Comprehensive evaluation of isoprenoid biosynthesis regulation in *Saccharomyces cerevisiae* utilizing the Genome Reporter Matrix™

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Abstract **Gene expression profiling is rapidly becoming a mainstay of functional genomic studies. However, there have been relatively few studies of how the data from expression profiles integrate with more classic approaches to examine gene expression. This study used gene expression profiling of a portion of the genome of *Saccharomyces cerevisiae* to explore the impact of blocks in the isoprenoid biosynthetic pathway on the expression of genes and the regulation of this pathway. Approximately 50% of the genes whose expression was altered by blocks in isoprenoid biosynthesis were genes previously known to participate in the pathway. In contrast to this simple correspondence, the regulatory patterns revealed by different blocks, and in particular by antifungal azoles, was complex in a manner not anticipated by earlier studies.**—Dimster-Denk, D., J. Rine, J. Phillips, S. Scherer, P. Cundiff, K. DeBord, D. Gilliland, S. Hickman, A. Jarvis, L. Tong, and M. Ashby. **Comprehensive evaluation of isoprenoid biosynthesis regulation in *Saccharomyces cerevisiae* utilizing the Genome Reproter Matrix™. *J. Lipid Res.* 1999.** 40: **850–860.**

Supplementary key words isoprenoid pathway regulation • genome expression profiling • *Saccharomyces cerevisiae*

Eukaryotic cells utilize a group of structurally related compounds, the isoprenoids, for a vast array of cellular processes. These processes include structural composition of the lipid bilayer, electron transport during respiration, protein glycosylation, tRNA modification, and protein prenylation. All isoprenoids are synthesized via a pathway known variously as the isoprenoid pathway, mevalonate pathway, or sterol biosynthetic pathway. Although the bulk end product of the pathway is sterols, there are several branches of the pathway that lead to non-sterol isoprenoids (Fig. 1). Due to the involvement of isoprenoids in a variety of physiologically and medically important processes, a comprehensive understanding of the regulation of this pathway would offer many scientific and practical benefits.

The regulation of the isoprenoid biosynthetic pathway is known to be complex in all eukaryotic organisms examined, including the budding yeast *Saccharomyces cerevisiae* (1–3). The overriding principle for the regulation of this pathway is multiple levels of feedback inhibition. This feedback regulation is keyed to multiple intermediates and appears to act at numerous steps of the pathway, involving changes in transcription, translation, and protein stability. Additionally, the availability of molecular oxygen, required for sterol and heme biosynthesis, also regulates the expression of genes at key steps of the pathway (4, 5). The emerging picture is that the isoprenoid pathway has numerous points of regulation that act to control overall flux through the pathway as well as the relative flux through various branches of the pathway. Given this complexity, it can be difficult to understand the rationale for any given instance of regulation of this pathway, unless it is viewed within the context of the entire pathway (2).

We have developed the ability to perform and analyze the level of gene expression from essentially every gene of an organism's genome simultaneously. The experiments reported here use Acacia's yeast Genome Reporter Matrix™ (GRM) which consists of a collection of reporter gene fusions to >95% of the protein coding genes of *S. cerevisiae*. To examine the regulation of the isoprenoid pathway in a more comprehensive fashion, the GRM technology was used to evaluate the expression of all genes in the pathway using reporter constructions that detect changes in both the transcription and translation of genes. Because natural selection operates on a selected outcome rather than on a particular molecular mechanism, profiling strategies that detect regulatory changes at several molecular mechanism contribute to a broader view of how regulatory circuits have evolved.

Abbreviations: GRM, Genome Reporter Matrix™; PCR, polymerase chain reaction; MCS, multiple cloning site; ORF, open reading frame.

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MATERIALS AND METHODS

Strains and media conditions

The strain of *Saccharomyces cerevisiae* used in this study, ABY11 (*MATa leu2Δ1 ura3-52*), was derived from S288c. GRM arrays were grown at 30°C on solid casamino acid medium (Difco) with 2% glucose and 0.5% UltraPure Agarose (Gibco BRL). The medium was supplemented with additional amino acids and adenine (Sigma) at the following concentrations: adenine and tryptophan at 30 μg/ml; histidine, methionine, and tyrosine at 20 μg/ml; leucine and lysine at 40 μg/ml. Stock solutions of the supplements were made at 100× concentrations in water. Yeast cells were transformed with the reporter plasmids by the lithium acetate method (6, 7).

