecm22p. In the sequences for BD44 and some of the other primers, the underlining indicates mutations introduced by QuikChange.

Primers BD48 and -49 (5′-GTATCGCCGGAATTCCCGGGGCTGAGTGA CAATGCCATCATG-3′ and its reverse complement) were used to delete the coding sequence for Ecm22p amino acids 1 to 101 in pJR2590 and pJR2587, resulting in pJR2591 and pJR2592, respectively.

By use of primers BD21 (5′-CGAATACAGTGGAGGTGGTGA̲TATGCAT ATGATGCTAGATTTCCTCG-3′) and BD22 (5′-CGAGGAAATCTAGCATC ATATGCATAT̲CACCACCTCCACTGTATTCG-3′), the *UPC2-1* mutation was introduced into pJR2570 to -2572, resulting in plasmids pJR2577 to -2579, respectively.

By use of primers BD23 (5′-GATGAGTATAGTGGAGGTGGTGA̲TATGC ATATGATGCTGGATTTCTTAGG-3′) and BD24 (5′-CCTAAGAAATCCAG CATCATATGCATAT̲CACCACCTCCACTATACTCATC-3′), the *ECM22-1* mutation was introduced into pJR2585 to -2587, resulting in plasmids pJR2593 to -2595, respectively.

pJR2316 was a *pERG2::lacZ* reporter described previously (40).

pJR2564 was a plasmid containing full-length *UPC2* and its promoter. *UPC2* was gap repaired into pRS426 (34) and then subcloned into pRS415. pJR2602 is pJR2564 with the *UPC2-1* mutation. It was created using primers BD21 and -22. pJR2653 was a plasmid coding for a truncated Upc2p (amino acids 1 to 152) that was created by introducing a stop codon at aa 583 by use of primers BD52 and BD53 in parental plasmid pJR2564.

pJR2338 was a plasmid containing full-length *ECM22* and its promoter. The BamHI fragment containing ECM22 and its promoter was cut from genomic DNA and ligated into pRS415. The sequence coding the triple hemagglutinin tag was inserted into the *ECM22* sequence at the spot coding aa 276.

pJR2644 is pJR2338 with the *ECM22-1* mutation, created using primers BD23 and -24 (see above). pJR2603 to -2608 were all created using the primers listed below to introduce mutations that would result in various amino acid substitutions at position 888 of *UPC2* in pJR2564. BD95 and -96 (5′-CAGTGGAGGT GGT̲AAT̲ATGCATATGATGCTAG-3′ and its reverse complement) were used

to create pJR2603 (G888N). BD97 and -98 (5′-CAGTGGAGGTGGT<u>GCT</u>AT GCATATGATGCTAG-3′ and its reverse complement) were used to create pJR2604 (G888A). BD99 and -100 (5′-CAGTGGAGGTGGT<u>GAG</u>ATGCATA TGATGCTAG-3′ and its reverse complement) were used to create pJR2605 (G888E). BD101 and -102 (5′-CAGTGGAGGTGGT<u>CAG</u>ATGCATATGCTA CTAG-3′ and its reverse complement) were used to create pJR2606 (G888Q). BD103 and -104 (5′-CAGTGGAGGTGGT<u>GTT</u>ATGCATATGATGCTAG-3′ and its reverse complement) were used to create pJR2607 (G888V). BD112 and -113 (5′-CAGTGGAGGTGGT<u>AGG</u>ATGCATATGATGCTAG-3′ and its reverse complement) were used to create pJR2608 (G888R).

β-Galactosidase assays. β-Galactosidase assays were performed essentially as previously described (6).

Analysis of protein levels. Strains were grown to mid-log phase, and whole-cell extracts were prepared as described previously (12). Extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Gal4p fusion proteins were immunoblotted with antibodies against the Gal4p DNA-binding domain (ClonTech) and 3-phosphoglycerate kinase (Molecular Probes). TAP-tagged proteins were immunoblotted with anti-3-phosphoglycerate kinase (Molecular Probes) and with an anti-Flag antibody (rabbit; Sigma) to detect the TAP tag. Immunoblots shown in Fig. 2A and E were scanned and quantified using the Li-Cor Odyssey imaging system.

Chromatin immunoprecipitations. Chromatin extracts were prepared essentially as described previously (24). Sonication yielded average DNA sizes of 400 to 1,000 bp. Two hundred microliters of chromatin extract was used for immunoprecipitation (IP), and 20 μl was used for input control preparation. For experiments using Gal4p fusion proteins (see Fig. 2D and 3C), IPs were performed with 25 μl of salmon sperm DNA/protein G agarose beads (Upstate) prebound to 9 μg of anti-Gal4p DNA-binding domain antibodies. For TAP-tagged proteins (Fig. 2B), IPs were performed using 30 μl immunoglobulin G-Sepharose (Amersham Biosciences). Typical IPs were performed overnight at 4°C. Immune complexes were washed as previously described (24). Chromatin complexes were released, cross-links were reversed, and both immunoprecipitated and input DNAs were prepared as described previously (3). For the Gal4 fusion experiments, PCRs were performed using *GAL7* (5′-CTCACAA CTATTGCGAAGCGC-3′ and 5′-CAGCTTGGCTATTTTGTGAACAC-3′) and *Ty1* (5′-CAGGCATGCAATCCAATACC-3′ and 5′-CGCTCCACTTTGTT TCAATCC-3′) primers for 30 cycles with 0.5 U of AmpliTaq Gold polymerase (Roche) and 10 pmol of each oligonucleotide. Following electrophoresis on agarose gels, PCR products were stained with Syto 60 (Molecular Probes) and scanned and quantified using the Li-Cor Odyssey imaging system. For chromatin IPs using TAP-tagged proteins, DNA enrichment was analyzed by use of real-time PCR and SYBR Green fluorescence on a Stratagene MX3000 real-time PCR system. Real-time PCR was performed using primers to the *ERG3* promoter (5′-GACGCCTTTTGTTGCGATTGTCG-3′ and 5′-CAGCAACAACA ATACCCGATCGC-3′) and to ACT1 (5′-GGCATCATACCTTCTACAACGA ATTG-3′ and 5′-CTACCGGAAGAGTACAAGGACAAAAC-3′) and DNA derived from whole-cell extracts as a standard.

