MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5427–5432 Vol. 16, No. 10
0270-7306/96/$04.00+0

Transcriptional Regulation by Ergosterol in the Yeast *Saccharomyces cerevisiae*

STEVEN J. SMITH,† JAMES H. CROWLEY, AND LEO W. PARKS*

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615

Received 9 February 1996/Accepted 9 July 1996

Sterol biosynthesis in the yeast *Saccharomyces cerevisiae* is an energy-expensive, aerobic process, requiring heme and molecular oxygen. Heme, also synthesized exclusively during aerobic growth, not only acts as an enzymatic cofactor but also is directly and indirectly responsible for the transcriptional control of several yeast genes. Because of their biosynthetic similarities, we hypothesized that ergosterol, like heme, may have a regulatory function. Sterols are known to play a structural role in membrane integrity, but regulatory roles have not been characterized. To test possible regulatory roles of sterol, the promoter for the *ERG3* gene, encoding the sterol C-5 desaturase, was fused to the bacterial *lacZ* reporter gene. This construct was placed in strains making aberrant sterols, and the effect of altered sterol composition on gene expression was monitored by β-galactosidase activity. The absence of ergosterol resulted in a 35-fold increase in the expression of *ERG3* as measured by β-galactosidase activity. The level of *ERG3* mRNA was increased as much as ninefold in *erg* mutant strains or wild-type strains inhibited in ergosterol biosynthesis by antifungal agents. The observed regulatory effects of ergosterol on *ERG3* are specific for ergosterol, as several ergosterol derivatives failed to elicit the same controlling effect. These results demonstrate for the first time that ergosterol exerts a regulatory effect on gene transcription in *S. cerevisiae*.

Ergosterol (Fig. 1), the primary membrane sterol found in yeast cells, serves a structural role in cellular membranes similar to that of cholesterol (Fig. 1) in mammalian systems. While sterols aid in the maintenance of the membrane environment, other roles have been observed for these lipid molecules. In mammalian systems, much work has focused on sterol-mediated gene regulation and sterol transport (7, 17, 36, 37). Examples of sterol-dependent gene regulation have been identified in mammalian systems, but the study of sterol-dependent genetic control in the yeast *Saccharomyces cerevisiae* has been less successful. Work from our laboratory demonstrated that ergosterol and other sterols having both a C-22═23 unsaturation and a C-24 methylation (Fig. 1) had the ability to regulate endogenous sterol biosynthesis, presumably by a feedback mechanism (11). Therefore, cholesterol and its derivatives would not be expected to inhibit sterol biosynthesis in yeast cells, as these sterols cannot be converted to ergosterol or be modified to contain a side chain C-22═23 unsaturation or C-24 methylation by the yeast enzymes (11). The enzymes responsible for these reactions, Erg5p and Erg6p, do not recognize cholesterol or cholesterol derivatives as substrates. Erg5p then must require C-24-methylated and/or C-24═25-unsaturated sterol, while Erg6p must require C-24═25 unsaturated sterol.

Structural features of the sterol molecule required for growth of a sterol-auxotrophic organism and involved in the regulation of sterol esterification have been reported elsewhere (22, 24). Yeast strains containing an *erg3*::*LEU2* allele make a sterol (ergosta-7,22-dienol) with an ergosterol-like side chain but lacking the C-5═6 unsaturation of ergosterol. We predicted that sterol biosynthesis might be regulated in a similar manner by both ergosta-7,22-dienol and ergosterol (Fig. 1), since structural features of the sterol side chain afford the greatest control of yeast sterol biosynthesis (11). Yeast cells not only differentiate between structurally dissimilar sterol molecules in the regulation of ergosterol biosynthesis (12) but also preferentially sequester nonergosterol sterols for esterification to fatty acids (21). In addition, when yeast cells are inhibited in sterol biosynthesis, they preferentially target ergosterol to the mitochondria despite producing a mixture of ergosterol and other sterols (30). These esterified sterols are then stored in lipid droplets until required for further membrane biogenesis. Previous work, then, has shown that in yeast cells, sterol has control over its own biosynthesis and intracellular localization.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the major regulatory point in mammalian sterol biosynthesis, is transcriptionally and posttranscriptionally controlled by intracellular levels of cholesterol (17). Control of sterol biosynthesis in yeast cells by the regulation of HMG-CoA reductase has also been observed (12, 14, 23). The Hmg1p-mediated sterol synthesis (HMG-CoA reductase in *S. cerevisiae*) is regulated by the end product, ergosterol (12), and by intracellular heme (41). In this work, we examine the effect of ergosterol on the transcriptional expression of a gene in the ergosterol biosynthetic pathway in *S. cerevisiae*.

Transcriptional control in *S. cerevisiae* has been studied by using gene promoters fused to the bacterial *lacZ* reporter gene, allowing a chromagenic measure of transcriptional levels (27, 33). In this study, the transcriptional regulation of the *ERG3* gene, encoding the C-5 sterol desaturase, was studied in a variety of ergosterol mutant strains that produce nonergosterol molecules as their principal sterol. In addition, inhibitors of sterol biosynthesis were used to perturb the normal synthesis of ergosterol as another means to determine how sterol modification may affect expression of the *ERG3* gene. We demon-

* Corresponding author. Mailing address: Department of Microbiology, Box 7615, North Carolina State University, Raleigh, NC 27695-7615. Phone: (919) 515-7860. Fax: (919) 515-7867. Electronic mail address: parks@mbio.ncsu.edu.

† Present address: Gladstone Institute of Cardiovascular Disease, San Francisco, CA 94141.

