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Transcriptional Regulation of the Two Sterol Esterification Genes in the Yeast *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae **transcribes two genes, *ARE1* and *ARE2*, that contribute disproportionately to the esterification of sterols. Are2p is the major enzyme isoform in a wild-type cell growing aerobically. This likely results from a combination of differential transcription initiation and transcript stability. By using *ARE1* and *ARE2* promoter fusions to *lacZ* reporters, we demonstrated that transcriptional initiation from the *ARE1* promoter is significantly reduced compared to that from the *ARE2* promoter. Furthermore, the half-life of the *ARE2* mRNA is approximately 12 times as long as that of the *ARE1* transcript. We present evidence that the primary role of the minor sterol esterification isoform encoded by *ARE1* is to esterify sterol intermediates, whereas the role of the *ARE2* enzyme is to esterify ergosterol, the end product of the pathway. Accordingly, the *ARE1* promoter is upregulated in strains that accumulate ergosterol precursors. Furthermore, *ARE1* and *ARE2* are oppositely regulated by heme. Under heme-deficient growth conditions, *ARE1* was upregulated fivefold while *ARE2* was down-regulated. *ARE2* requires the *HAP1* transcription factor for optimal expression, and both *ARE* genes are derepressed in a *rox1* (repressor of oxygen) mutant genetic background. We further report that the *ARE* genes are not subject to end product inhibition; neither *ARE1* nor *ARE2* transcription is altered in an *are* mutant background, nor does overexpression of either *ARE* gene alter the response of the *ARE-lacZ* reporter constructs. Our observations are consistent with an important physiological role for Are1p during anaerobic growth when heme is limiting and sterol precursors may accumulate. Conversely, Are2p is optimally required during aerobiosis when ergosterol is plentiful.**

The conjugation of sterols and fatty acids is a critical homeostatic response by all eukaryotic cells to an excess of either resource. This intracellular esterification reaction is mediated by enzymes known collectively as O-acyltransferases and provides an important storage depot and detoxification process by which to overcome the membrane perturbations that accrue from elevated sterol or free fatty acid levels. Thus, the uptake, synthesis, and conjugation of these metabolites are subject to multiple levels of regulation. In mammalian cells, sterol and fatty acid biosynthesis and receptor-mediated lipoprotein uptake are controlled primarily at the transcriptional level by the sterol regulatory element binding protein, a positive transcription factor which is inactive when sterols and fatty acids exceed cellular requirements (7). Sterol biosynthesis is further regulated posttranslationally, by phosphorylation and proteasomal degradation (18). Each mechanism causes metabolic down-regulation in response to excess cholesterol or fatty acids. By contrast, the sterol esterification reaction is up-regulated by elevated cellular cholesterol or fatty acids (14). The major mode of regulation of the mammalian acyl coenzyme A (CoA): cholesterol acyltransferases arises from allosteric binding of the sterol substrates, particularly cholesterol and oxysterol (11, 12, 15). However, they are also regulated transcriptionally (36, 40, 47).

ACAT1 is the founding member of the O-acyltransferase gene family that now extends to multiple organisms (17, 43). In the model eukaryote *Saccharomyces cerevisiae*, a paradigm has been identified whereby, within the same cell, more than one form of the enzymes are expressed (42, 49). The ACAT-related enzymes of yeast encoded by the *ARE1* and *ARE2* genes differentially determine the sterol ester pools of the cell. Deletion of both genes is required to produce a cell lacking sterol esterification activity (49, 51). However, under normal growth conditions, the approximate contributions of the *ARE1* and *ARE2* gene products to the total sterol ester mass are 25 and 75%, respectively, as assessed by the phenotypes produced by single *ARE* gene disruptions (2, 49). In mammals, two *ACAT* genes exist, *ACAT1* and *ACAT2* (1, 10, 35). In induced-mutant mouse models, sterol esterification is determined by *ACAT1* in all tissues except the liver and intestine, where *ACAT2*-mediated activity predominates (8, 10, 31). In humans, *ACAT2* is expressed primarily in hepatocytes and enterocytes while *ACAT1* is ubiquitous (34). *ACAT1* accounts for the majority of sterol esterification in most human cells, with the exception of those of intestinal origin, where *ACAT2* appears to be the major contributor (13). Thus, the paradigm persists that in hepatocytes and enterocytes, *ACAT1* and *ACAT2* are both

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TABLE 1. Strains used for analysis of *ARE1* and *ARE2* regulation

Strain	Genotype	Source
BWG1-7a	*MAT***a** *ade1-100 his4-519 leu2-112 ura3-52*	L. Guarente
BWG/*erg2*	*MAT***a** *ade1-100 his4-519 leu2-112 ura3-52 erg2*Δ::*LEU2*	M. Bard
BWG/*erg3*	*MAT***a** *ade1-100 his4-519 leu2-112 ura3-52 erg3*Δ::*LEU2*	M. Bard
BWG/*erg6*	*MAT***a** *ade1-100 his4-519 leu2-112 ura3-52 erg6*Δ::*LEU2*	M. Bard
JR527	*MAT***a** *ura3-52 his3*Δ*200 ade2-101 lys2-801 met*	J. Rine
JR1159	*MAT***a** *ura3-52 his3*Δ*200 ade2-101 lys2-801 met hmg1*::*LYS2*	J. Rine
JR1160	*MAT***a** *ura3-52 his3*Δ*200 ade2-101 lys2-801 met hmg2*::*HIS3*	J. Rine
TKY22	*MAT***a** *leu2-3,112 ura3-52 ade1 trp1*::*hisG hem1*Δ	T. Keng
LPY22	*MAT***a** *ade1-100 his4-519 leu2-112 ura3-52 hap1*Δ::*LEU2*	L. Guarente
RZ53-6	*MAT*α *trp1-289 leu2-3,112 ura3-52 ade1-100*	R. Zitomer
RZ53-6/*rox1*	*MAT*α *trp1-289 leu2-3,112 ura3-52 ade1-100 rox1*Δ::*LEU2*	R. Zitomer
SCY059	*MAT*α *his3-11,15 leu2-3,112 trp1-1 ura3-1 kan1-100 ade2 met14*Δ *are1*::*HIS3 are2*::*LEU2*	S. Sturley
SCY060	*MAT*α *his3-11,15 leu2-3,112 trp1-1 ura3-1 kan1-100 ade2-1 are1*::*HIS3*	S. Sturley
SCY061	*MAT***a** *his3-11,15 leu2-3,112 trp1-1 ura3-1 kan1-100 met14*Δ *are2*::*LEU2*	S. Sturley
SCY062	*MAT***a** *his3-11,15 leu2-3,112 trp1-1 ura3-1 kan1-100*	S. Sturley
SCY983	*MAT***a** *ura3-52 leu2-3 rpb1-1*	M. Culbertson

