Positive and negative regulation of a sterol biosynthetic gene (*ERG3*) in the post-squalene portion of the yeast ergosterol pathway

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Abstract Regulation of sterol biosynthesis in the terminal portion of the pathway represents an efficient mechanism by which the cell can control the production of sterol without disturbing the production of other essential mevalonate pathway products. We demonstrate that mutations affecting early and late steps in sterol homeostasis modulate the expression of the *ERG3* gene: a late step in sterol biosynthesis in yeast. Expression of *ERG3* is increased in response to a mutation in the major isoform of HMG CoA reductase which catalyzes the rate-limiting step of sterol biosynthesis. Likewise, mutations in non-auxotrophic ergosterol biosynthetic genes downstream of squalene production (*erg2, erg3, erg4, erg5*, and *erg6*) result in an up-regulation of *ERG3* expression. Deletion analysis of the *ERG3* promoter identified two upstream activation sequences: UAS1, which when deleted reduces *ERG3* gene expression 3–4-fold but maintains sterol regulation and UAS2, which when deleted further reduces *ERG3* expression and abolishes sterol regulation. The recent isolation of two yeast genes responsible for the esterification of intracellular sterol (*ARE1* and *ARE2*) has enabled us to directly analyze the relationship between sterol esterification and *de novo* biosynthesis. Our results demonstrate that the absence of sterol esterification leads to a decrease in total intracellular sterol and *ERG3* is a target of this negative regulation.

Key words: Ergosterol; *ERG3*; Promoter; Sterol esterification

1. Introduction

Sterols are essential cellular molecules which function to regulate membrane fluidity, permeability, the activity of membrane-bound enzymes and growth rate. Ergosterol (ergosta-5,7,22-trien-3β-ol) is the predominant fungal sterol and the biosynthetic pathway leading to its formation is now well defined (see [1] for review). However, the regulation of sterol biosynthesis, within the post-squalene portion of the pathway, has received little attention.

The *ERG3* gene in yeast encodes a C-5 sterol desaturase which introduces a Δ^5-bond in the B-ring of ergosterol. C-5 sterol desaturase is a multicomponent enzyme system which is cyanide sensitive, requires iron and molecular oxygen for activity and is coupled to the NAD(P)H-cytochrome *b* / cytochrome b_5 reductase microsomal electron transport system [2]. This enzymatic activity has been conserved throughout evolution as demonstrated by the presence of C-5 sterols in all organisms from the fungal, plant and animal kingdoms. We have shown previously by gene-disruption that *ERG3* is non-essential for the aerobic viability of *Saccharomyces cerevisiae* [3]. Haploid yeast cells harboring a null allele of *ERG3* accumulate the sterol intermediate episterol (ergosta-7,22-dien-3β-ol) which can functionally substitute for ergosterol in the membrane. Although the mutant does not require the addition of exogenous sterol for viability, it does exhibit reduced growth rate, decreased transformation efficiencies and increased permeability to cations such as Ca^{2+} [4]. While we have shown *ERG3* to be non-essential in an otherwise wild-type background, Smith and Parks [5] have demonstrated that *ERG3* is essential in a heme-deficient background. In another report, Nes and Dhanuka [6] have shown that sterol molecules containing a Δ^5-bond or having the capability of being converted to a Δ^5 sterol regulate the ability of a *hem3 erg12* double mutant (GL7) to synthesize ergosterol. These observations suggest that *ERG3* may be a regulatory target for sterol biosynthesis in the terminal portion of the ergosterol biosynthetic pathway.

In the present study, we examine the transcriptional regulation of *ERG3* by expression of the *Escherichia coli lacZ* gene, under the control of the *ERG3* promoter, in wild-type and mutant strains of *S. cerevisiae*. We show that *ERG3* is subject to regulation by mutations in the ergosterol biosynthetic pathway which alter intracellular sterol composition; and by mutations in the *ARE* genes which alter the ratio of free to esterified sterol. In addition, we identify by deletion promoter analysis two *cis*-acting regulatory regions present in the *ERG3* promoter, one which mediates activation of the *ERG3* gene under normal growth conditions and a second region of the *ERG3* promoter which is partially required for *ERG3* up-regulation in response to a block in the ergosterol biosynthetic pathway.