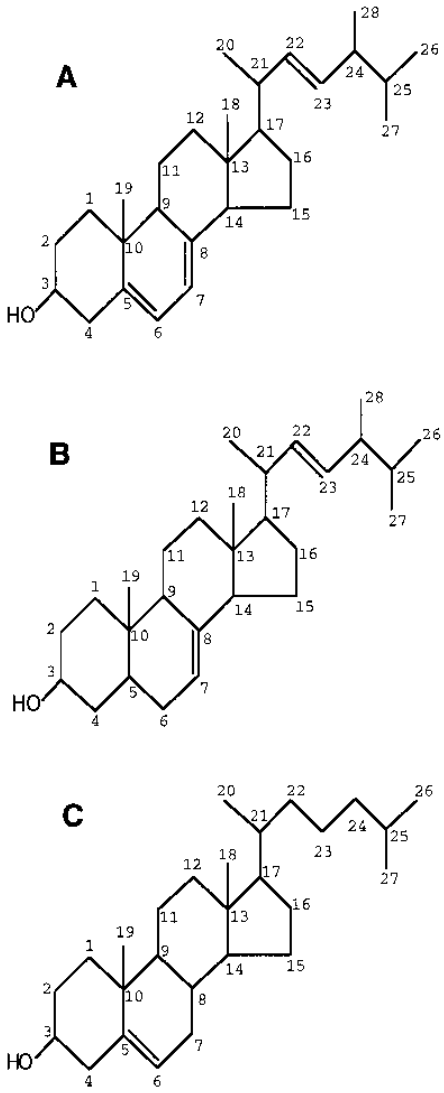

FIG. 1. Sterols relevant to this work. (A) Ergosterol; (B) ergosta-7,22-dienol; (C) cholesterol.

strate that ergosterol can regulate gene expression at the level of transcription in *S. cerevisiae*.

MATERIALS AND METHODS

Materials. The Difco products yeast extract, Bacto Agar, Tryptone, dextrose, and yeast nitrogen base (YNB; without amino acids) were obtained from Fisher Scientific. Extraction solvents, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), isopropyl-thio-β-D-galactoside (IPTG), and inorganic chemicals were also from Fisher Scientific. Amino acids, nucleic acids, *o*-nitrophenyl-β-D-galactopyranoside, 5-fluoro-orotic acid, 2-mercaptoethanol, tergitol, ketoconazole, and polyethylene glycol were obtained from Sigma Chemical Co. Restriction DNA endonucleases were purchased from Bethesda Research Laboratories, Inc., and New England Biolabs. Nitrocellulose filters were obtained from Gelman Sciences (Baxter Scientific). *Pfu* DNA polymerase and the Sequenase 2.0 kit were from Stratagene. The radiolabeled nucleotide, [α-^{32}P]dCTP, was obtained by Amersham Life Science Inc. for use in making radiolabeled DNA probes. Lovastatin was a gift from A. Alberts (Merck Research Laboratories, Rahway, N.J.), and fenpropimorph was a gift from BASF (Ludwigshafen, Germany).

Media and culture conditions. Yeast extract-peptone-dextrose, selective defined medium with YNB, prespore, and sporulation media were made as described previously (33). YNB was enriched with necessary amino acids and nucleic acids at 40 μg/ml for strain and plasmid selection. X-Gal plates for *S. cerevisiae* were made by the recipe of Ushinsky and Keng (42).

LB medium, used for culturing *Escherichia coli*, contained ampicillin (50 μg/ml) when appropriate for plasmid selection (35). Bacto Agar was used at a concentration of 2% for all solid media. *E. coli* and *S. cerevisiae* were grown at 37 and 28°C, respectively.

Strains and strain construction. All *S. cerevisiae* strains used in these experiments and their origins are listed in Table 1. Genetic crosses were performed by conventional techniques, and tetrad segregants were obtained by ascus dissection (33). *E. coli* DH5αF′ was used for plasmid amplification (18).

The congenic yeast strains 463-1C and 463-1D were the progenitors to most strains used in this work. SY13 was constructed by crossing SY12A with 463-1D. The strain 463-1C was transformed with a 1.6-kb restriction fragment containing the *LEU2* gene and then backcrossed to 463-1D to obtain the tetrad isolate SY114, which served as a Leu$^+$ control strain in these studies. As research has demonstrated the importance of using congenic yeast strains when making direct comparisons of β-galactosidase activity data (9), efforts were taken through backcrossing to be certain that strains used in this work differed only in the gene(s) of interest. Strain LPY11 was isolated from an initial cross of BKY48-5C with 463-1C followed by backcrosses to 463-1C. Strain LPY30, containing the *erg5::LEU2* null allele, was isolated by the micro-homology-mediated PCR targeting technique of Manivasakam et al. (25). A 42-bp homologous oligonucleotide was made on the basis of the −207 to −165 region upstream of the *ERG5* gene (38). An additional 46-bp primer was made to the +892 to +938 region just downstream of the stop codon for *ERG5*. Each primer contained 20 bp of the corresponding ends of the *LEU2* gene, and these two primers were used in a PCR, with the *LEU2* gene on YEp351 (19) serving as the template. The resulting PCR product was then used in a one-step gene disruption (34). The sterol profile of strain LPY30 was consistent with a disrupted sterol C-22 desaturase, and a Southern blot confirmed the disruption of the *ERG5* gene (data not shown). Strain SY20 was originally isolated in a mutagenesis screen for resistance to filipin (31).

Plasmids and plasmid construction. The vectors used in this work for subcloning and promoter expression experiments are listed in Table 1. Structures of all subclones and promoter-*lacZ* fusions constructed for these experiments are presented in Fig. 2. In all promoter fusions constructed, the ATG initiation codon was the only region of the coding sequence present.