RESULTS

Upc2p and Ecm22p contained discrete transcriptional activation domains. To identify the transcriptional activation domains of Ecm22p and Upc2p, various segments of *ECM22* and *UPC2* were fused in frame to the sequence encoding the DNA-binding domain of *GAL4*. The Gal4p DNA-binding domain, which is of the same structural class as those of Ecm22p and Upc2p, binds its recognition sites in the promoters of galactose-induced genes such as *GAL1*, *-7*, and *-10* but will activate transcription only if fused to, or associated with, a functional transcriptional activation domain (21, 27). The ability of each fusion to activate transcription was tested in a strain containing an integrated reporter in which the *GAL7* promoter directed the expression of the *lacZ* gene (*pGAL7::lacZ*) and lacked the endogenous *GAL4* gene.

When fused with the *GAL4* DNA-binding domain, the region of *UPC2* encoding the smallest C-terminal fragment tested was sufficient to activate transcription at a level nearly 28-fold above that of the *GAL4* DNA-binding domain alone

(Fig. 1B, pJR2570). Segments of *UPC2* that did not contain this C-terminal region produced either no increase or only small increases in transcription. Oddly, full-length *UPC2* fused with the *GAL4* DNA-binding domain was a poor transcriptional activator (Fig. 1B, pJR2572). The poor activation of the full-length fusion protein could result from the competition between the *GAL7* promoter for the Gal4p DNA-binding domain and many SRE-containing promoters for the intrinsic DNA-binding domains of Upc2p (aa 1 to 100). However, the ability of smaller fragments of Upc2p to activate transcription more profoundly than larger fragments missing the intrinsic DNA-binding domain (compare pJR2570 with pJR2576 in Fig. 1B) also suggested that other portions of Upc2p might regulate its intrinsic activation capability. All fusion constructs produced fusion protein, and fusions that resulted in activation did not produce more fusion protein than nonactivating fusions did (Fig. 3D and data not shown).

Despite over 70% identity with the Upc2p carboxyl terminus, the corresponding region of Ecm22p activated transcription at a level only eightfold above that of the Gal4p DNA-binding domain alone (Fig. 1C, pJR2585). A fragment of Ecm22p (amino acids 102 to 479) activated transcription at a 13-fold-higher level (Fig. 1C, pJR2591), and this central fragment and the C-terminal fragment together (aa 102 to 814) activated transcription at a level almost 90-fold over that the DNA-binding domain alone (Fig. 1C, pJR2592). As with *UPC2*, the fusion containing full-length *ECM22* was unable to activate transcription to such a high level (Fig. 1C, pJR2587). Additional protein fusions in which the C termini of Upc2p and Ecm22p were further subdivided produced no detectable protein, precluding further refinement of the activation domain boundaries (data not shown).

Mechanism of induction by Ecm22p and Upc2p. When cells are treated with lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase, sterols are depleted and the expression of several *ERG* genes, including *ERG2* and *ERG3*, is induced (5, 10, 36, 37, 40). The induction of *ERG2* and *ERG3* in response to lovastatin depends on Upc2p and Ecm22p (40). Microarray expression profiles show that lovastatin treatment results in increased transcription of *UPC2* but not of *ECM22* (19). To test whether the induction of *ERG2* and *ERG3* was a result of increased levels of Ecm22p or Upc2p protein or of the increased effectiveness of each protein, TAP-tagged versions of Upc2p and Ecm22p were used to measure the levels of these proteins upon induction. In untreated cells, there was 40% more Ecm22p than Upc2p. Following treatment with lovastatin, Upc2p levels increased fourfold, whereas levels of Ecm22p decreased to about a third of the untreated level (Fig. 2A). Thus, in the case of Upc2p, it was possible that increased Upc2p levels could explain the induction of *ERG2* and *ERG3* expression by lovastatin-mediated sterol depletion. However, induction mediated by Ecm22p must reflect increased effectiveness of the protein to explain induction in the face of decreasing protein levels.

Chromatin immunoprecipitations were performed on untagged, *UPC2-TAP*, and *ECM22-TAP* strains grown in the presence or absence of lovastatin to ascertain whether occupancy at the *ERG3* promoter increased under inducing conditions. Under noninducing conditions, there was very little enrichment of Upc2p at the *ERG3* promoter compared to the
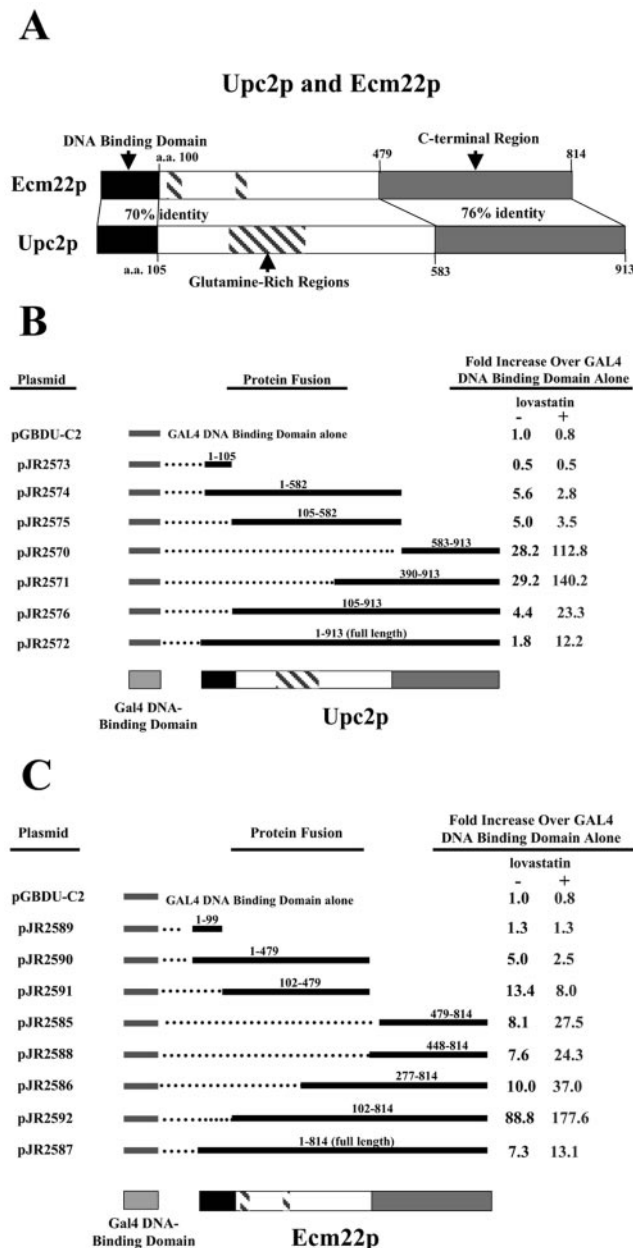
A