expressed in the same cell and yet contribute differentially to the esterification of sterols.

The expression of multiple genes for the sterol esterification reaction in a single cell must confer a selective advantage, given its retention throughout evolution. This could reflect differences in subcellular localizations, responses to the environment, or substrate specificity. In yeast, the Are proteins are both localized to the endoplasmic reticulum but exhibit marked substrate preferences (48, 53). In this study, we confirm that, in terms of contribution to the sterol ester mass in yeast, the *ARE1* gene product primarily esterifies intermediates in the sterol biosynthetic pathway such as lanosterol, whereas *ARE2* is responsible for esterification of the end product ergosterol. Furthermore, we demonstrate that the *ARE* genes are differentially regulated in response to alterations in sterol metabolism. The *ARE1* gene is up-regulated by the accumulation of pathway intermediates and heme deficiency, whereas in the latter case, *ARE2* is repressed. This would be physiologically relevant under anaerobic growth conditions. We propose that the regulated removal of biosynthetic pathway intermediates before they either become toxic or participate further in the production of the end product represents a novel form of sterol homeostasis that may be common to all eukaryotic cells.

MATERIALS AND METHODS

Strains, growth conditions, *ARE* expression constructs, and transformations. Yeast strains (Table 1) were grown at 30°C in a mixture of yeast extract, peptone, and 2% glucose (YEPD) or complete synthetic medium (0.67% yeast nitrogen base, 2% glucose; CSM [3, 9]) with appropriate nutrients omitted as required for plasmid selection. Supplementation with adenine at 40 mg/liter was done when adenine auxotrophic strains were used for analysis of β-galactosidase activity. Yeast strains and *Escherichia coli* strain DH5α were transformed and maintained as previously described (3, 23). The ergosterol biosynthesis inhibitor fenpropimorph was added to the growth media at 0.5 μM at the time of culture inoculation, reducing the growth rate by 50%. Once the culture reached a density of 5×10^6 cells/ml, fenpropimorph was added at a final concentration of 25 μM and the culture was incubated for an additional 18 h before harvesting. Expression plasmids for *ARE1* (YEp3-16 and pADH5-36) and *ARE2* (YEpARE2 and pS5-ARE2) have been described elsewhere (20, 49).

Sterol extraction and analysis. Total sterols (free and esterified) were extracted and quantified from yeast cells as previously described (5, 32). Yeast cells were grown in 100 ml of minimal medium for 36 h in a 30°C water bath with shaking at 250 rpm. Nitric acid-washed glass beads (425 to 600 μm) were added at 0.5 volume, the tubes were vortexed twice for 30 s, and this was followed by sequential additions of 4 ml of methanol, 2 ml of chloroform, and 2 ml of 0.9% (wt/vol) NaCl with frequent vortexing. The chloroform phase containing extracted sterols was removed, dried under nitrogen, and resuspended in a small volume of methylene chloride and streaked on F_{254} precoated silica gel thin-layer chromatography (TLC) plates (E. Merck, Darmstadt, Germany). TLC was performed in methylene chloride ($CHCl_2$), and the lipids were visualized by using 0.01% (wt/vol) berberine. Free sterols and steryl esters were scraped from the TLC plate, and the sterols were separated from the silica by resuspension in methylene chloride and vacuum filtration. Steryl esters were saponified overnight, at room temperature in the dark, in 1 ml of 6% (wt/vol) KOH in methanol. Hydrolyzed esters were extracted in *n*-heptane. Free sterol and ester fractions were quantified by gas chromatography. Sterols were separated on a Hewlett-Packard 5890 series II gas chromatograph with a capillary column (15 m by 0.25 mm by 0.25 μm [film thickness]; Hewlett-Packard HP5) programmed from 195 to 300°C. The temperature was initially 195°C for 3 min; it was then increased at 5.5°C/min to a final temperature of 300°C, at which it was held for 4 min. The linear velocity was 30 cm/s, nitrogen was used as the carrier gas, and injections were run in the splitless mode. Sterol fractions were resuspended in 1 ml of *n*-heptane, and 1 μl was coinjected with 0.1 μg of cholesterol, which was used as an internal standard. The area of each peak was compared to the area of the cholesterol peak to determine the amount of each sterol present. Each sample was injected twice, and the value reported was the average of the two injections. The dry weight was measured prior to extraction, so the total amount of sterol per gram of cells was determined. Samples of 1 to 3 μl, dissolved in *n*-heptane, were injected, and sterol composition was determined on the basis of retention times relative to the retention times of known sterol standards.