To make plasmids containing the *ERG3* promoter, oligonucleotides homologous to a region 900 bp 5′ of the *ERG3* translational start site, and also an oligonucleotide homologous to the *ERG3* translational start site region, were obtained. These oligonucleotides and plasmid pSN302 (40), having the entire *ERG3* gene and promoter, were used in PCR to obtain the *ERG3* promoter fragment. The PCR product was cut with *Eco*RI and *Bam*HI and ligated into YIp353 (27), forming plasmid pSN301. Sequencing was performed to be certain

TABLE 1. Yeast strains and plasmids used in this work

Strain or plasmid	Genotype or description	Source or reference
Strains		
LPY11	**a** *erg6::LEU2 leu2 ura3-52 his3*	This laboratory
BKY48-5C	α *erg6::LEU2 leu2 ura3-52*	15
SCV1	**a** *erg24::LEU2 leu2 ura3-52 his4*	This laboratory
463-1C	**a** *leu2 ura3-52 his3 trp1Δ1*	K.T.[a]
463-1D	α *leu2 ura3-52 his3 trp1Δ1*	K.T.
SY12A	**a** *erg3::LEU2 leu2 ura3-52 his4*	40
SY13	**a** *erg3::LEU2 ura3-52 his3 trp1Δ1*; congenic to 463-1C	This work
SY20	**a** *erg5-1 leu2 ura3-52 his 3 trp1Δ1*; congenic to 463-1C	This laboratory
LPY30	**a** *erg5::LEU2 ura3-52 his3*; congenic to 463-1C	This laboratory
SY114	**a** *LEU2 ura3-52 his 3 trp1Δ1*; congenic to 462-1C	This work
Plasmids		
pSN302	pUC19 with *ERG3*	40
pSN208	YEp352 with *ERG3*	40
pSN206	pSN208 with *HIS3*	This work
pSN301	*ERG3-lacZ* fusion, 890-bp *Eco*RI-*Bam*HI PCR promoter fragment in YIp353	This work
pSN303	*ERG3-lacZ* fusion, 500-bp *Eco*RI-*Bam*HI PCR promoter truncated fragment in pSN301	This work
pSN305	*ERG3-lacZ* fusion in YIp353	This work
YIp353		27

[a] K.T., Kelly Tatchell, Louisiana State University Medical School, Shreveport.

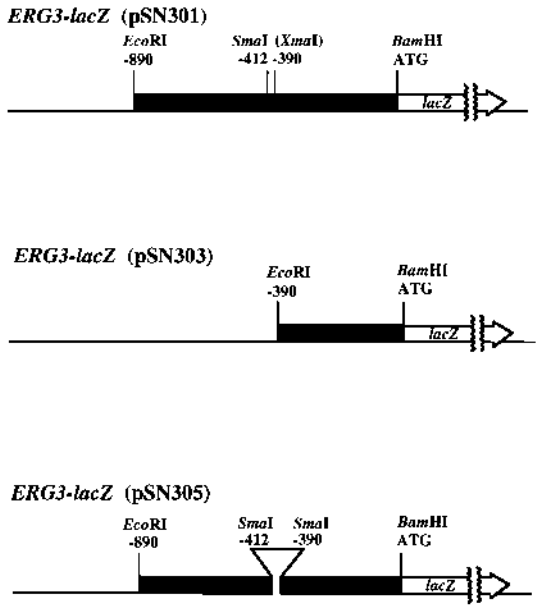

FIG. 2. Plasmid constructions used in this work. All promoters were fused to the bacterial *lacZ* reporter gene of a YIp vector. The plasmids were linearized and integrated in the genome at *ura3* as described in Materials and Methods.

of the PCR reaction fidelity, and the promoter sequence was found to be identical to previously published sequences (3). To produce plasmid pSN303, pSN301 was digested with *Eco*RI and *Xma*I, and the ends were filled in by using T4 DNA polymerase and deoxynucleoside triphosphates and then religated. pSN305 was formed by digesting pSN301 with *Sma*I, removing the intervening 22 bp, and then religating the fragments.

Plasmid pSN206 was constructed by using *Bam*HI to linearize the episomal (2 μm) plasmid pSN208 (40), containing the entire *ERG3* promoter and coding sequence. The *HIS3* gene, isolated on a 1.8-kb *Bam*HI restriction fragment, was then ligated into linearized pSN208, resulting in the autonomously replicating plasmid pSN206.

Transformations. Yeast transformations were performed by either a lithium acetate (16) or electroporation (6) procedure. Following transformation, cells were plated onto defined medium lacking leucine, histidine, or uracil. *E. coli* was transformed by a $CaCl_2$ procedure (35).

Recombinant DNA techniques. Except where indicated, all DNA manipulations, including plasmid isolations, restriction enzyme digestions, ligations, sequencing, PCR, and agarose gel electrophoresis, were done according to previously described protocols (35). Restriction fragments were retrieved from agarose gels by using a GeneClean kit as instructed by the manufacturer (Bio 101, Vista, Calif.). Oligonucleotides for PCR primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).

Preparation and analysis of sterols. Sterols were extracted and analyzed by using established procedures (22, 28, 39). Following backcrosses, sterol analyses were performed on cultures from individual tetrad isolates to verify the genotype by determining the sterols produced.