Upc2p and Ecm22p

B

Plasmid	Protein Fusion	Fold Increase Over GAL4 DNA Binding Domain Alone	
		lovastatin	
		−	+
pGBDU-C2	GAL4 DNA Binding Domain alone	1.0	0.8
pJR2573	1-105	0.5	0.5
pJR2574	1-582	5.6	2.8
pJR2575	105-582	5.0	3.5
pJR2570	583-913	28.2	112.8
pJR2571	390-913	29.2	140.2
pJR2576	105-913	4.4	23.3
pJR2572	1-913 (full length)	1.8	12.2

Gal4 DNA-Binding Domain   Upc2p

C

Plasmid	Protein Fusion	Fold Increase Over GAL4 DNA Binding Domain Alone	
		lovastatin	
		−	+
pGBDU-C2	GAL4 DNA Binding Domain alone	1.0	0.8
pJR2589	1-99	1.3	1.3
pJR2590	1-479	5.0	2.5
pJR2591	102-479	13.4	8.0
pJR2585	479-814	8.1	27.5
pJR2588	448-814	7.6	24.3
pJR2586	277-814	10.0	37.0
pJR2592	102-814	88.8	177.6
pJR2587	1-814 (full length)	7.3	13.1

Gal4 DNA-Binding Domain   Ecm22p

FIG. 1. (A) Upc2p and Ecm22p show strong sequence similarity. They are 70% identical in the DNA-binding domains and 76% identical in the C-terminal regions. Both proteins also have glutamine-rich regions. (B) The C-terminal region of Upc2p contained an activation domain, and fusions containing this region could be induced by lovastatin. Plasmids encoding the indicated protein fusions were transformed into the *pGAL7::lacZ* reporter strain, PJ69-4a. The resulting strains were grown in the presence or absence of 5 μg/ml lovastatin. Strains were grown to mid-log phase and harvested, and β-galactosidase activity was tested as described in Materials and Methods. Values for all β-galactosidase assays in this study represent averages of at least three experiments each. Values are shown as increases (*n*-fold) over that of the Gal4p DNA-binding domain alone grown under noninducing conditions. β-Galactosidase activity of the Gal4p DNA-binding domain averaged 21.6 Miller units across all experiments. (C) The Ecm22p C-terminal region also contained an activation domain and could be induced by lovastatin. Plasmids encoding the indicated protein fusions were transformed into PJ69-4a and tested as described for panel B.

untagged control. However, enrichment increased 16-fold following treatment with lovastatin. In contrast, Ecm22p was enriched sevenfold under noninducing conditions, but this enrichment virtually disappeared after induction with lovastatin (Fig. 2B).

As Gal4 fusion proteins were expressed from the *ADH1* promoter, which is not induced by lovastatin (19), these fusions provided an opportunity to determine whether induction by Upc2p could also occur independently of increases in Upc2p levels. These fusions also allowed the determination of whether the response to low sterol levels was mediated by a specific region of Upc2p or Ecm22p and whether that region was distinct from the activation domain. All Gal4-Ecm22p and Gal4-Upc2p fusion proteins that contained the carboxyl-terminal activation region also increased transcription in response to lovastatin treatment; those which did not contain this region caused no increase (Fig. 1B and C). The Gal4p DNA-binding domain fused to its own activation domain did not increase transcription in response to lovastatin (data not shown). Therefore, by this analysis, activation and regulation were mediated by the carboxyl-terminal third of both proteins.

To provide an independent test of whether activation and regulation were indeed mediated by the carboxyl-terminal region in a full-length protein, this region was removed from Upc2p. The truncated protein activated *ERG2* expression to only one-third of the level for the full-length protein under noninducing conditions (Fig. 2C). Induction of *ERG2* following treatment with lovastatin was virtually eliminated, with the small amount of residual induction likely the result of increased transcription of *UPC2* (see above).

To determine whether the increase in the occupancy of a regulated promoter by Upc2p following induction still occurred in the absence of an increase in protein, chromatin immunoprecipitations were performed on strains containing fusion proteins. Gal4p fusions containing the smallest active C-terminal fragment of Upc2p or Ecm22p were tested in the presence or absence of lovastatin. In the case of the Gal4-Upc2 fusion protein, *GAL7* promoter DNA was enriched to a greater degree in immunoprecipitates from cells grown in lovastatin (Fig. 2D), indicating a greater level of occupancy of the promoter by the protein. Interestingly, the Gal4-Ecm22 fusion showed no change in promoter occupancy after lovastatin induction (Fig. 2D). As expected, increased protein levels were not observed for either fusion protein in the presence of lovastatin; in fact, levels of Gal4-Upc2p decreased (Fig. 2E). Hence the activation/regulatory regions of the two proteins had rather different behaviors. The Ecm22p fusion protein became more active on a per-molecule basis when bound to the promoter. In contrast, the Upc2 fusion protein was recruited to the promoter upon induction, even when that promoter was recognized by the Gal4 DNA-binding domain.