2. Materials and methods

2.1. Strains, growth conditions, and transformation methods

The wild-type yeast strain, BWG1-7a (*MAT***a** *ade1-100 his4-519 leu2-112 ura3-52*), was provided by Dr. L. Guarente, Massachusetts Institute of Technology, and used to create a panel of ergosterol biosynthetic mutants, *erg2* (C-8 sterol isomerase), *erg3* (C-5 sterol desaturase), *erg4* (C-24 sterol reductase), *erg5* (C-22 sterol desaturase), and *erg6* (C-24 sterol methyltransferase) disrupted with the *LEU2* gene by the one-step gene disruption technique of Rothstein [7] and verified by sterol analysis. J. Rine provided JRY527 (*MAT***a** *ura3 52 his3Δ200 ade2 101 lys2 801 met*$^-$) and the isogenic HMG CoA reductase mutants JRY1159 (*hmg1::LYS2*) and JRY1160 (*hmg2::HIS3*). Yeast wild-type and *are* mutants, disrupted at one or both *ARE* genes: *ARE1 ARE2* (*MAT***a** *his3-11 his3-15 leu2-3,112 trp1-1 ura3-1 kan1-100*), *are1are2* (*MAT*α *his3-11 his3-15 leu2-3,112 trp1-1 ura3-1 kan1-100 ade2-1 met14Δ are1::HIS3 are2::LEU2*), *ARE1are2* (*MAT*α *his3-11 his3-15 leu2-3,112 trp1-1 ura3-1 kan1-100 met14Δ are2::LEU2*), and *are1ARE2* (*MAT***a** *his3-11 his3-15 leu2-3,112 trp1-1 ura3-1 kan1-100 ade2-1 are1::HIS3*) were used to study

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the effect of sterol esterification on *ERG3* expression. Yeast were grown in YEP medium containing 2% glucose (YEPD) [8]. Selective media containing 2% glucose (SC) were based upon synthetic complete medium as described by Sherman et al. [8] with appropriate nutrients omitted where required to maintain plasmid selection.

Yeast strains were transformed using the lithium/cesium acetate method as described previously [9]. *E. coli* (DH5α) was transformed as described previously [10].

2.2. Construction of ERG3 promoter-lacZ fusion gene

The *ERG3* promoter was amplified by PCR using pIU304 as template DNA and sequence specific PCR primers, MB4 (5′ GGGGGGATCCATATCTCAAATCTAGACGAAT 3′) and MB5 (5′ GGGGGGAATTCTCCCCTCGAGGTCCTGCTTTGAGTCG 3′). MB4 contains a *Bam*HI restriction enzyme recognition sequence and MB5 contains an *Eco*RI restriction enzyme recognition sequence to facilitate subcloning of the amplified DNA at the *Bam*HI and *Eco*RI sites of the *lacZ* containing vector, pYLZ-6 [11], to yield pIU565. pYLZ-6 contains the *lacZ* gene lacking the first eight coding amino acids adjacent to a MCS allowing for insertion of promoter fragments to drive expression of *lacZ*. PCR primers were designed to amplify 891 base pairs of the *ERG3* promoter and the *ERG3* initiation codon only. No other *ERG3* coding amino acids were included in the fusion.