β-Galactosidase analyses. Yeast strains containing the *ERG3-lacZ* fusion were isolated by first fusing the promoter and ATG start codon of the *ERG3* gene to a plasmid-borne promoterless *lacZ* gene. The promoter construct, pSN301, and its derivatives (Fig. 2) were used to examine the expression of the promoters in various genetic backgrounds. All plasmids containing promoter-*lacZ* fusions were linearized within the *URA3* gene by using either *Nco*I or *Stu*I and integrated into the yeast chromosome at the *ura3* locus. The chromosomal integrations of these plasmids eliminated the inherent variability of centromeric or episomal plasmid copy number during β-galactosidase assays. After strains were transformed with the appropriate integrating plasmid, transformants were selected and backcrossed to the parental strain at least twice, and two to three tetrad isolates with the desired genotype were used for each assay.

Quantitative β-galactosidase broth assays were performed according to established procedures (26, 33). For performing assays, 5.0-ml cultures were grown in YNB broth containing nutritional supplements necessary for growth. The cultures were grown to a cell density of 1×10^7 to 2×10^7 cells per ml, as measured by a direct count using a hemocytometer. Cells were harvested, washed in breaking buffer, and frozen until the assays were performed. Prior to performance of the assays, the cells were broken in a MiniBeadbeater (Biospec Products, Bartlesville, Okla.), and the supernatant was removed for use in the assays. The tetrad isolates analyzed were grown in triplicate under identical conditions, and three assays were performed on each. The protein concentration of each cellular extract was determined by the use of the Pierce (Rockford, Ill.) bicinchoninic acid protein assay kit. The equation used to calculate β-galactosidase activity was as previously reported (33). Results from the assays fell within 20% of the mean. To assess the statistical significance of the β-galactosidase data, all results have been reported as the average activity plus or minus the 95% confidence interval.

mRNA analysis. Total RNA was extracted from yeast strains by the hot phenol extraction method described by Collart and Oliviero (13) except that a sodium acetate buffer (50 mM sodium acetate, 10 mM EDTA adjusted to pH 5.0 with acetic acid) (20) with 0.5% sodium dodecyl sulfate (SDS) was used instead of the TES buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS; adjusted to pH 7.5). Cultures were pregrown overnight in SC medium at 30°C and inoculated to fresh medium to an optical density at 600 nm of 0.5. Cultures were then harvested during the mid-logarithmic stage of growth (optical density at 600 nm of 1.0) prior to the hot phenol extraction. For Northern (RNA) blot analysis, total RNA was separated by agarose-formaldehyde gel electrophoresis (8). Slot blot analysis was carried out as described by Sambrook et al. (35), using a Bio-Dot SF apparatus (Bio-Rad, Hercules, Calif.). All blotting was done with GeneScreen nylon membranes (DuPont-NEN Research Products, Boston, Mass.), and all hybridizations were performed by using the 50% formamide prehybridization and hybridization solutions and protocol described by Sambrook et al. (35). Blots were probed first with a radiolabeled *ERG3* DNA probe; prior to probing with *ACT1*, the blots were stripped according to the membrane manufacturer's directions. Radioactive probes to the entire coding regions of the *ERG3* and *ACT1* genes were generated by using the Boehringer Mannheim (Indianapolis, Ind.) random primed DNA labeling kit. Quantitation of slot blots was done with a Molecular Dynamics (Sunnyvale, Calif.) PhosphorImager.

Where inhibitors of ergosterol biosynthesis were used to examine mRNA levels, 10-ml cultures of SY114 were grown overnight in synthetic complete medium and inoculated as described above with the addition of the indicated subinhibitory concentrations of either ketoconazole, fenpropimorph, or lovastatin. Cultures were grown to an optical density at 600 of 1.0 prior to total RNA extraction, and the incubation time required to reach this optical density was not significantly different among untreated or treated cultures.

RESULTS

To examine the effect of sterol on *ERG3* expression, the *ERG3* promoter was fused to the bacterial *lacZ* reporter gene. Plasmids bearing the promoter-*lacZ* fusions were integrated into the yeast genome at the chromosomal *ura3* locus. Plasmid integration allows the genetic construction to be stably maintained with a constant copy number. For the examination of transcriptional regulation of the *ERG3* gene, we used plasmid pSN301, which contains 890 bp directly upstream of *ERG3* and the *ERG3* ATG start codon fused to the *lacZ* reporter gene (Fig. 2). Tetrad isolates of SY13 and SY114 containing the integrated pSN301 were examined for β-galactosidase activity. The activity in the *erg3*::*LEU2* strain was 35-fold higher than that in the *ERG3* strain (Table 2), indicating that this defect in sterol biosynthesis alters the transcription of the *ERG3* gene. Cultures of SY13 with an integrated pSN301 were grown in media with and without exogenously supplied ergosterol to determine if sterol in the growth medium could reverse the increase in transcriptional levels. As anticipated, there were no apparent differences in the cultures (data not shown), since yeast cells are unable to acquire significant amounts of sterol from the growth medium under aerobic conditions (2).

We sought to demonstrate that the difference in expression of the *ERG3* promoter between strains SY13 and SY114 was due only to the presence of the *erg3*::*LEU2* allele in the otherwise congenic strains. SY13, containing the integrated pSN301(*ERG3-lacZ*), was transformed with the episomal plasmid pSN206, which contains the wild-type *ERG3* allele. The functional *ERG3* allele allowed production of ergosterol and caused a reduction of β-galactosidase activity to wild-type levels (6.0 Miller units). This observed β-galactosidase activity was actually less than that seen in SY114, which is likely a reflection of the high copy number of this plasmid, resulting in multiple copies of the *ERG3* gene in the cell.