Transcriptional activation was increased by *UPC2-1.* *UPC2-1* is a semidominant mutation (G888D) that results in increased aerobic sterol uptake (8, 26). This mutation does not mimic a *upc2* null allele but may produce a Upc2p with increased activity (8). The extensive identity in the C-terminal regions of Upc2p and Ecm22p allowed an equivalent mutation, G790D, to be made in *ECM22* (33). *UPC2-1* and its *ECM22* equivalent, *ECM22-1*, were each introduced into a *upc2Δ ecm22Δ* double-mutant strain and tested for their abilities to
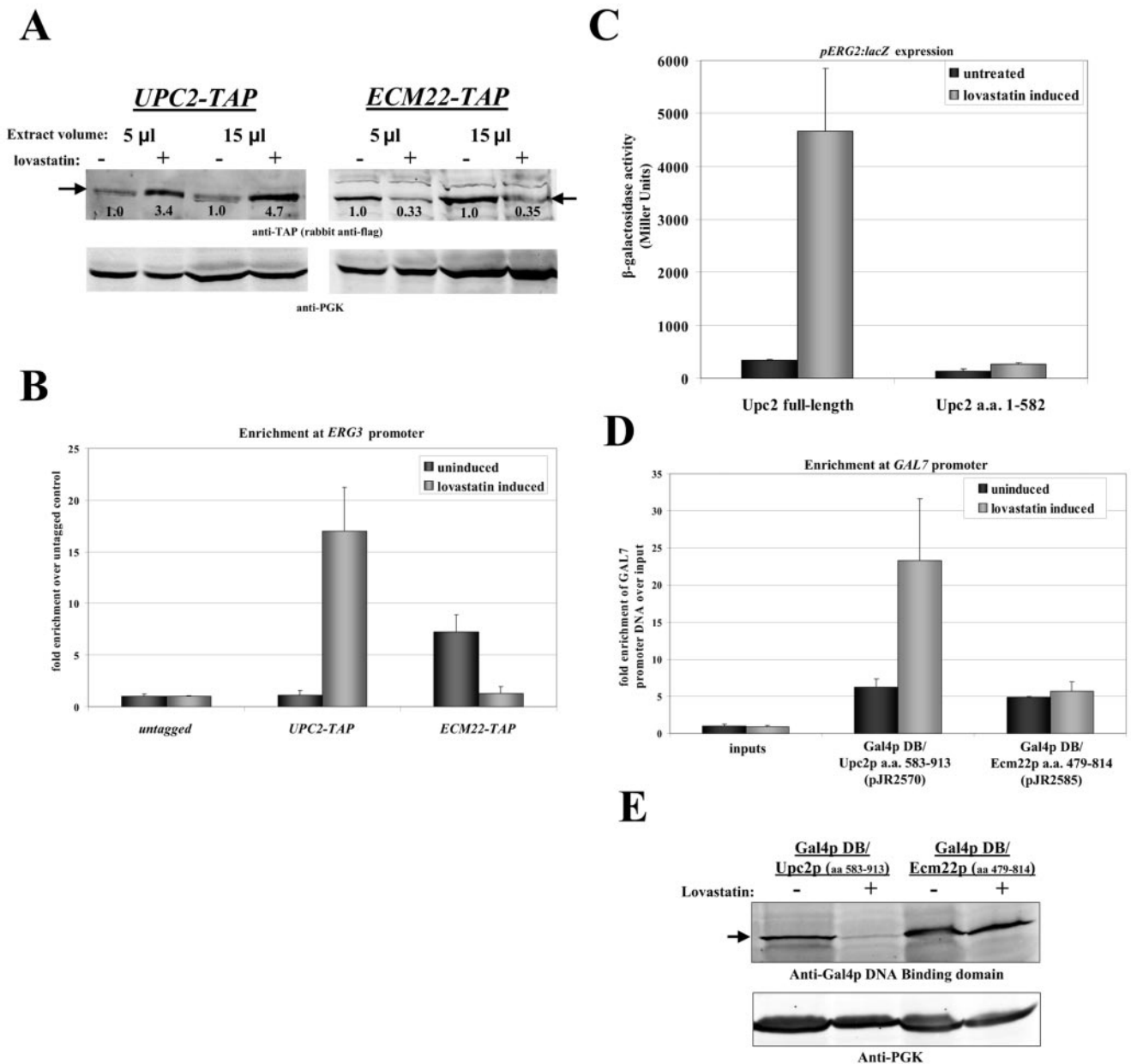
FIG. 2. (A) Levels of Upc2p increased and levels of Ecm22p decreased in response to lovastatin. Whole-cell extracts were prepared from JRY7865 (*UPC2-TAP*) and JRY7866 (*ECM22-TAP*) grown in the presence or absence of 30 μg/ml lovastatin. Protein levels were analyzed by SDS-PAGE followed by immunoblotting as described in Materials and Methods. Arrows indicate tagged proteins. Relative protein levels were quantified using the Li-Cor Odyssey imaging system. Numbers indicate changes (*n*-fold) in induced protein levels relative to uninduced protein levels. (B) In the presence of lovastatin, the levels of Upc2p-TAP increased at *ERG3* promoters, whereas the levels of Ecm22p-TAP decreased. Chromatin immunoprecipitations were performed as described in Materials and Methods. Real-time PCR was used to quantitate the levels of *ERG3* promoter DNA and *ACT1* control DNA. The ratio of *ERG3* promoter DNA to *ACT1* DNA was calculated for each sample. Bars indicate the averages of three reactions and the increases in the levels of enrichment seen in Upc2p-TAP and Ecm22p-TAP experiments over the untagged control level. (C) The carboxy-terminal region of Upc2p is important for the activation of *ERG2* and its induction by lovastatin. Plasmids containing full-length *UPC2* (pJR2564) or *UPC2* coding only for aa 1 to 582 (pJR2653) were transformed into a *upc2Δ ecm22Δ* strain (JRY7181) that contained a *pERG2*::*lacZ* reporter (pJR2316). Strains were grown in the presence or absence of 5 μg/ml lovastatin, harvested, and assayed for β-galactosidase activity as described in Materials and Methods. (D) In the presence of lovastatin, the levels of a Gal4p/Upc2p fusion increased at the *GAL7* promoter. Chromatin immunoprecipitations were performed as described in Materials and Methods. Bands were quantified using the Li-Cor Odyssey imaging system, and the ratio of *GAL7* promoter DNA to control DNA (Ty1) was calculated for each PCR. Bars indicate the enrichment of these ratios from the immunoprecipitations over that of the input DNA and are the average values from at least three PCRs. (E) Treatment with lovastatin did not increase the levels of fusion protein. The *Gal7*::*lacZ* reporter strain (PJ69-4a) transformed with the indicated plasmids was grown to log phase in the presence or absence of 5 μg/ml lovastatin. Whole-cell extracts were prepared, subjected to SDS-PAGE, and immunoblotted as described in Materials and Methods. The arrow indicates fusion proteins.
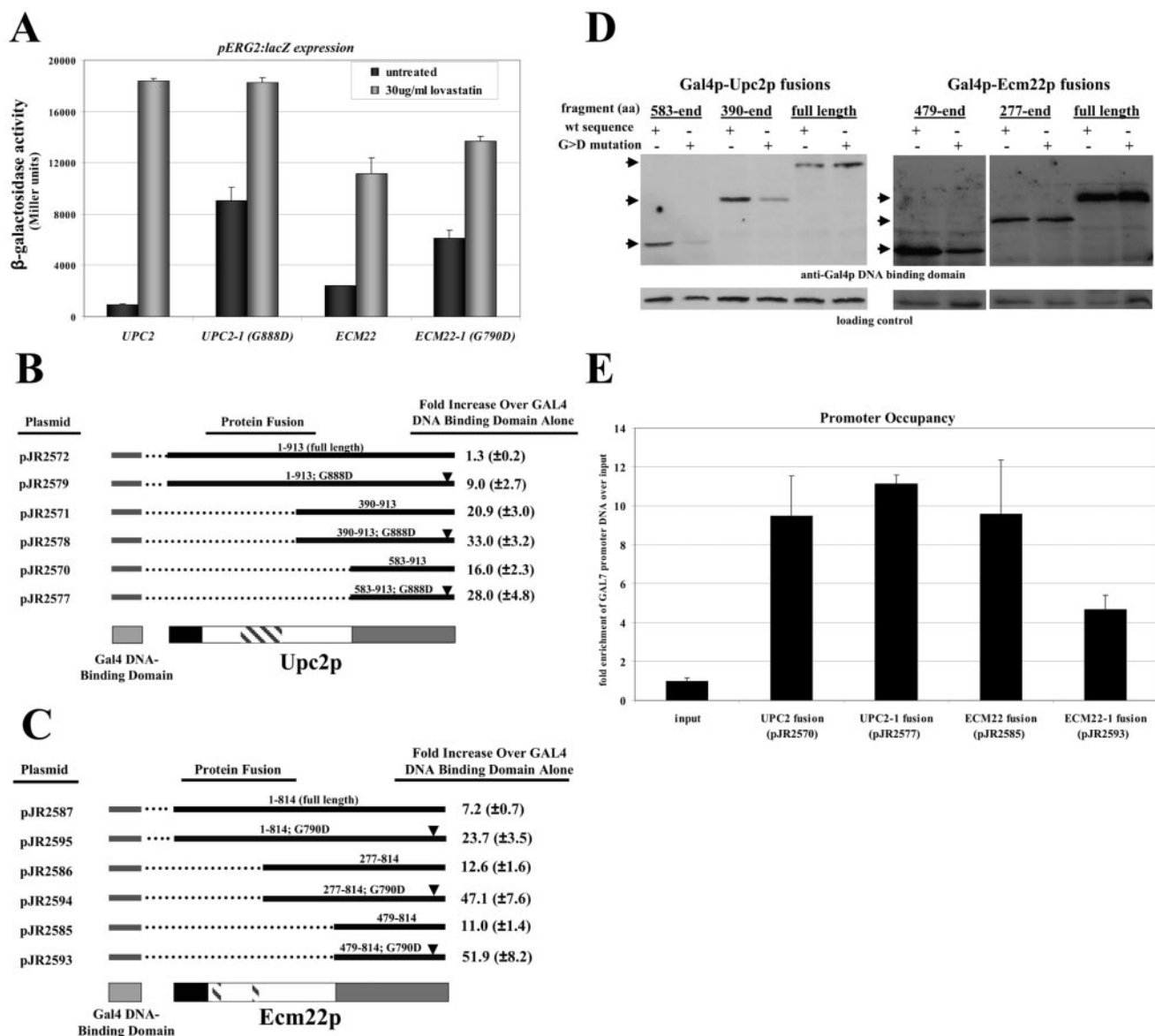
FIG. 3. (A) The *UPC2-1* and *ECM22-1* mutations increased uninduced levels of *ERG2* expression. *UPC2* (pJR2564), *UPC2-1* (pJR2602), *ECM22* (pJR2338), and *ECM22-1* (pJR2644) were each transformed into a *upc2Δ ecm22Δ* strain (JRY7181) that contained a *pERG2::lacZ* reporter (pJR2316). Strains were grown in the presence or absence of 30 μg/ml lovastatin, harvested, and assayed for β-galactosidase activity as described in Materials and Methods. (B) The *UPC2-1* mutation increased transcriptional activation. Plasmids encoding the indicated protein fusions were tested as described for Fig. 1B. (C) The *ECM22-1* mutation also increased transcriptional activation. Plasmids encoding the indicated protein fusions were tested as described for Fig. 1B. (D) The *UPC2-1/ECM22-1* mutation did not increase protein levels. Plasmids encoding the indicated protein fusions and transformed into PJ69-4a were grown to mid-log phase. Whole-cell extracts were prepared, subjected to SDS-PAGE, and immunoblotted as described in Materials and Methods. Arrows indicate fusion proteins. (E) The *UPC2-1/ECM22-1* mutation did not increase the level of transcription factor at the promoter. Chromatin immunoprecipitations were performed as described in Materials and Methods. Bands were quantified using the Li-Cor Odyssey imaging system, and the ratio of *GAL7* promoter DNA to control DNA (Ty1) was calculated for each PCR. Bars indicate the enrichment of these ratios for the immunoprecipitations compared to that of the input DNA. Each bar represents at least three reactions.