Deletion mutants of pIU565 were created either using naturally occurring restriction enzyme sites within the *ERG3* promoter or by PCR (see Fig. 1). pIU567 is deleted for one of the *Sma*I sites and the 18 bp between the two *Sma*I sites. pIU572 contains a 521 bp *Xho*I-*Sma*I deletion of the *ERG3* promoter and pIU573 contains a 385 bp *Sma*I-*Xba*I deletion of the *ERG3* promoter. PCR was used to generate a set of five nested deletion mutants which possessed identical 3′ ends (and thus were identical at the *ERG3*promoter-*lacZ* fusion junction) and varied at the 5′ ends only. The 3′ PCR primer (MB4) remained constant in each PCR reaction and different 5′ primers were used to create various end-points. pIU304 was used as template DNA for all PCR reactions (see Fig. 1 for position of PCR primers). MB4 contains the *Bam*HI restriction enzyme recognition sequence and each of the 5′ primers contains the *Eco*RI restriction enzyme recognition sequence, to facilitate subcloning of the amplified DNA into the MCS of pYLZ-6. The authenticity of each PCR product was verified by DNA sequencing using the Sequenase 2.0 DNA sequencing kit from USB and sequencing primers, YLZ6-1 (5′ CAATACGCAAACCGCCTG 3′) and YLZ6-2 (5′ AGGCGATTAAGTTGGGTA 3′) which are homologous to pYLZ-6 vector sequences flanking the *ERG3* promoter insert.

2.3. β-Galactosidase enzyme assay

Following transformation of an appropriate yeast strain with an *ERG3*promoter-*lacZ* fusion plasmid, two randomly picked transformants were grown overnight in SC medium lacking uracil to maintain plasmid selection. Cultures were harvested at an OD_{660} of 0.85–1.0. β-Galactosidase enzyme assays were performed on cell extracts as described in [8]. Each *ERG3*promoter-*lacZ* fusion construct was assayed in two separate transformants, in duplicate, over 3 days. Values reported are the average of three assays along with the standard deviation. A *gcn4-lacZ* fusion gene [12] was used as a control to verify that the regulatory effect was specific for *ERG3*.

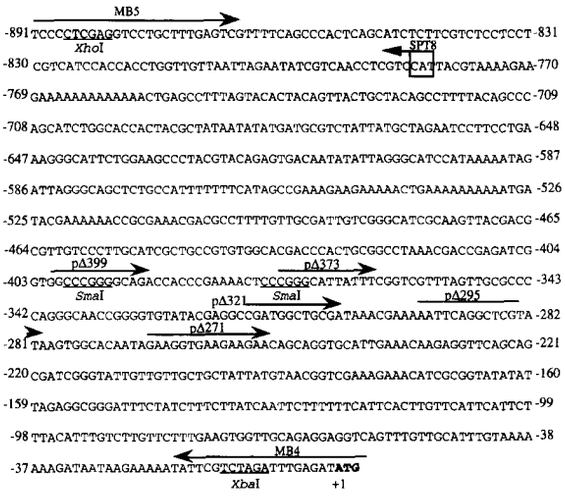


Fig. 1. Sequence of the *ERG3* promoter including the initiation codon (indicated in bold) and 891 bp upstream. PCR primers used to amplify segments of the promoter are indicated by arrows and named for the deletion end-point created. The actual sequence of MB4 is the complement of the sequenced overlined.

2.4. Quantitative sterol analysis

Total sterols were extracted as described by Molzahn and Woods [13]. Sterols were separated on a Hewlett-Packard 5890 series II gas chromatograph (GC) with a capillary column (Hewlett Packard-HP5) of 15 m × 0.25 mm × 0.25 μm film thickness programmed from 195 to 300°C. The initial temperature was 3 min at 195°C, then an increase at 5.5°C/min until the final temperature was reached and held for 4 min. The linear velocity was 30 cm/s using nitrogen as the carrier gas and injections were run in the splitless mode. 1 μl of sample dissolved in heptane was injected and the sterol composition was determined based on retention times relative to retention times of known standards. To calculate the percentage of sterol per mg of cell mass, 10 ml of the original culture was harvested by vacuum filtration onto a preweighed 0.45 μm Whatman filter. The cells were dried in a 75°C oven overnight, followed by desiccation at room temperature for 4 h. Weight of the cells was taken immediately upon opening the desiccator. The amount of each individual sterol was calculated based on the area under each peak of the chromatograph relative to a

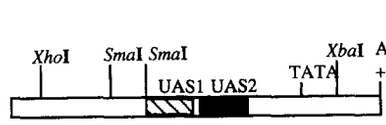
Table 1
Expression of the *ERG3-lacZ* fusion gene in an ergosterol biosynthetic mutant background