In an attempt to localize the region of the *ERG3* promoter responsible for the ergosterol-dependent regulation, two dele-

TABLE 2. Promoter modifications and the expression of *ERG3*

Yeast strain	Relevant genotype	Promoter fusion	Plasmid integrant	Sterol produced[a]	Enzyme activity[b] (mean ± SD)
SY114	*ERG3*	*ERG3-lacZ*	pSN301	$\Delta^{5,7,22}$	40 ± 7.7
SY13	*erg3*::*LEU2*	*ERG3-lacZ*	pSN301	$\Delta^{7,22}$	1,860 ± 232
SY114	*ERG3*	*ERG3*-Δ(−890 to −390)-*lacZ*	pSN303	$\Delta^{5,7,22}$	1,220 ± 98.1
SY114	*ERG3*	*ERG3*-Δ(−412 to −390)-*lacZ*	pSN305	$\Delta^{5,7,22}$	554 ± 44.9
SY13	*erg3*::*LEU2*	*ERG3*-Δ(−890 to −390)-*lacZ*	pSN303	$\Delta^{7,22}$	3,317 ± 715
SY13	*erg3*::*LEU2*	*ERG3*-Δ(−412 to −390)-*lacZ*	pSN305	$\Delta^{7,22}$	1,030 ± 151

[a] $\Delta^{5,7,22}$, ergosta-5,7,22-trienol (ergosterol); $\Delta^{7,22}$, ergosta-7,22-dienol.
[b] β-Galactosidase enzyme activity expressed in Miller units (26, 33).

tions of the promoter were constructed. Initially, plasmid pSN301 was cut with *Eco*RI and *Xma*I, blunt ended, and religated, removing 500 bp of the *ERG3* promoter. The resulting plasmid, pSN303 (Fig. 2), was linearized within the *URA3* gene with *Nco*I, and SY13 and SY114 were transformed. β-Galactosidase assays were performed on *ERG3*- and *erg3*::*LEU2*-containing strains with this integrated plasmid (Table 2). A greatly elevated level of expression from the *ERG3* promoter was seen in each strain, suggesting that a regulatory region had been deleted in the construction of pSN303. We then removed a smaller region of the *ERG3* promoter, bordered by a pair of *Sma*I (*Xma*I) sites 390 and 412 bp upstream of the *ERG3* coding region. Upon cleavage and religation, the resulting plasmid, pSN305 (Fig. 2), differs from pSN301 only in the absence of the 22-bp segment between the *Sma*I sites. pSN305 was linearized within the *URA3* gene with *Nco*I and transformed into SY13 and SY114. Increased transcriptional levels were seen in β-galactosidase assays (Table 2), which indicates that the excised region may be involved in sterol-dependent regulation.

Because a difference in transcriptional levels from the *ERG3* promoter was observed when the sterol composition was changed, we examined its expression from the integrated pSN301 in strains containing mutations in other sterol biosynthetic genes. Strains with mutations in *ERG5* (sterol C-22 desaturase), *ERG6* (sterol C-24 methyltransferase), and *ERG24* (sterol C-14 reductase) were used. These mutants allowed the ability of particular sterol structural features to regulate *ERG3* transcriptional levels to be examined. In all of the mutant strains (Table 3), the β-galactosidase levels were approximately 35-fold higher than those in an ergosterol-producing wild-type yeast strain. Any sterol produced other than ergosterol resulted in an increase in *ERG3* transcriptional levels. Northern analyses were performed to verify the apparent loss of regulatory control of *ERG3* expression in mutant strains producing nonergosterol sterols. Northern analysis confirmed expression of an mRNA transcript approximately 1.2 kb in length that hybridized with the *ERG3* DNA probe (Fig. 3A). Quantitation of *ERG3* mRNA abundance by slot blot analysis revealed that *ERG3* expression was ninefold higher in strains containing a null mutation in either the *ERG6* or *ERG24* gene (Fig. 3B). Strains containing a null mutation in the *ERG5* gene, however, showed only a fourfold increase in expression of the *ERG3* gene.

Ergosterol synthesis in yeast cells can be altered by genetic

TABLE 3. Effect of sterol modification on expression of *ERG3* in strains carrying pSN301 (*ERG3-lacZ*)

Yeast strain	Relevant genotype	Sterol produced[a]	Enzyme activity[b] (mean ± SD)
SY114	*ERG3 ERG5 ERG6 ERG24*	$\Delta^{5,7,22}$	40 ± 7.7
SY13	*erg3*::*LEU2 ERG5 ERG6 ERG24*	$\Delta^{7,22}$	1,860 ± 232
SCV1	*ERG3 ERG5 ERG6 erg24*::*LEU2*	$\Delta^{8,14}$	1,826 ± 239
LPY11	*ERG3 ERG5 erg6*::*LEU2 ERG24*	$\Delta^{5,7,22,24}$	1,799 ± 240
SY20	*ERG3 erg5-1 ERG6 ERG24*	$\Delta^{5,7}$	1,948 ± 224

[a] $\Delta^{5,7,22}$, ergosta-5,7,22-trienol (ergosterol); $\Delta^{7,22}$, ergosta-7,22-dienol; $\Delta^{8,14}$, ergosta-8,14-dienol; $\Delta^{5,7,22,24}$, cholesta-5,7,22,24(25)-tetraenol; $\Delta^{5,7}$, ergosta-5,7-dienol.
[b] β-Galactosidase enzyme activity expressed in Miller units (26, 33).