Construction of promoter-*lacZ* fusion plasmids. *ARE1* and *ARE2* promoter regions were amplified by PCR using genomic DNA from strain W303 as the template and sequence-specific primers KP-3 (5′ GGGGGGAATTCCGTCCA TGGTCACACCGTCC 3′) and KP-4 (5′ GGGGGGATCCATTCTTGCAATC TGTTTTGG 3′) for *ARE1* and KP-1 (5′ GGGGGGAATTCGGTACCCAAA ATTCAAGCCTT 3′) and KP-5 (5′ GGGGGGATCCATGGTTGTGTTTGTT ATTGT 3′) for *ARE2.* Each set of primers was designed with *Eco*RI and *Bam*HI recognition sequences to facilitate cloning into the *lacZ* reporter plasmid pYLZ-6 (22) to yield p*ARE1-lacZ* (pIU1107) and p*ARE2-lacZ* (pIU1113). Plasmids pIU1107 and pIU1113 were used to construct targeted chromosomal integrations. To create an integrating plasmid from pIU1113, the *CEN6* region was deleted by digestion with *Sca*I and replaced with the *Sca*I sequence from pRS306 (41), yielding pIU1116. The presence of a *Sca*I site in the promoter of *ARE1* prevented the use of this strategy, so its integration was accomplished by removing the *ARE2* promoter from pIU1116 and replacing it with the *ARE1* promoter sequence from pIU1107, generating pIU1115. Deletion plasmids were made from the integrating vectors pIU1115 and pIU1116. A truncated *ARE1* promoter was made by digestion with *Eco*RI and *Eco*RV, deleting 500 bp of promoter sequence from pIU1115. The resulting fusion plasmid, pIU1160, contained 500 bp of *ARE1* sequence proximal to the ATG start codon. Part of the promoter region of *ARE1* (220 bp upstream of the start codon) was amplified by PCR from pIU1115 using *Pfu* polymerase and the primers KP-4 and Are1-1 (5′ GGGGG GAATTCGTATGTGCTGCTCATCTC 3′). The amplified fragment was di-

gested with *Eco*RI and *Bam*HI, purified, and ligated into *Eco*RI- and *Bam*HI-digested pIU1115, to which the promoter sequence had been removed, yielding pIU1146. The deletion of 589 bp of *ARE2* distal promoter sequence was done by restriction digestion of pIU1116 with *Eco*RI and *Bgl*II. The *Eco*RI cohesive ends were blunted by using Klenow fragment and religated, generating pIU1141 containing 411 bp of *ARE2* promoter sequence proximal to the ATG codon. Each plasmid used in this study was sequenced by using primers YLZ6-1 (5′ CAATACGCAAACCGCCTG 3′) and YLZ6-2 (5′ AGGCGATTAAGTTGG GTA 3′).

RNA hybridizations and measurements of mRNA decay. Total RNA was prepared from yeast by using a hot acidic phenol extraction method (3). Equal amounts (15 μg) of total RNA were resolved in 1.2% formaldehyde agarose gels and transferred to nylon membranes by using conventional procedures (3). DNA probes specific for *ARE1* and *ARE2* were chosen close to the 5′ terminus, since this is the region least conserved between the genes. A 518-bp probe for *ARE1* (nucleotides 45 to 564) was made by digesting the construct *ARE1*/pGEX-3X with *Bam*HI (Z. Guo and S. L. Sturley, unpublished data). A 498-bp probe for *ARE2* (nucleotides 45 to 542) was made by digesting the construct *ARE2*/pGEX-3X with *Bam*HI and *Eco*RI (20). The probes were radiolabeled with [^{32}P]dCTP (Prime-it; Stratagene) and used in hybridizations at 65°C in ExpressHyb buffer (Clontech). The membrane was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature and in 0.1% SSC–0.1% SDS at 60°C. mRNA decay rates were measured by using strain SCY983 (a gift of M.Culbertson), which carries a temperature-sensitive mutation in RNA polymerase II (33). Briefly, SCY983 was grown in 100-ml cultures (0.5×10^7 cells) and the temperature of the culture was abruptly adjusted from 24 to 36°C by adding an equal volume of YEPD medium at 48°C and then transferring the culture flask to a shaker bath at 36°C. Aliquots of the culture were removed after 0 to 100 min, and cell pellets were frozen on dry ice. Total cellular RNA was extracted from the frozen cells, suspended in sterile water, and stored at −80°C.

Protein electrophoresis and immunoblotting. Microsomes from various yeast strains were prepared and resolved by denaturing gel electrophoresis (5 μg of total protein per lane) by using 10% polyacrylamide in the presence of 0.1% SDS. The proteins were electroblotted to nitrocellulose, blocked in 5% nonfat milk in 20 mM Tris-HCl–137 mM NaCl–0.1% Tween 20 (TBST), and probed at 3.4 μg/ml with chicken immunoglobulin Y (IgY) antibody generated against the NH_2-terminal 180 residues of Are2p (20) in TBST–1% nonfat milk for 1 h. Detection of the immune complexes was attained by using horseradish peroxidase-conjugated secondary anti-chicken IgY antibody (Promega) and the ECL Western blotting detection reagent (Amersham).

β-Galactosidase enzyme assays. Strains to be assayed were transformed with the described plasmids linearized at *Stu*I to target integration at the endogenous *URA3* locus. In each case, two independent transformants were grown overnight in CSM lacking uracil. Cultures were harvested at an optical density at 600 nm of 0.7 to 0.8 (≈1.5×10^7 cells/ml). β-Galactosidase assays from the promoter-*lacZ* fusion genes (39) were performed on total protein extracts prepared from duplicate colonies in three independent experiments. Protein concentrations were assessed by using the Bradford dye-binding assay (6).