Genotype[a]	β-Galactosidase activity			
	ERG3-lacZ (pIU565)	Ratio of erg mutant/WT	*gcn4-lacZ*[b] (p180)	Ratio of erg mutant/WT
BWG1-7a (wt)	123 ± 22	1.0	33 ± 4.7	1.0
erg6	315 ± 75	2.6	35 ± 3.8	1.06
erg2	713 ± 56	5.8	19 ± 5.0	0.58
erg3	770 ± 112	6.3	21 ± 2.0	0.64
erg5	627 ± 78	5.1	21 ± 3.2	0.64
erg4	274 ± 16	2.2	35 ± 6.0	1.06
JRY527 (wt)	66 ± 17	1.0	100 ± 37	1.0
hmg1	284 ± 45	4.3	99 ± 9.0	0.99
hmg2	67 ± 1	1.0	130 ± 30	1.3

Quantitation of β-galactosidase enzyme activity from the *ERG3*promoter-*lacZ* fusion was carried out as described in Section 2. β-Galactosidase activity is nmol ONPG hydrolyzed/min per mg protein and is the average of values obtained from two independent transformants assayed in duplicate over 3 days.

[a]BWG1-7a, erg2, erg3, erg4, erg5, and erg6 mutants are isogenic strains. JRY527, hmg1, and hmg2 are isogenic strains. *HMG1* encodes the major isoform of HMG CoA reductase in yeast contributing 83% of enzymatic activity in a vegetatively growing cell [16].

[b]*gcn4-lacZ* serves as a control to verify that the regulatory effect is specific to *ERG3*.



Plasmid name	Deletion end point	β-galactosidase Activity					
		BWG1-7a	*erg2*	Ratio of mutant/WT	JR527	*hmg1HMG2*	Ratio of mutant/WT
pIU565	-891	123 +/- 22	713 +/- 56	5.8	61 +/- 12	284 +/- 45	4.7
p-Δ399	-399	104 +/- 21					
pIU567	-397 -373	142 +/- 27					
pIU572	-397 -11	9 +/- 0.2					
pIU573	-885 -373	188 +/- 40					
p-Δ373	-373	148 +/- 35					
p-Δ321	-321	52 +/- 9	363 +/- 77	7.0	25 +/- 8.0	104 +/- 28	4.2
p-Δ295	-295	33 +/- 5					
p-Δ271	-271	26 +/- 6	43 +/- 11	1.6	23 +/- 4.0	32 +/- 6.0	1.4
pYLZ-6		5 +/- 0.2					

Fig. 2. Deletion analysis of the *ERG3* promoter. β-Galactosidase enzyme activity was quantitated from *ERG3* promoter deletion mutants which were fused to *lacZ*, and transformed into the wild-type yeast strains, BWG1-7a and JRY527, and two ergosterol biosynthetic mutants, blocked at *erg2* and *hmg1*. Specific activity is nmol ONPG hydrolyzed/min per mg protein and is the average of two independent transformants assayed in duplicate over 3 days.

known amount of sterol standard loaded onto the GC using the Hewlett-Packard sterol quantitation program. Each sample was injected twice and the values averaged.