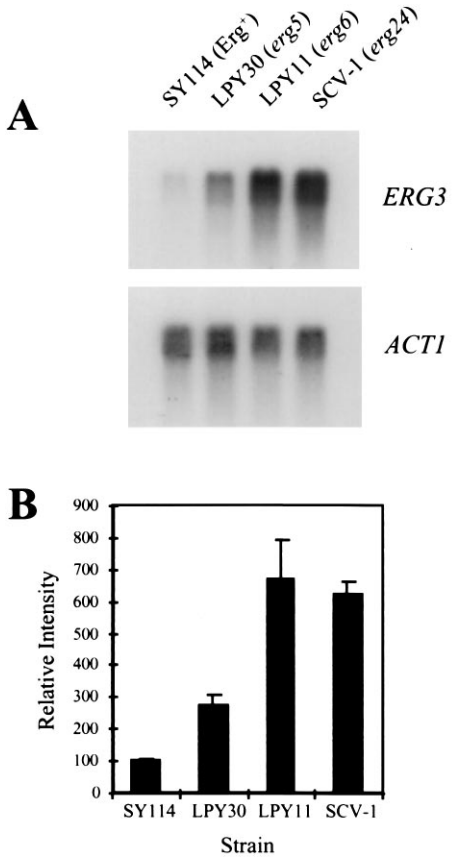

FIG. 3. Effects of mutations in *ERG5*, *ERG6*, and *ERG24* on levels of *ERG3* mRNA. Ten micrograms of total mRNA from each strain was loaded into an agarose-formaldehyde gel for Northern analysis (A). For quantitation of *ERG3* mRNA levels, slot blot analysis was used (B), with 20 μg of total RNA loaded per slot. Error bars represent the standard deviation from three independent samples of each strain. For both analyses, *ACT1* mRNA levels were determined as a control for RNA loading errors. Autoradiograms of the Northern blots were scanned by using a Relisys Image Scanner and Adobe Photoshop version 2.5 for Macintosh.

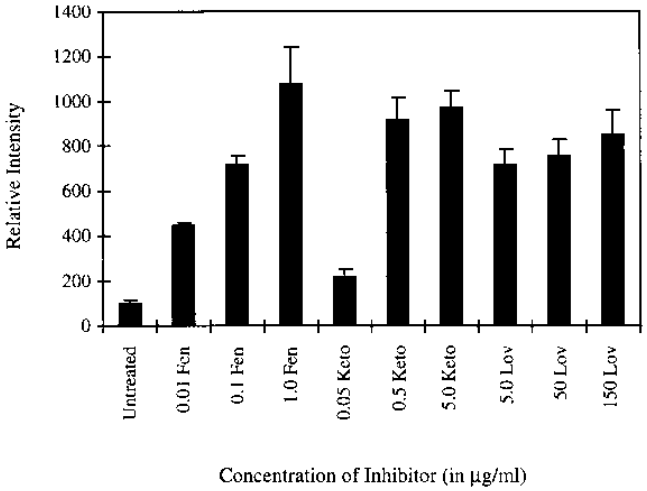

FIG. 4. Effects of sterol biosynthesis inhibitors on levels of *ERG3* mRNA. Twenty micrograms of RNA from each sample was loaded into each well for slot blot analysis. Blots were first probed with an *ERG3* DNA probe, and quantitation of the radioactive signal was carried out as described in Materials and Methods. Error bars represent the standard deviation from three independent samples for each condition. Blots were stripped and reprobed with an *ACT1* DNA probe as a control, and the standard deviation among all samples was within 10% of the mean, with no individual sample exceeding 2 standard deviations from the mean. The inhibitors used were fenpropimorph (Fen), ketoconazole (Keto), and lovastatin (Lov).

mutation, as described above, or by the use of sterol biosynthesis inhibitors. There are a variety of antifungal agents that block ergosterol biosynthesis in yeasts and other fungi, and these inhibitors cause the fungal cells to produce sterols other than ergosterol (10). Ketoconazole inhibits the sterol C-14 demethylase, resulting in the synthesis of C-14 methyl sterols (43). Fenpropimorph, a member of the morpholine antifungal family, primarily inhibits the sterol C-14 reductase, resulting in the accumulation of ergosta-8,14-dienol (ignosterol) in treated cells (4, 5). Lovastatin (mevinolin) blocks HMG-CoA reductase (1), which is much earlier in the ergosterol biosynthetic pathway, and treated cells are simply deprived of sterol at inhibitory concentrations (23). Upon treatment of a wild-type strain (SY114) with increasing amounts of these inhibitors, an increase in *ERG3* mRNA levels was evident (Fig. 4), with the increase in *ERG3* mRNA levels approaching the level of expression seen in strains producing aberrant sterol through genetic manipulation. Thus, it appears that any sterol produced, other than ergosterol, results in an increase in *ERG3* expression at the level of transcription.

DISCUSSION

Although ergosterol was discovered in yeast cells over a century ago, its principal role has been recognized only as a major membrane structural component. Ergosterol has been reported to account for up to 10% of the total dry weight of some fungi, but 1 to 2% is typical (29). Ergosterol has been identified as a part of yeast membrane structures, although other roles have been anticipated. Experiments by Rodriguez et al. (32) established that there are at least four distinct roles for sterols in yeast cells, each differing in the qualitative and quantitative features of the sterol that satisfies a given role. The C-22═23 unsaturation and the C-24 methylation have been shown to exert an overall regulatory effect on the synthesis of ergosterol (11). In addition, virtually all mutants in sterol biosynthesis accumulate excess sterol and store it as steryl ester (21). These observations led to the idea that ergosterol is able to exert some regulatory effects, although the regulatory examples are poorly defined. The present experiments were undertaken to explore possible specific regulatory effects of ergosterol.