RESULTS

Transcriptional activity of *ARE1* and *ARE2* promoters. We and others have demonstrated a marked imbalance in the contribution of the yeast *ARE* gene products to total sterol esterification within the cell (50, 51, 53). To assess whether this reflects expression differences at the transcriptional level, we quantified the promoter activity of *ARE1* and *ARE2* in wild-type cells by using promoter-*lacZ* fusions. We constructed several fusions with various 5′ untranslated regions from each of the genes to determine the minimal sizes of the promoters that exhibit optimal activity (Table 2). The sequences of the *lacZ* fusion plasmids pIU1115, pIU1160, and pIU1146 (*ARE1*) and pIU1116 and pIU1141 (*ARE2*) were confirmed at the nucleotide level relative to the sequences in the *Saccharomyces* genome database (http://genome-www.stanford.edu/Saccharomyces/). To minimize differences in plasmid copy number or stability, we digested each fusion plasmid with *Stu*I for integration at the *URA3* locus. β-Galactosidase activity was assessed by conventional methods. We confirmed that the patterns of expression for each fusion were similar in multiple integrants and in cells carrying fusions expressed autosomally (data not shown).

TABLE 2. β-Galactosidase activities conferred by *ARE1-lacZ* and *ARE2-lacZ* fusions on wild-type strain BWG 1-7a

Plasmid[b] (promoter length, bp)	β-Galactosidase activity in BWG1-7a[a]	
	ARE1-lacZ	*ARE2-lacZ*
pIU1116 (1,000)		87 + 3
pIU1141 (411)		167 + 18
pIU1115 (1,000)	1.2 + 0.2	
pIU1160 (500)	1.7 + 0.2	
pIU1146 (220)	1.7 + .3	

[a] β-Galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein and is the average of two independent transformants assayed in duplicate over 3 days.
[b] The control plasmid lacking an *ARE* promoter insert typically gave a β-galactosidase activity of 0.5 U or less (21).

ARE2-driven β-galactosidase expression increased in the integrants containing the minimal promoter region, suggesting the presence of transcription-repressing sequences between positions −411 and −1000 of the *ARE2* gene. This repressive effect may reflect the overlap of this construct with the 3′ region of the neighboring open reading frame YNR018w. The 411-bp promoter fusion (in pIU1141) that exhibited the greatest activity was thus chosen for subsequent experiments. In promoter deletion experiments for *ARE1*, all of the promoter lengths tested (220 bp to 1 kb) conferred comparable β-galactosidase activities. The 220-bp promoter fusion from pIU1146 was used for all subsequent experiments because it did not contain DNA sequence from other genes on chromosome III. The β-galactosidase activity of the *ARE1* promoter construct was consistently and significantly lower than that conferred by the *ARE2* promoter. This is in accordance with the 15 to 25% contribution to the esterification activity of a wild-type strain that can be accounted for by the Are1 enzyme.

To confirm the fidelity of the reporter gene fusions, we also assessed the steady-state levels of *ARE* gene transcripts in wild-type cells by Northern RNA hybridizations by using probes labeled to similar specific activity and by comparison to expression of the *ACT1* gene encoding actin. *ARE1* transcripts were consistently observed at significantly lower levels than the *ARE2* transcript (Fig. 1, zero time point). Furthermore, the *ARE1* gene transcript was significantly less stable than the *ARE2* transcript (Fig. 1). By using a temperature-sensitive mutation in the largest subunit of RNA polymerase II (*rpb1-1*), we studied the decay of the *ARE* transcripts over 4 h after a rapid shift to the nonpermissive temperature. The *ARE1* transcript displayed a half-life of approximately 5 min, whereas the half-life of *ARE2* was approximately 60 min. Thus, the differences in *ARE*-mediated sterol esterification activity under normal aerobic growth conditions can most simply be explained by the relative abundance of the specific transcripts. This results from the combined effects of differences in transcription initiation and transcript stability for each gene.

Overexpression of Are1p and Are2p and steryl ester quantification. To assess whether the *ARE*-encoded enzymes differentially contribute to sterol esterification at the post-

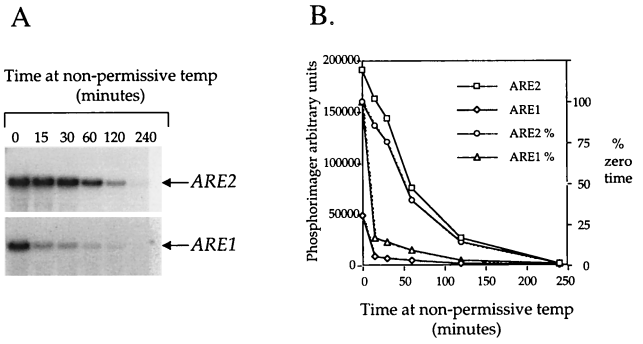

FIG. 1. Stability measurements of *ARE1* and *ARE2* transcripts. Strain SCY983 (temperature sensitive for RNA polymerase II [33]) was grown to mid-logarithmic phase at 24°C and rapidly shifted to the nonpermissive temperature (36°C). Total RNA was extracted from samples withdrawn at the indicated times and processed for hybridization analysis with a *ARE1*- or *ARE2*-specific probe. (A) Autoradiograph of RNA blot hybridization. (B) Graphical representation of data collected from a phosphorimager of panel A. Data are presented as total counts (arbitrary phosphorimager units) or as a percentage of the zero time point value.