3 Results and discussion

3.1. A block in the ergosterol biosynthetic pathway up-regulates *ERG3* expression

Thus far, regulation of ergosterol biosynthesis has focused on enzymatic steps in the pre-lanosterol portion of the pathway. Casey et al. [14] have presented evidence for the ability of ergosterol to regulate its own biosynthesis via a negative feedback mechanism. However, the exact nature of this regulatory mechanism was not clear. We were interested in determining whether or not a block in the ergosterol biosynthetic pathway would effect the expression of *ERG3*. To examine this, a panel of isogenic ergosterol biosynthetic mutants was transformed with pIU565 (wild-type *ERG3* promoter-*lacZ* fusion gene) and assayed for β-galactosidase activity under aerobic growth conditions. The results presented in Table 1 illustrate that a block in the ergosterol biosynthetic pathway results in an increase in *ERG3* expression. Interestingly, each ergosterol mutation results in a different degree of *ERG3* up-regulation, suggesting that the cell is capable of modulating the degree of *ERG3* activation in response to the accumulation of distinct sterol intermediates. Mutations in *erg2* and *erg3* both result in a 6-fold increase in *ERG3* expression. The *erg2* mutation in *S. cerevisiae* is non-auxotrophic for sterol and results in an accumulation of sterol intermediates such as ergosta-5,8,22-3β-ol, and ergosta-8-en-3β-ol [15] and the *erg3* mutation leads to an accumulation of ergosta-7,22-dien-3β-ol [3]. Similarly, a mutation in the *HMG1* gene encoding the major isoform of HMG-CoA reductase [16] results in a 4-fold increase in *ERG3* expression. Loss of the *HMG2* gene results in no increase in *ERG3* expression. *hmg1* and *hmg2* mutants show a 50 and 20% decrease, respectively, in end product ergosterol (data not shown). These decreases are not sufficient to result in an ergosterol requiring phenotype and furthermore, more than half of cellular ergosterol is stored as esters in microlipid droplets.

3.2. The *ERG3* promoter contains at least one UAS required for regulated expression of *ERG3*

To identify *cis*-acting sequences necessary for regulated expression of *ERG3*, a series of *ERG3* promoter-deletion mutants was created and fused to *lacZ*. β-Galactosidase enzyme

Table 2
Quantitation of β-galactosidase enzyme activity from the *ERG3*promoter-*lacZ* fusion gene in sterol esterification mutant backgrounds

Genotype[a]	β-Galactosidase activity			
	ERG3-lacZ	Ratio of mutant/wt	*gcn4-lacZ*[b]	Ratio of mutant/wt
Wild-type	85 ± 25	1.0	15 ± 0.7	1.0
are1are2	31 ± 9.2	0.35	33 ± 2.1	2.2
ARE1are2	49 ± 11	0.53	14 ± 2.1	0.9
are1ARE2	89 ± 22	0.98	33 ± 4.2	2.2

Quantitation of β-galactosidase enzyme activity from the *ERG3*promoter-*lacZ* fusion was carried out as described in Section 2. β-Galactosidase activity is nmol ONPG hydrolyzed/min per mg protein and is the average of values obtained from two independent transformants assayed in duplicate over 3 days.
[a] All four strains are isogenic except at the specific *ARE* locus indicated.
[b] The *gcn4-lacZ* fusion gene served as a control to verify that the esterification effect was specific to *ERG3*.

Table 3
Determination of the percentage of each sterol present within the total intracellular sterol fraction

Genotype	% total cellular sterol represented by ergosterol and nonergosterol intermediates				
	Erg	Lano	Zymo	Feco	Epi
ARE1ARE2	55.0	1.6	15.0	11.0	14.5
are1are2	86.6	0.7	0.0	0.75	8.9
are1ARE2	67.0	1.6	12.0	4.1	12.0
ARE1are2	66.0	0.7	13.4	9.0	9.0

[a]Values represent the percentage of the total sterol pool represented by each individual sterol. Each experiment was repeated three times and the data presented is of a typical experiment. Erg, ergosterol; Lano, lanosterol; Zymo, zymosterol; Feco, fecosterol; Epi, episterol.

activity generated from the mutant promoters was compared to that of the wild-type *ERG3* promoter-*lacZ* fusion, in the wild-type yeast strain. Each fusion plasmid was transformed separately into BWG1-7a and transformants were grown for maximal expression, i.e. in SC-uracil medium (for plasmid maintenance), high aeration and to log phase of growth. Analysis of β-galactosidase activity from the *ERG3* promoter-*lacZ* deletion mutants, in the ergosterol biosynthetic mutants, *erg2* and *hmg1*, compared to their isogenic wild-type strains, BWG1-7a and JRY527, respectively, revealed a *cis*-acting region, UAS1, which is necessary for full up-regulation of *ERG3* in response to a block in the ergosterol pathway (Fig. 2). Deletion of UAS1 (located between −373 and −321 nt upstream of the *ERG3* initiation codon) causes a 2–3-fold decrease in β-galactosidase enzyme activity in a wild-type strain but sterol regulation is maintained (expression of *ERG3* in *erg2* and *hmg1* strains with UAS1 deleted is still 4–7-fold greater than in *ERG2* and *HMG1* strains (Fig. 2)). A second upstream activation site, UAS2, defined as a deletion of 50 nt downstream of UAS1, results in a further reduction in *ERG3* expression, 2–8-fold (depending upon genetic background) and deletion of this UAS eliminates sterol regulation.