The structural gene involved in ergosterol biosynthesis, *ERG3*, was selected as a possible target for regulation by sterol. The promoter for *ERG3* was fused in frame to the bacterial reporter gene, *lacZ*, and introduced on integrative plasmids. Plasmid integration allowed each fusion to be stably maintained, eliminating a possible problem of copy number differences when autonomously replicating plasmids were used. Following integration of the plasmid, several transformants were selected, analyzed for β-galactosidase activity, and then backcrossed to the parental strain. The congenic strains obtained were used to study the effects of sterol alterations. Sterol composition was altered by introducing mutations in the sterol biosynthetic pathway or through inhibition of ergosterol biosynthesis by antifungal agents. Each sterol mutant was verified for altered sterol content before the strain was used in the experiments.

Ergosterol-mediated regulation of transcription on the promoter for the structural gene *ERG3* was quite dramatic. This gene encodes the sterol C-5 desaturase, which catalyzes the final modification of the sterol nucleus in the synthesis of ergosterol. The absence of ergosterol caused a 35-fold elevation in the level of transcription of the *ERG3* gene, as determined by β-galactosidase activity from expression of the *ERG3-lacZ* fusion. Modifications of the promoter structure resulted in substantial alterations in the sterol regulatory effects on the transcription of *ERG3*, indicating that regions 390 bp upstream of the *ERG3* coding region contain regulatory sequences for the control of *ERG3* expression.

The sterol modifications associated with the mutations in the *ERG3*, *ERG5*, *ERG6*, and *ERG24* mutant genes caused a similar increase in expression of *ERG3*. These results suggest that changes in structural features of ergosterol, such as the C-5═6 unsaturation, the C-22═23 unsaturation, the C-24 methylation, and the reduction of the double bond at C-14, oppose the ability of the resulting sterol molecules to perform feedback regulation that is normally mediated by ergosterol. Interestingly, we have found consistently that mutations in the *ERG5* gene, resulting in the production of ergosta-5,7-dienol, cause a much smaller increase in *ERG3* mRNA levels than in strains with mutations in other *ERG* genes or in cells treated with sterol biosynthetic inhibitors. Strains with a mutation in *ERG5* produce no ergosterol, and so we are left with the possibility that the structural features of ergosta-5,7-dienol resemble those of ergosterol to the point that the $\Delta^{5,7}$ sterol may partially satisfy the ergosterol-specific regulatory function. This partial regulation was seen only in Northern analyses of mRNA levels, as *ERG3* expression measured by analyses of *ERG3-lacZ* fusions indicated a more complete loss of regulation. This would appear to reveal a limitation in the use of *ERG3-lacZ* fusions to determine most accurately the effects of the physiological state of the cell on *ERG3* expression. Nevertheless, alteration of sterol content via sterol biosynthesis inhibitors corroborates the notion that any perturbation in the normal ergosterol content will lead to the increased expression of the *ERG3* gene. The transcriptional regulation by ergosterol described here opens the door to further work examining the regulatory relationship of ergosterol with that of heme and oxygen.

It is interesting that although *ERG3* transcription is regulated by ergosterol, the altered sterols produced by strains deficient in sterol C-5 desaturase, C-22 desaturase, C-24 meth-

yltransferase, or C-14 reductase are unable to perform this function. This finding suggests that this regulation is very specific for the ergosterol molecule, as structurally related sterols and precursors are not sufficient. This specificity implies that ergosterol performs an important function that cannot be performed by similar sterols. Understanding ergosterol regulation of gene expression could help define the functions of this very energy expensive molecule.

ACKNOWLEDGMENTS

This research was supported in part by a grant from the National Institutes of Health (DK37222) and the North Carolina Agricultural Research Service.

We thank Kelly Tatchell for providing yeast strains used in this study and Alan Myers and Kelly Tatchell for providing plasmids used in this work. In addition, the we thank John McMillan for performing DNA sequencing and Frank Leak and Lisa Palermo for construction of strains used in this study.