activate an *ERG2*::*lacZ* reporter gene. Both *UPC2-1* and *ECM22-1* increased *ERG2* expression over the level of expression caused by *UPC2* and *ECM22* (Fig. 3A), suggesting that the mutations either increased the intrinsic activation abilities of these two proteins, increased the level of each protein, or reduced their sensitivities to a negative regulator. To discriminate among these three possibilities, we tested *ERG2* expres-

sion after lovastatin treatment, asking whether the mutant proteins exhibited the same induced levels or, alternatively, the same induction ratios from a higher baseline as those of the wild-type proteins. In the presence of lovastatin, the mutant and wild-type forms of each protein induced *ERG2* expression to approximately the same induced levels, but did so from different starting points (Fig. 3A). Hence, the mutations did

not affect the intrinsic activation ability of either protein; rather they appeared to partially relieve Ecm22p and Upc2p functions from something that limited their activities.

To characterize the *UPC2-1/ECM22-1* mutations further, these mutations were introduced into a subset of the *GAL4* fusions described above, all of which were expressed from the *ADH1* promoter. These fusions activated transcription to levels two to seven times higher than those of their wild-type counterparts (Fig. 3B and C). These increases were not caused by increased levels of the fusion proteins. Indeed, mutation-containing fusions actually produced slightly less protein than their nonmutant counterparts (Fig. 3D).

As protein levels did not increase in the mutant proteins, increased transcriptional activation in uninduced cells could be achieved in either of two ways. The mutation could cause transcription factors at the promoter to have greater potency per molecule, activating transcription more effectively. Alternatively, the mutation could result in more transcription factor bound to the promoter even in uninducing conditions. To distinguish between these possibilities, occupancies of the promoter by mutant and nonmutant fusion transcription factors were tested with chromatin immunoprecipitation. As expected for fusion proteins with the Gal4p DNA-binding domain, *GAL7* promoter DNA was enriched in the immunoprecipitated DNA. For both Ucp2p and Ecm22p fusion proteins, the mutant form of the protein showed no greater level of occupancy of the *GAL7* promoter than the nonmutant form did (Fig. 3E). Indeed, the *ECM22-1* fusion protein exhibited a reduced occupancy relative to that of the wild-type *ECM22* fusion. Hence, the *UPC2-1* and *ECM22-1* mutations increased the activity of the fusion protein in uninduced cells, presumably by providing relief from some negative regulator, since the induced levels caused by mutant and wild-type Ecm22p and Upc2p were essentially identical.