transcriptional level, the contribution of transcription was minimized by overexpressing the *ARE1* and *ARE2* genes by using the multicopy vectors Yep3-16 (*ARE1*) and Yep352Are2 (*ARE2*) in an *are1*Δ *are2*Δ deletion strain. We first addressed whether yeast can overexpress *ARE1* and *ARE2* and accumulate steryl esters and whether the enzymes esterify the same or different sterols. Free sterols and steryl esters were extracted and quantified from 100-ml cultures grown in CSM containing additional uracil for 36 h. Free sterol levels were not statistically significantly different in *are1*Δ *are2*Δ mutants, regardless of which gene was expressed (Tables 3 and 4). Overexpression of either gene in *are1*Δ *are2*Δ mutants showed little difference in the total amount of steryl ester accumulated. It is possible that the accumulation of steryl ester is regulated by substrate supply. Quantitative sterol analysis was then performed with an *are1*Δ *are2*Δ mutant strain (SCY059) overexpressing either *ARE1* (Table 3) or *ARE2* (Table 4) to quantify the sterol type accumulating in the free and ester fractions. Cells were grown in 100-ml cultures of CSM containing additional uracil for 36 h and harvested. Free sterol and steryl ester amounts were determined as percentages of the dry weight. Overexpression of *ARE1* or *ARE2* had no significant effect on free sterol composition; ergosterol was the major sterol produced in the free sterol fraction. By contrast, the ester fraction of cells overexpressing *ARE1* show a marked accumulation of sterol intermediates, specifically lanosterol and, to a lesser extent, zymosterol. Lanosterol represents 15.5% of the total ester fraction, whereas ergosterol represents only 19% when *ARE1* is overexpressed. In this genetic background or in a wild-type background, lanosterol is converted to the end product ergosterol or other sterol intermediates since it is not esterified to levels greater than 1% of the total esters. Cells overexpressing *ARE2* favor esterification of ergosterol (39% of the total ester fraction), while lanosterol esterification is again minor (1% of the ester fraction). Although Are1p and Are2p clearly esterify all of the sterol types analyzed, these data suggest possible substrate preferences in vivo. Are1p preferentially esterified sterol intermediates, especially lanosterol, whereas ergosterol was the preferred substrate for Are2p

TABLE 3. Quantification of sterols and sterol esters during overexpression of *ARE1* in YE_p3-16 (strain SCY059 [*are1*Δ *are2*Δ])

Compound(s)	Free sterols[a] (% of free compounds)	Esters[b] (% of total esters)	SE/FS ratio[c]
Lanosterol	7.6 ± 14 (1.2)	484 ± 159 (15.5)	63
Zymosterol	4.1 ± 8.6 (0.6)	1,321 ± 284 (42.3)	322
Fecosterol	63 ± 9.2 (10.2)	329 ± 70 (10.5)	5
Episterol	36 ± 9 (5.8)	396 ± 120 (12.6)	11
Ergosterol	504 ± 125 (82.2)	598 ± 199 (19.1)	1
Total sterols	614 ± 138 (100)	3,128 ± 798 (100)	5

[a] Sterols (micrograms per gram [wet weight] of cells ± the standard deviation) from three separate cultures were extracted, and the average value is reported.
[b] Mass of sterols (micrograms per gram [wet weight] of cells ± the standard deviation) in the esterified form after saponification (three separate cultures were extracted, and the average value is reported).
[c] The SE/FS ratio represents the proportion of steryl ester (SE) relative to that remaining unesterfied (FS).

TABLE 4. Quantification of sterols and steryl esters during overexpression of *ARE2* in Yep352Are2 (strain SCY059 [*are1*Δ *are2*Δ])

Compound(s)	Free sterols[a] (% of free compounds)	Esters[b] (% of total esters)	SE/FS ratio[c]
Lanosterol	1.8 ± 4.9 (0.3)	22.9 ± 8.6 (0.8)	13
Zymosterol	24 ± 27 (4.7)	1,029 ± 249 (34.9)	43
Fecosterol	56.4 ± 14 (11.1)	467 ± 143 (15.8)	8
Episterol	47 ± 24 (9.2)	289 ± 96 (9.8)	6
Ergosterol	380 ± 198 (74.7)	1,140 ± 302 (38.7)	3
Total sterols	509 ± 251 (100)	2,948 ± 714 (100)	6

[a] Sterols (micrograms per gram [wet weight] of cells ± the standard deviation) from three separate cultures were extracted, and the average value is reported.
[b] Mass of sterols (micrograms per gram [wet weight] of cells ± the standard deviation) in the esterified form after saponification (three separate cultures were extracted, and the average value is reported).
[c] The SE/FS ratio represents the proportion of steryl ester (SE) relative to that remaining unesterfied (FS).

Regulation of *ARE* gene expression in response to impaired ergosterol biosynthesis. The observation that overexpression of *ARE1* and *ARE2* causes accumulation of different steryl esters led us to question whether these genes may also respond to different sterol signals. We were interested in determining whether early or late sterol pathway mutants, and thus, the accumulation of intermediates in these strains, would affect the transcription of *ARE1* and *ARE2*. To assess the effects of sterol intermediate accumulation in late sterol pathway mutants, an isogenic panel of ergosterol biosynthetic deletion mutants transformed with linearized plasmids pIU1141 and pIU1146 was assayed for β-galactosidase activity under aerobic conditions (Table 5). To assess the effects of a lesion in the essential gene *ERG24*, the ergosterol biosynthesis inhibitor fenpropimorph was used. Fenpropimorph targets both the sterol C_{14} reductase (*ERG24*) and the C_8 sterol isomerase (*ERG2*), but the C_{14} reductase is before the isomerase in the pathway.

ARE1 promoter activity increased in the *erg2*Δ (3.8-fold), *erg3*Δ (4.1-fold), and *erg6*Δ (3.3-fold) late pathway mutants, suggesting that *ARE1* expression is modulated in response to accumulation of ergosterol intermediates (Table 5). *ARE2* did not display significant transcriptional changes in response to the same mutations, suggesting that accumulation of sterol

TABLE 5. β-Galactosidase activity conferred by *ARE1-lacZ* and *ARE2-lacZ* promoter fusions in response to lesions or pharmacological inhibition of the ergosterol biosynthetic pathway