REFERENCES

1. **Alberts, A. W., J. Chen, G. Kuron, V. Hunt, C. Huff, C. Hoffman, J. Rothrock, M. Lopez, H. Joshua, A. Patchett, R. Monaghan, S. Currie, E. Stapley, G. Albers-Schonberg, J. Hirchfield, K. Hoogsteen, J. Liesch, and J. Springer.** 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl CoA reductase and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. USA **77:**3957–3961.
2. **Andreasen, A. A., and T. J. B. Stier.** 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J. Cell. Comp. Physiol. **41:**23–36.
3. **Arthington, B. A., L. G. Bennett, P. L. Skatrud, C. J. Guynn, T. J. Barbuch, C. E. Ubright, and M. Bard.** 1991. Cloning, disruption and sequence of the gene encoding yeast C-5 sterol desaturase. Gene **102:**39–44.
4. **Baloch, R. I., and E. I. Mercer.** 1987. Inhibition of sterol 8-7 isomerase and 14 reductase by fenpropimorph, tridemorph, and fenpropidin in cell-free enzyme systems from *Saccharomyces cerevisiae*. Phytochemistry **26:**663–668.
5. **Baloch, R. I., E. I. Mercer, T. E. Wiggins, and B. C. Baldwin.** 1984. Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph, and fenpropidin. Phytochemistry **23:**2219–2226.
6. **Becker, D. M., and L. Guarente.** 1991. High-efficiency transformation of yeast by electroporation. Methods Enzymol. **194:**182–186.
7. **Brown, M. S., and J. Goldstein.** 1986. A receptor-mediated pathway for cholesterol homeostasis. Science **232:**34–47.
8. **Brown, T.** 1993. Analysis of RNA by Northern and slot blot hybridization. Curr. Protoc. Mol. Biol. **1:**4.9.1–4.9.14.
9. **Brown, T. A., and B. L. Trumpower.** 1995. Strain-dependent variation in carbon source regulation of nucleus-encoded mitochondrial proteins of *Saccharomyces cerevisiae*. J. Bacteriol. **117:**1380–1382.
10. **Burden, R. S., D. T. Cooke, and G. A. Carter.** 1989. Inhibitors of sterol biosynthesis and growth in plants and fungi. Phytochemistry **28:**1791–1804.
11. **Casey, W. M., J. P. Burgess, and L. W. Parks.** 1991. Effect of sterol side-chain structure on the feed-back control of sterol biosynthesis in yeast. Biochim. Biophys. Acta **1081:**279–284.
12. **Casey, W. M., G. A. Keesler, and L. W. Parks.** 1992. Regulation of partitioned sterol biosynthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **174:**7283–7288.
13. **Collart, M. A., and S. Oliviero.** 1993. Preparation of yeast RNA. Curr. Protoc. Mol. Biol. **2:**13.12.1–13.12.5.
14. **Dimster-Denk, D., M. K. Thorsness, and J. Rine.** 1994. Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Saccharomyces cerevisiae*. Mol. Biol. Cell **580:**655–665.
15. **Gaber, R. F., D. M. Copple, B. K. Kennedy, M. Vidal, and M. Bard.** 1989. The yeast gene *ERG6* is required for normal membrane function by is not essential for biosynthesis of the cell-cycle-sparking sterol. Mol. Cell. Biol. **9:**3447–3456.
16. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
17. **Goldstein, J., and M. S. Brown.** 1990. Regulation of the mevalonate pathway. Nature (London) **343:**425–430.
18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
19. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:**163–167.
20. **Köhrer, K., and H. Domdey.** 1991. Preparation of high molecular weight RNA. Methods Enzymol. **194:**398–405.
21. **Lewis, T. A., G. A. Keesler, G. P. Fenner, and L. W. Parks.** 1988. Pleiotropic mutations in *Saccharomyces cerevisiae* affecting sterol uptake and metabolism. Yeast **4:**93–106.
22. **Lorenz, R. T., W. M. Casey, and L. W. Parks.** 1989. Structural discrimination in the sparking function of sterols in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **171:**6169–6173.
23. **Lorenz, R. T., and L. W. Parks.** 1990. Effects of lovastatin (mevinolin) on sterol levels and on activity of azoles in *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **34:**1660–1665.
24. **Lorenz, R. T., and L. W. Parks.** 1991. Involvement of heme components in sterol metabolism of *Saccharomyces cerevisiae*. Lipids **26:**598–603.
25. **Manivasakam, P., S. C. Weber, J. McElver, and R. H. Schiestl.** Microhomology mediated PCR targeting in *Saccharomyces cerevisiae*. Nucleic Acids Res. **23:**2799–2800.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty.** 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene **45:**299–310.
28. **Parks, L. W., C. D. K. Bottema, R. J. Rodriguez, and T. A. Lewis.** 1985. Yeast sterols: yeast mutants as tools for the study of sterol metabolism. Methods Enzymol. **111:**333–346.
29. **Parks, L. W., and W. M. Casey.** 1995. Physiological implications of sterol biosynthesis in yeast. Annu. Rev. Microbiol. **49:**95–116.
30. **Parks, L. W., C. McLean-Bowen, F. R. Taylor, and S. Hough.** 1978. Sterols in yeast subcellular fractions. Lipids **13:**730–735.
31. **Parks, L. W., S. J. Smith, and J. H. Crowley.** 1995. Biochemical and physiological effects of sterol alterations in yeast—a review. Lipids **30:**227–230.
32. **Rodriguez, R. J., C. Low, C. D. K. Bottema, and L. W. Parks.** 1985. Multiple functions for sterols in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **837:**336–343.
33. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Rothstein, R.** 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. **194:**281–301.
35. **Sambrook, J., E. R. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Sanchez, H. B., L. Yieh, and T. F. Osborne.** 1995. Cooperation by sterol regulatory element-binding protein and Sp1 in sterol regulation of low density lipoprotein receptor gene. J. Biol. Chem. **270:**1161–1169.
37. **Sheng, Z., H. Otani, M. S. Brown, and J. L. Goldstein.** 1995. Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver. Proc. Natl. Acad. Sci. USA **92:**935–938.
38. **Skaggs, B. A., J. F. Alexander, C. A. Pierson, K. S. Schweitzer, K. T. Chun, C. Koegel, R. Barbuch, and M. Bard.** 1996. Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome *P*-450 involved in ergosterol biosynthesis. Gene **169:**105–109.
39. **Skipski, B. P., A. F. Smolove, R. C. Sullivan, and M. Barclay.** 1965. Separation of lipid classes by thin layer chromatography. Biochim. Biophys. Acta **106:**386–396.
40. **Smith, S. J., and L. W. Parks.** 1993. The *ERG3* gene in *Saccharomyces cerevisiae* is required for the utilization of respiratory substrates and in heme-deficient cells. Yeast **9:**1177–1187.
41. **Thorsness, M., W. Schafer, L. D'ari, and J. Rine.** 1989. Positive and negative transcriptional control by heme of genes encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**5702–5712.
42. **Ushinsky, S. C., and T. Keng.** 1994. A novel allele of *HAP1* causes uninducible expression of *HEM13* in *Saccharomyces cerevisiae*. Genetics **136:**819–831.
43. **Vanden Bossche, H. W. Lauwers, G. Willemsens, P. Marichal, F. Cornelissen, and W. Cools.** 1984. Molecular basis for the antimycotic and antibacterial activity of N-substituted imidazoles and triazoles: the inhibition of isoprenoid biosynthesis. Pestic. Sci. **15:**188–198.