The *UPC2-1* mutation substituted an acidic residue for an uncharged one (G888D), suggesting that the mutation either created or enhanced an acidic activation domain. Therefore, we tested whether the introduction of an acidic residue at this position was crucial to the mutant phenotype by substituting six other amino acids for glycine at amino acid 888 in full-length *UPC2* and testing the effect on *ERG2::lacZ* expression. Any substitute larger than alanine resulted in an increase of *ERG2* expression similar to that of the original aspartic acid substitution, regardless of the charge on the substituted amino acid (Fig. 4). Hence, size, not charge, was the determinant of phenotype at this position.

What molecule(s) controls Ecm22p and Upc2p activity? The increase in Ecm22p and Upc2p activities in response to lovastatin treatment presumably reflects a response to reduced levels of either ergosterol or some intermediate in ergosterol biosynthesis. In simple models, one of these molecules could directly inhibit Ecm22p and Upc2p such that depletion of the molecule leads to more-active transcription factors. If so, mutations in various steps of the ergosterol biosynthetic pathway might reveal whether ergosterol itself or an intermediate in the biosynthetic pathway of ergosterol is responsible for the effect. The early enzymes of the ergosterol biosynthesis pathway are encoded by essential genes, but the final few steps in the pathway are nonessential and, hence, amenable to this analysis (Fig. 5A). The effects of disrupting the late steps of ergosterol
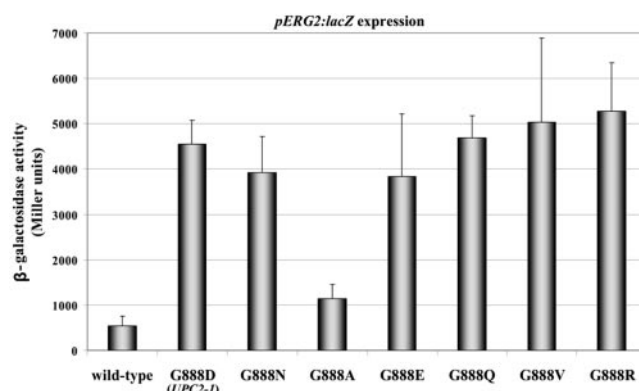

FIG. 4. Substitution of any amino acid larger than alanine at glycine 888 resulted in increased transcription. *UPC2* (pJR2564) and *UPC2* with mutations resulting in the indicated amino acid substitutions (pJR2602 to pJR2608) were each transformed into JRY7181 (*upc2Δ ecm22Δ*) along with a *pERG2::lacZ* reporter (pJR2316). Strains were grown to mid-log phase and assayed for β-galactosidase activity as described for Fig. 3A.

biosynthesis on Upc2p/Ecm22p-regulated genes were tested by measuring expression of an *ERG2::lacZ* reporter in the relevant mutants.

Although all *erg* gene deletions tested resulted in some increase in *ERG2* expression, the levels of response varied widely (Fig. 5B). The magnitude of the response did not correlate with the order of steps in the pathway, and no response rivaled the magnitude of induction by lovastatin. Moreover, *ERG2* expression in all strains could be further induced by treatment with lovastatin (Fig. 5B). These data indicated that ergosterol itself could not be the only molecule capable of regulating the activity of Upc2p and Ecm22p.

To check whether the increase in *ERG2* expression observed in an *erg3Δ* strain still required Upc2p or Ecm22p, *erg3Δ upc2Δ*, *erg3Δ ecm22Δ*, and *erg3Δ upc2Δ ecm22Δ* strains were constructed and tested with an *ERG2::lacZ* reporter. Increased *ERG2* expression relative to that of *upc2Δ* and *ecm22Δ* single-deletion strains was observed for both the *erg3Δ upc2Δ* and *erg3Δ ecm22Δ* double-deletion strains (Fig. 5C). In an *erg3Δ upc2Δ ecm22Δ* triple-deletion strain, however, no increase in *ERG2* expression over that of the *upc2Δ ecm22Δ* double-deletion strain was observed, indicating that the increase in *ERG2* expression in an *erg3Δ* strain required either Upc2p or Ecm22p.

DISCUSSION

The goal of this work has been to understand how the activities of Upc2p and Ecm22p, dual transcription factors regulating ergosterol synthesis in yeasts and other fungi, are regulated. Because genetic redundancy cannot be maintained in the absence of selection, we reasoned that there must be some significant difference in the actions of these two proteins to allow their maintenance to be selected for in the 20 million or so years of *Saccharomyces* divergence.