Strain or condition	β-Galactosidase activity (fold change relative to wild type)[d]	
	ARE1-lacZ	*ARE2-lacZ*
Wild-type BWG 1-7a[a]	1.6 ± 0.2 (1.0)	186 ± 17 (1.0)
erg2 mutant	6.0 ± 0.9 (3.8)	184 ± 30 (1.0)
erg3 mutant	6.5 ± 0.5 (4.1)	246 ± 25 (1.3)
erg6 mutant	5.2 ± 0.9 (3.3)	199 ± 30 (1.1)
Fenpropimorph added[b]	3.7 ± 0.3 (2.3)	339 ± 30 (1.8)
Wild-type JR 527[c]	1.7 ± 0.3 (1.0)	80 ± 11 (1.0)
hmg1 mutant	4.2 ± 0.5 (2.5)	156 ± 20 (2.0)
hmg2 mutant	1.5 ± 0.2 (0.9)	103 ± 16 (1.3)

[a] The *erg* mutants were created in the BWG 1-7a background.
[b] Fenpropimorph was added to strain BWG 1-7a at 0.5 μM, the cells were grown to 50% and then inhibited by the addition of 25 μM fenpropimorph, and the cells grew for an additional 18 h.
[c] JR527 is the wild-type strain isogenic to the *hmg1* and *hmg2* mutations.
[d] β-Galactosidase activity is reported as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein and is the average of two independent transformants assayed in duplicate over 3 days.

TABLE 6. β-Galactosidase activity conferred by *ARE1-lacZ* and *ARE2-lacZ* fusions on strains either bearing deletions in the endogenous *ARE* genes or overexpressing *ARE1* or *ARE2*

Genotype	β-Galactosidase activity (fold change relative to wild type)[a]	
	ARE1-lacZ	*ARE2-lacZ*
ARE1 ARE2 (wild type)	2.1 ± 0.3 (1.0)	140 ± 16 (1.0)
*are1*Δ *are2*Δ	2.0 ± 0.2 (0.95)	158 ± 18 (1.1)
*ARE1 are2*Δ	2.0 ± 0.3 (0.95)	177 ± 36 (1.3)
*are1*Δ *ARE2*	2.1 ± 0.3 (1.0)	154 ± 28 (1.1)
ARE1 ARE2/pS5	1.6 ± 0.3 (1.0)	126 ± 13 (1.0)
ARE1 ARE2/pADH5-36	2.0 ± 0.3 (1.3)	
ARE1 ARE2/pS5ARE2		150 ± 14 (1.2)

[a] β-Galactosidase activity is reported as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein and is the average of two independent transformants assayed in duplicate over 3 days.

intermediates is not a regulatory signal for *ARE2* expression. Both *ARE1* and *ARE2* expression increased in BWG 1-7a cells inhibited with fenpropimorph. Yeast cells inhibited with fenpropimorph accumulate ignosterol (4), an intermediate in sterol biosynthesis. These results were confirmed in Northern hybridizations (not shown).

We were also interested in the transcription of *ARE1* and *ARE2* in strains lacking the *HMG1* and *HMG2* genes encoding isoforms of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. *HMG1* encodes the major isoform for this activity, which is rate limiting for isoprenoid biosynthesis (16) and is oxygen regulated. HMG2p activity is increased during oxygen limitation (21). Expression of both *ARE1-* and *ARE2-lacZ* fusions in an *hmg1* mutant increased twofold over the wild-type level, while no significant changes were observed in the *hmg2* mutant (Table 5).

Transcription of *ARE1* and *ARE2* due to changes in esterification of sterol. To determine whether the *ARE1* and *ARE2* genes are subject to end product regulation, isogenic strains SCY059 (*are1*Δ *are2*Δ), SCY060 (*are1*Δ), SCY061 (*are2*Δ), and SCY062 (wild type) were transformed with pIU1141 and pIU1146 and assayed for β-galactosidase activity. Plasmids pADH (vector), pADH5-36 (*ARE1*), and pS5ARE2 were transformed into SCY059 (*are1*Δ *are2*Δ) to test whether overexpression of the *ARE* genes would alter transcription. These plasmids use the alcohol dehydrogenase (*ADH1*) promoter in place of the endogenous promoter to drive expression of *ARE1* and *ARE2*. The *ADH1* promoter is constitutively active and should not be subject to regulation. No significant differences in transcription of *ARE1* or *ARE2* were observed in the *are* mutant strains or when either gene was overexpressed (Table 6). The unchanged gene expression in response to mutations in the *ARE* genes, in addition to the lack of response when esters are overproduced, suggests that *ARE1* and *ARE2* are not subject to end product feedback inhibition at the level of transcription.

Divergent effects of heme and oxygen on *ARE1* and *ARE2* expression. Heme is required for sterol synthesis and as a cofactor for the cytochrome P450 enzymes lanosterol 14α-demethylase and sterol C-22 desaturase and is associated as a cytochrome b_5 cofactor with the C-5 desaturase and C-24 sterol methyl oxidase. Heme may also have a role in sterol esterification since δ-aminolevulinic acid (δ-ALA) synthase mutants (*hem1*) supplemented with δ-ALA displayed a fourfold increase in steryl ester synthase activity compared to heme de-

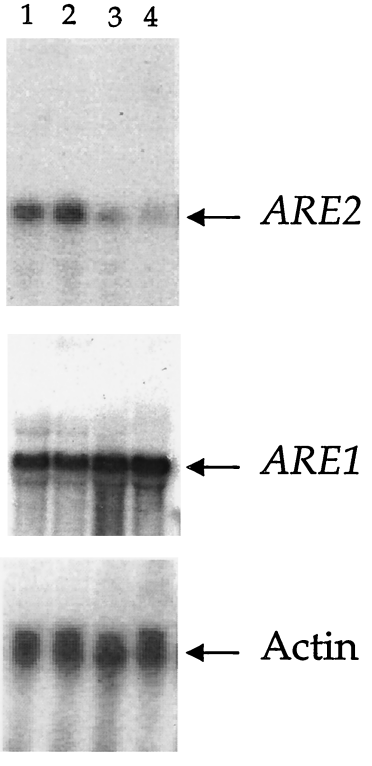

FIG. 2. RNA blot hybridization analysis of *ARE1* and *ARE2* gene expression in *hem1* mutants. Strain TKY22 was grown under heme-sufficient (50 μg of δ-ALA per ml; lanes 1 and 2) or heme-depleted (0.5 μg of δ-ALA per ml; lanes 3 and 4) conditions. Total RNA was extracted and analyzed by RNA blot hybridization with an *ARE1-* or *ARE2*-specific probe. The message from the *ACT1* gene encoding actin was used as a loading control.