3.3. A disruption of the sterol esterification genes, ARE1 and ARE2, results in a decrease in ERG3 expression

Intracellular sterol can exist in two forms, either free, or as esters, conjugated to long chain fatty acids. Steryl esters are the storage form of sterol while free sterols are available to be incorporated into the plasma membrane, their principle site of action. These two pools are interchangeable and regulation of the interconversion is required to maintain the essential amount of free sterol [17]. The esterification of intracellular sterols in all mammalian species is mediated by acyl-CoA:-cholesterol acyltransferase (ACAT), a membrane-bound enzyme which is dependent on acyl-CoA as a fatty acid donor [18]. Yang et al. [19] have recently identified two yeast genes, ACAT related enzyme 1 (*ARE1*) and ACAT related enzyme 2 (*ARE2*) required for esterification of intracellular sterols in yeast. *ARE2* encodes the major isozyme and is responsible for the majority of sterol esterification in a vegetatively growing cell. Disruption of both *ARE1* and *ARE2* results in a viable cell with no detectable esterified sterol. In addition, Yang et al. [19] have shown that the inability to esterify intracellular sterols down-regulates the sterol biosynthetic pathway as determined by [^{14}C]acetate incorporation into the lipid pool. To determine if *ERG3* is a target of down-regulation, we assayed for β-galactosidase activity in the *ARE* wild-type and *are* mutant strains transformed with pIU565. The results, presented in Table 2, indicate that *ERG3* expression is down-regulated 3-fold in the absence of intracellular sterol esterification (*are1are2*). There is a more modest decrease (1.9-fold) in *ERG3* expression in the absence of the major *ARE* gene, *ARE2*, and no significant difference in *ERG3* expression in the absence of the minor isoform, Are1p, compared to wild-type cells.

3.4. In the absence of sterol esterification, the concentration of intracellular sterol is decreased

We have shown that *ERG3* expression is decreased in the absence of sterol esterification. Based on this observation, we were interested in quantifying the amount of intracellular sterol present in the *are* mutants compared to the isogenic wild-type strain. Results indicated that the *are1are2* double mutant accumulates 33% less total sterol per mg of dry cell weight than a wild-type cell. This result is consistent with a down-regulation of the sterol biosynthetic pathway. The inability of the cell to esterify excess sterol implies that the cell must down-regulate endogenous synthesis to maintain the critical low level of free sterol. We then considered the ratio of ergosterol to ergosterol intermediates, based on the gas-chromatographic (GC) separation, and found that the *are1are2* double mutant possessed the greatest ratio of ergosterol to intermediates compared to the wild-type strain or either single mutant (see Table 3). A plausible explanation for this finding is that the absence of sterol esterification renders all intermediates accessible to the sterol biosynthetic enzymes and thus complete conversion to ergosterol is not prevented.

In summary, we have shown that *ERG3* expression is up-regulated in response to a block in the ergosterol biosynthetic pathway (i.e. *erg2, erg3, erg5*) or a decrease in end-product sterol due to a mutation in the major HMG-CoA reductase encoding gene, *HMG1*. We also demonstrate that *ERG3* expression is down-regulated in the absence of esterification and provides the first evidence for direct transcriptional regulation of a late sterol biosynthetic gene in response to sterol esterification defects.

These results support the involvement of a sterol sensor which is capable of first detecting the absence of ergosterol and/or the presence of sterol intermediates within the membrane, and second, detecting the level of free ergosterol and mediating the regulation of de novo ergosterol synthesis to maintain a critical concentration of free sterol. These results also suggest that in mammalian cells, enzymatic steps distal to squalene, currently undefined at the molecular level, will very probably play an important role in cholesterol regulation.

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