The carboxyl-terminal regions of Upc2p and Ecm22p share extensive sequence identity. This carboxyl-terminal region is also conserved among the Upc2p and Ecm22p homologs in
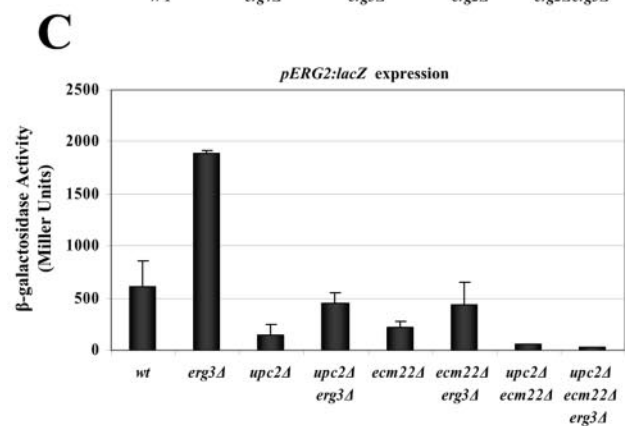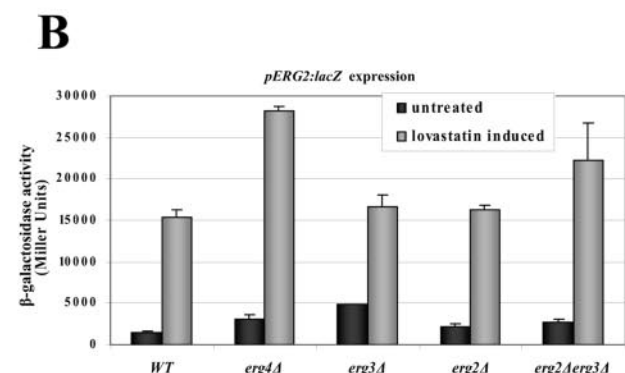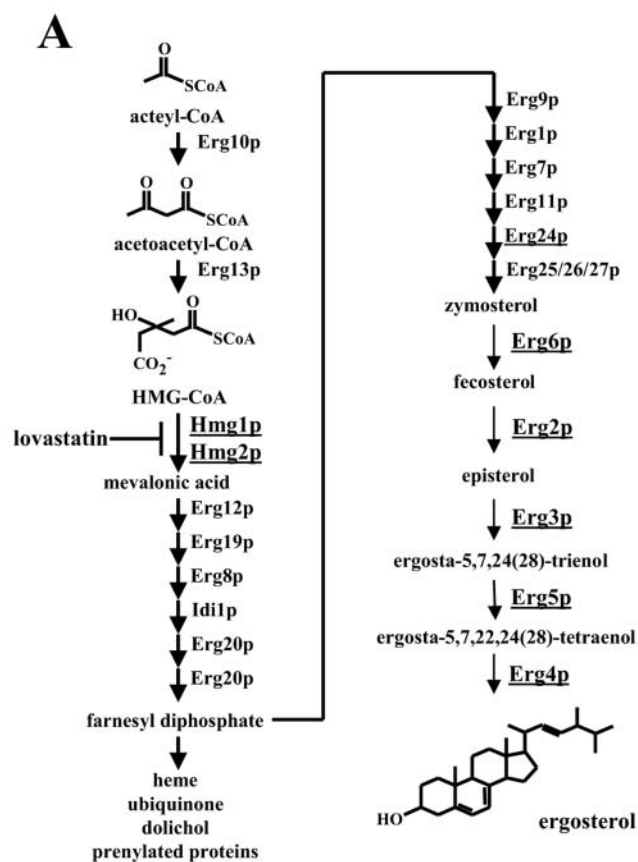
FIG. 5. (A) The ergosterol biosynthetic pathway. The first half of the pathway, shown on the left, leads to the production of farnesyl

sensu stricto strains (7, 22; see also http://www.yeastgenome .org). The more distantly related fungi *Candida albicans* and *Ashbya gossypii* have only one *UPC2/ECM22* ortholog each (9, 28, 35; see also http://genolist.pasteur.fr/CandidaDB /genome.cgi and http://agd.unibas.ch/Ashbya_gossypii/), which also share high levels of identity with Ecm22p and Upc2p in the carboxyl-terminal region. Although this region has no recognizable similarity to any characterized protein domains, the conservation in this region across fungal species and the results presented here indicate that this region may represent a discrete domain.

This study revealed that the carboxyl-terminal regions of both Upc2p and Ecm22p contained transcriptional activation domains. Fusions of this region to the Gal4p DNA-binding domain were able to activate transcription at a Gal4p-regulated promoter (Fig. 1B and C). This activation domain contains a large faction of acidic residues, suggesting it has an acidic activation domain, as do other transcription factors in this family (14, 29). It is unclear why the carboxyl termini of Upc2p and Ecm22p differ in their abilities to activate transcription in uninduced cells (28-fold versus 8-fold) despite such extensive identity (Fig. 1B and C) and similar levels of fusion protein (Fig. 2E). The small differences in sequence may lead to different levels of intrinsic activities, or they may lead to different sensitivities to regulation.

Transcriptional activation capacity was increased in both Upc2p and Ecm22p by the *UPC2-1/ECM22-1* mutation. This mutation did not lead to increased protein levels (Fig. 3D) or greater levels of occupancy at the promoter (Fig. 3E). Indeed, in most cases the mutations decreased the level of the fusion protein, suggesting that the mutant protein either was a more potent activator or had become less sensitive to inhibition. Although this glycine-to-aspartic acid mutation resonated with the ability of mutations that increased acidity to increase transcriptional activation in Gal4p (14), this explanation did not apply to this particular mutation. Any amino acid substitute larger than alanine at this position resulted in the same phenotype, regardless of the charge (Fig. 4). Perhaps any bulky side group at this position can disrupt the binding of some repressive protein or inhibitory small molecule. The reduced level of the mutant protein could reflect stabilization offered by a repressor/inhibitor that was reduced by the *UPC2-1* and *ECM22-1* mutations.

Regulation of Upc2p and Ecm22p through this carboxyl-terminal region was also evident when cells were treated with lovastatin. Full-length Upc2p could induce *ERG2* expression by severalfold after induction by lovastatin, but Upc2p lacking

diphosphate, which, in addition to sterol production, also contributes to the biosynthesis of several nonsterol products, including heme and ubiquinone, whereas the second half of the pathway, shown on the right, is specific to sterol production. Protein products of nonessential genes are underlined. The action of lovastatin is also represented. (B) Deletion of nonessential *ERG* genes led to various degrees of increased *ERG2* expression. A *pERG2::lacZ* reporter (pJR2316) was transformed into the indicated strains. The strains were grown to mid-log phase and assayed for β-galactosidase activity. (C) Increased *ERG2* expression in *erg3Δ* strains required Upc2p or Ecm22p. A *pERG2::lacZ* reporter (pJR2316) was transformed into the indicated strains. The strains were then assayed for β-galactosidase activity.
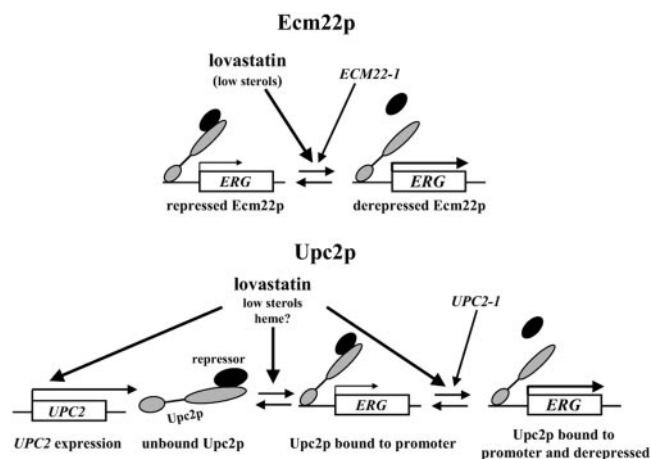
FIG. 6. A model for Upc2p and Ecm22p regulation. Sterol depletion and the *ECM22-1* mutation caused decreased promoter occupancy by Ecm22p but increased transcriptional activation. In contrast, low sterol levels caused increases in Upc2p protein levels, an increase in the probability of each protein to be at a promoter, and greater potency of each bound Upc2p.

this carboxyl-terminal region could not (Fig. 2C). Gal4 protein fusions that contained the carboxyl-terminal region of Upc2p or of Ecm22p could induce reporter expression in response to lovastatin (Fig. 1B and C). The level of fusion proteins did not increase in the presence of lovastatin (Fig. 2E), indicating that the observed increase in transcription resulted from more-active protein rather than from more protein.