TABLE 7. β-Galactosidase activity conferred by *ARE1-lacZ* and *ARE2-lacZ* fusions during changes in heme or due to mutations in transcription factors *HAP1* and *ROX1*

Strain (condition or genotype)	β-Galactosidase activity (fold change relative to wild type)[c]	
	ARE1-lacZ	*ARE2-lacZ*
TKY22 (50 μg of δ-ALA/ml)	1.8 ± 0.1 (1.0)	85 ± 17 (1.0)
TKY22 (0.5 μg of δ-ALA/ml)	9.3 ± 0.2 (5.2)	9.0 ± 1.5 (0.11)
BWG 1-7a[a]	1.9 ± 0.2 (1.0)	155 ± 19 (1.0)
LPY22 (*hap1*)	1.7 ± 0.3 (0.9)	25 ± 5.0 (0.16)
RZ53-6[b]	0.91 ± 0.17 (1.0)	43 ± 4.7 (1.0)
RZ53-6/*rox1*	4.6 ± 0.87 (5.1)	130 ± 20 (3.0)

[a] The *hap1* mutant was created in the BWG 1-7a background.
[b] RZ53-6 is the wild-type strain isogenic to the *rox1* mutant.
[c] β-Galactosidase activity is reported as nanomoles of *o*-nitrophenyl-β-D-galatopyranoside hydrolyzed per minute per milligram of protein and is the average of two independent transformants assayed in duplicate over 3 days.

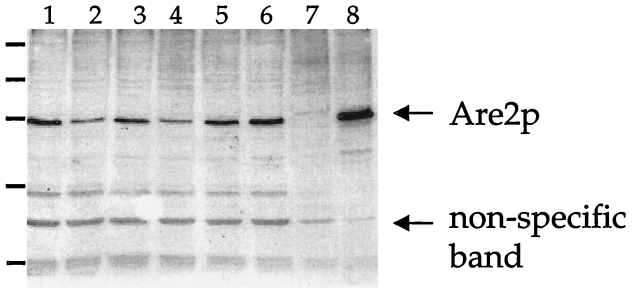

FIG. 3. Regulation of Are2p due to alterations in heme metabolism or mutations in the *HAP1* and *ROX1* transcription factors. Microsomes from wild-type (BWG, lane 1; RZ253–6, lane 5), *hap1* (lane 2), *rox1* (lane 6), or *hem1* (lanes 3 and 4 with 50 or 0.5 μg of δ-ALA per ml, respectively) cells grown to mid-logarithmic phase were prepared and resolved by denaturing gel electrophoresis (5 μg of total protein per lane). The proteins were transferred to nitrocellulose and probed with chicken IgY antibody generated against the NH_2-terminal 180 residues of Are2p (20). Detection of the immune complexes was attained by using a horseradish peroxidase-conjugated secondary anti-chicken IgY antibody (Promega) and the ECL Western blotting detection reagent (Amersham). Lanes 7 and 8 represent microsomes from *are1*Δ *are2*Δ cells carrying a vector control or *ARE2* on YEpARE2. The indicated nonspecific band serves as a loading control.

pleted cells (24). In strains lacking the *HEM1* gene (TKY22 [25]), supplementation with 50 μg of δ-ALA, the enzymatic product of Hem1p, per ml simulates a wild-type *HEM1* sterol profile. By contrast, the addition of 0.5 μg of δ-ALA per ml allows slow growth, low levels of ergosterol, and elevated amounts of lanosterol (45). TKY22 cells supplemented with 50 μg of δ-ALA per ml were compared to those exhibiting the *hem1* mutant sterol phenotype (0.5 μg of δ-ALA per ml). While *ARE2* was repressed by growth under δ-ALA-limiting conditions, the *ARE1* gene was clearly induced (Fig. 2). These results were verified by the introduction of the *ARE1* and *ARE2* reporter constructs into strain TKY22. *ARE2*-mediated expression was decreased ninefold in a *hem1* δ-ALA-deficient background, whereas *ARE1-lacZ* activity increased over fivefold in the same situation (Table 7). TKY22 cells grown in 50 μg of δ-ALA per ml have a wild-type sterol profile, accumulating ergosterol as the major sterol (45.6%). Strain TKY22 grown in 0.5 μg of δ-ALA per ml accumulates 16% lanosterol and markedly decreased levels of ergosterol in the total sterols (4.2%). Increased lanosterol esterification could reflect the up-regulation of *ARE1* in the *hem1* strain.

Heme is also required for activation of the transcriptional activator Hap1p (heme-activated protein) in response to oxygen (19). Hap1p regulates many genes involved in oxygen-requiring processes such as cytochrome synthesis, sterol biosynthesis, fatty acid biosynthesis, and oxidative stress response (52). Heme also mediates repression of hypoxic genes during aerobic growth by increased expression of the *ROX1* transcriptional repressor (46). Transcription of *ROX1* is regulated by the Hap1 activator. During aerobic growth, Rox1p binds to promoters of hypoxic genes, repressing their transcription. During anaerobiosis, expression of *ROX1* is decreased and hypoxic gene transcription is derepressed. To test the role of these transcription factors in the regulation of the *ARE* genes, we measured the activity of the promoter fusions in a strain lacking *HAP1* or *ROX1*. Strains BWG1-7a (wild type) and LPY22 (*hap1*Δ) are isogenic and were used to measure *ARE* transcription in a *hap1* deletion strain (37), while the effects of *ROX1* on the *ARE* genes were measured in the isogenic strains RZ53-6 (wild type) and RZ53-6/*rox1* (*rox1*Δ) (30). The effects of *HAP1* and *ROX1* on transcription of *ARE1* and *ARE2* are consistent with different effects of heme on *ARE1* and *ARE2* expression (Table 7). *ARE2* expression decreased sixfold in the *hap1* mutant. *ARE1* transcription was unchanged by the *hap1* mutation, consistent with the lack of a Hap1p consensus sequence in its promoter. Both *ARE1* and *ARE2* are upregulated by the absence of the transcriptional repressor *rox1* (fivefold and threefold, respectively).