These observations led to a model in which the carboxyl-terminal activation domains of Upc2p and Ecm22p are targeted by a repressor protein or small molecule that masks their intrinsic activation capabilities. According to this view, a change at amino acid 888 (for Upc2p) or at amino acid 790 (for Ecm22p) partially disrupts this regulation. In response to sterol depletion, this negative regulation is relieved, leading to greater levels of transcription of Upc2p/Ecm22p-induced genes (Fig. 6). Formally, it is not possible to distinguish whether sterol depletion results in derepression by the loss of a corepressor or direct activation of Ecm22p and Upc2p activity by the accumulation of some coactivator. It seems most likely that the primary signal indicating sterol depletion is derepression rather than the accumulation of a molecule that activates Upc2 and Ecm22. Since lovastatin inhibits HMG-CoA reductase, which catalyzes an early step and the first committed step in sterol biosynthesis (Fig. 5A), the only logical candidate for an activating ligand would be HMG-CoA.

The next step in dissecting how Upc2p and Ecm22p are regulated is finding the entity that presumably represses Upc2p and Ecm22p functions. One intriguing candidate is *MOX2*. The *mox2-2* mutation appears to relieve the negative regulation of Upc2p (2). This mutation increased uninduced, but not induced, levels of *ERG2* expression in a manner similar to the *UPC2-1* mutation (data not shown). To our knowledge, the *MOX2* gene has not yet been cloned but is an attractive candidate for playing a role in this process. *MOT3*, which also represses at least some *ERG* genes (17), is not likely to be the regulator of Upc2p and Ecm22p. There are no known Mot3p binding sites near the Gal4p binding sites at which Gal4-

Ecm22p and Gal4p-Upc2p fusions were activated upon sterol depletion.

Treatment with lovastatin results in high levels of induction of *ERG2* and *ERG3* via Upc2p and Ecm22p (40). Presumably, this induction is a response to lower sterol levels in the cell. When normal levels of specific sterols were perturbed by deleting nonessential *ERG* genes, *ERG2* expression was induced (Fig. 5B), indicating that Upc2p and Ecm22p activities were indeed responsive to sterols. However, the magnitude of the response depended upon which step was blocked. Some of the complexity in the response pattern might be explained by the nonlinear nature of the late ergosterol biosynthetic pathway. When late steps are blocked, other enzymes are still able to modify the sterol intermediate(s), leading to the accumulation of various nonergosterol sterols (16). It is possible that yeasts contain an ergosterol-binding sensor. If so, nonergosterol sterols would also appear to bind, but with various affinities, resulting in various degrees of response. Recently, it has been found that mammalian SCAP (SREBP activation protein), which is part of the cholesterol regulation pathway, and not the transcription factors SREBP1 or -2, binds directly to cholesterol (31). No such sterol-binding protein has been described for *Saccharomyces cerevisiae*, and no orthologous candidate exists. Alternatively, different sterols may lead to different membrane characteristics, which may, in turn, lead to different regulatory responses.

Perhaps the most surprising result from this work was the different ways in which Upc2p and Ecm22p behaved upon lovastatin treatment. As discussed above, the activation domains of both Upc2p and Ecm22p appear to become derepressed in response to lovastatin, increasing the potency of the activator. In addition, lovastatin treatment seems to have two additional effects on Upc2p. Full-length Upc2p levels are elevated under low-sterol conditions (Fig. 2A) consistent with the increase in *UPC2* transcription previously reported (19). Moreover, lovastatin treatment leads to a greater fraction of the *ERG3* promoters being occupied by Upc2p (Fig. 2B). The increase in promoter occupancy by Upc2p can occur independently of increased protein levels, as the fraction of Gal4-DBD-Upc2p fusions bound to *GAL7* promoters increased under inducing conditions, even in the absence of increased protein (Fig. 2D and E). Hence, there is both more Upc2p and a greater probability that each molecule will be bound to a target promoter. As the *UPC2* promoter itself contains an SRE, some or all of its increased level may result from self-activation. Importantly, both Ecm22p levels and the amount of Ecm22p bound to promoters decreased under inducing conditions (Fig. 2A and B). Yet, at least in the absence of Upc2p, Ecm22p is still able to increase the expression levels of its target genes. Thus, taken together, there appear to be three distinct effects that result from lovastatin treatment: the increased synthesis of an activator, an increased probability that activator molecules will be bound to a target, and the increased potency of activators bound to promoters. Upc2p exhibited all three effects, whereas Ecm22p exhibited only one (Fig. 6).

Lovastatin inhibits an early step in the ergosterol biosynthetic pathway (Fig. 5A). Because farnesyl diphosphate, an intermediate in the pathway, also contributes to the biosynthesis of nonsterol products, including heme, lovastatin treatment would lead to both sterol depletion and the depletion of these

nonsterol products. It is therefore possible that the induction of Upc2p and Ecm22p by lovastatin is not entirely the result of sterol depletion but is due to a combination of both sterol depletion and the depletion of another nonsterol product. Given Upc2p's role in the activation of anaerobically expressed genes (1, 2, 25, 38), one tantalizing possibility is that Upc2p responds both to low sterol levels and to the depletion of heme. Perhaps sterol depletion results in the derepression of the activation domains of both Upc2p and Ecm22p, whereas heme depletion triggers an increase in promoter occupancy by Upc2p, perhaps by both regulating the synthesis of Upc2p and Ecm22p and increasing the probability, on a per-molecule basis, that Upc2p will be bound to the promoter. There are several ways in which increased occupancy could be achieved independently of increasing the level of a transcription factor. Perhaps a portion of the Upc2p protein interferes with the DNA-binding domain in a nonspecific manner until it is depleted of some pathway intermediate, in a manner somewhat analogous to the way that Hsp90 binds to unliganded glucocorticoid receptors (30). Alternatively, inducing conditions may cause a release of Upc2p from sequestration, resulting in greater levels of promoter occupancy.

ACKNOWLEDGMENTS

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