We wished to confirm the effects of these alterations in gene transcription at the protein level and focused on the Are2 protein since it demonstrated striking changes in transcription profiles. By using a polyclonal antibody to the NH_2-terminal 180 residues of Are2p (20), we confirmed that the changes in transcript abundance produce a corresponding change in microsomal Are2 protein levels. The Are2 protein was more abundant in the presence of normal *HAP1* function and δ-ALA levels but was upregulated in the absence of the *ROX1* gene (Fig. 3).

DISCUSSION

Our results confirm and extend previous analyses indicating that the two yeast *ARE* genes have different physiological functions, especially in response to oxygen (46, 51). The most fundamental question is why yeast contains two sterol esterification genes and yet neither is essential for survival. Previous work using *are1* and *are2* strains indicate that the role of Are1p is to esterify sterol intermediates, principally lanosterol, the first sterol in the pathway, whereas Are2p principally esterifies the end product ergosterol (38, 48, 53). Our results are similar in that we overexpressed *ARE1*- and *ARE2*-containing plasmids in an *are1 are2* double deletion strain. Under the conditions reported here, the esterified-to-free ratio of lanosterol was fivefold greater in an *are1 are2* strain overexpressing *ARE1* relative to the same strain overexpressing *ARE2*. However, the esterified-to-free ratio of zymosterol in an *are1 are2* double

mutant overexpressing *ARE1* relative to the same strain overexpressing *ARE2* was only one-fourth. These results suggest that the role of *ARE1* under aerobic growth conditions is to limit conversion of lanosterol to zymosterol, thereby interrupting the pathway such that ergosterol precursors can be stored in microlipid droplets and mobilized to free sterols as required (28)

The role of *ARE1* in esterifying sterol intermediates is confirmed by our analysis indicating that accumulation of sterol intermediates in *erg2*, *erg3*, and *erg6* strains results in significant increases in *ARE1* expression (Table 5). *erg2* mutants accumulate sterol intermediates containing only Δ-8 sterols, *erg3* strains are unable to desaturate sterols at the C-5 position, and *erg6* mutants are unable to methylate the sterol side chain. All three strains are viable even though the end product ergosterol is not made. Although the accumulation of ergosterol intermediates gives rise to changes in *ARE* gene expression, a reduction in ergosterol, as seen in an *HMG1* mutant, also results in modest increases in both *ARE1* and *ARE2* expression. A mutation in the minor isoform of HMG-CoA reductase, *HMG2*, essentially does not affect *ARE* transcription.

Although an interruption in ergosterol biosynthesis or decreased cellular levels of ergosterol affect *ARE* gene transcription, mutations in the two *ARE* genes themselves have no effect on transcription. We found that *ARE1*-driven expression of the *lacZ* construct was unaltered in a wild-type, *are2*Δ, or *are1*Δ *are2*Δ strain or in a wild-type strain overexpressing *ARE1*. Similar results were observed for *ARE2* expression. Neither deletion nor overexpression of *ARE2* altered the expression of the *ARE2-lacZ* construct. The genes are thus not subject to end product regulation. Sterol synthesis is intimately dependent upon the cell's being heme competent. Both *ERG11* and *ERG5* encode cytochrome P450 enzymes required for C-14 demethylation and C-22 desaturation, respectively, and *ERG3* requires the cofactor cytochrome b_5 for desaturation at the C-5 position in the sterol molecule. The HMG-CoA reductase genes *HMG1* and *HMG2* are positively and negatively regulated by heme (45). It appears that a similar situation of contraregulation exists for *ARE1* and *ARE2*, which are induced or repressed by heme (Table 7). We demonstrated a sixfold decrease in *ARE2* expression in a *hap1* strain, and Thorsness et al. (45) demonstrated a 23-fold decrease in *HMG1* expression in a *hap1* strain. However, the promoter for neither gene predicts canonical *HAP1* DNA binding motifs (5′ CGGNNNTANCGG 3′ [27]). Similarly, the *ERG9* promoter lacks a *HAP1* recognition motif and yet a twofold decrease in *ERG9-lacZ* expression is seen in a *hap1* background (26). These results suggest either that the effects of the *HAP1* mutations are indirect or that novel *HAP1* binding domains exist in ergosterol biosynthetic genes. Lastly, our results indicate an increase in *ARE1* expression during anaerobiosis. This is consistent with data reported by Valachovic et al. (48) and also with a DNA microarray analysis of anaerobically growing cells which revealed an eightfold increase in *ARE1* message levels (44). We demonstrated that in a *rox1* mutant background, both *ARE1* and *ARE2* expression increased. The *ARE1* promoter does contain a canonical *ROX1* recognition sequence (3′ GCTATTGTTCGC 5′ [29]) located on the antisense strand at −135 bp upstream of the ATG. However, it is unclear how *ROX1* exerts an effect on the transcription of *ARE2*, although similar results were seen in an *ERG9-lacZ* fusion, which also lacks a *ROX1* recognition motif (26).

This investigation elucidates an important physiological role for Are1p during anaerobic growth when heme is limiting, as well as under conditions under which ergosterol is either not synthesized or made at less than wild-type levels. Conversely, we show that Are2p is optimally required during aerobiosis when ergosterol is plentiful.

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