The drug treatments were performed by adding the drugs directly to the liquefied medium during preparation. The HMG-CoA synthase inhibitor (L659-699) and the squalene synthase inhibitor (zaragozic acid) were provided by J. Bergstrom (Merck). The remaining drugs were purchased commercially. Stock solutions of drugs were prepared in 100% solvent (e.g., DMSO, methanol, or water). When required, the lactone forms of the statins were converted as previously described (8). The final concentration of each drug (and solvent) used is listed in Table 1.

Construction of reporter gene fusions

The regulatory region of each yeast gene was cloned into one of two vectors, pAB1 or pAB2. The vector pAB1 was constructed in the following manner. First, the polymerase chain reaction (PCR) was used to amplify the transcriptional terminator region from the gene *PGK1* using the oligonucleotides 5′-PGKTERM (5′-GATTGAATTCAATTGAAATCGATAG-3′) and 3P-PGKTERM (5′-CCGAGGCGCCGAATTTTCGAGTTAT-3′). The amplified fragment consists of the 263 base-pair region immediately downstream of the *PGK1* stop codon, and contains an *Eco*RI site at the 5′ end and a *Nar*I site at the 3′ end. These restriction sites were engineered into the two PCR primers (underlined sequences). The terminator was then cloned into YIplac211 (9) that had been linearized with *Eco*RI and *Nar*I, yielding pAB34. Next, the coding region of the green fluorescent protein (GFP) from *Aequoria victoria* (10) was amplified by PCR using the oligonucleotides 5P-GFP-ORF (5′-CATGTCTAGAGGAGAAGAACTTTTC-3′) and 3P-GFP-ORF (5′-CGCGAATTCCTATTTGTATAGTTCA-3′). Again, these oligonucleotides contain engineered *Xba*I and *Eco*RI sites at the 5′ and 3′ ends, respectively (underlined). This fragment was cloned into pAB34, linearized with *Xba*I and *Eco*RI, to produce pAB35. Finally, the GFP-PGK terminator fragment was moved into the episomal vector YEplac195 (9) as an *Xba*I/*Nar*I fragment, thereby producing pAB1.

The vector pAB2 is pAB1 with an altered multiple cloning site (MCS). The new MCS contains 8 base-pair recognition sites for three restriction enzymes. These larger 8 base-pair recognition sites occur less frequently throughout the yeast genome than the 6 base-pair sites present in the MCS of pAB1. Thus, the utilization of restriction enzymes that recognize 8 base-pair sequences to clone the various regulatory regions (engineered into the PCR primers used to amplify the regions) would minimize the occurrence of those sites within the regions themselves. To construct pAB2, pAB1 was linearized with *Xba*I and *Sph*I, dropping out the existing MCS, and an adapter containing the new MCS was ligated in. The adapter was made by hybridizing two oligonucleotides, 8Cutter (5′-CGGCGCGCCGCGGCCGCATGGCCGGCCAAT-3′) and 8CutEnd (5′-CTAGATTGGCCGGCCATGCGGCCGCGGCGCGCCGCATG-3′). This adapter has sites for the restriction enzymes *Fse*I, *Not*I, and *Asc*I (underlined).

The promoter regions were cloned utilizing PCR of genomic DNA prepared from a strain derived from S288c; JRY147 (*MATa SUC2 mal mel gal2 CUP1*). The promoter-specific primers were designed such that the proximal primer spanned the start codon of the specific gene and included a few (usually four) codons derived from the gene. The position of the distal primer was determined on a case-by-case basis depending on the distance to, and orientation of, the neighboring open reading frame (ORF) and the restriction sites present. Where the upstream ORF was positioned in a divergent orientation and within 1,200 base-pairs, the size of the promoter fragment amplified was adjusted such that all nucleotides up to, but not including, the start codon of the upstream ORF were present. In cases where the upstream ORF was situated in the same orientation, the amplified fragment was designed to extend into the coding region but not so as to include the start codon. The approximate sizes of the various regulatory regions present on the reporter plasmids presented in this study are given (Table 2). Both primers had restriction enzyme recognition sites engineered into the ends to allow the subsequent cloning of the PCR fragment into pAB1, or pAB2.

Determinations of reporter gene expression levels

The individual strains comprising the GRM were maintained as independent colonies (and cultures) in a 96-well format, in medium selecting for the *URA3*-containing reporter plasmid. Prior to each experiment, fresh dilutions of the reporter-containing strains were inoculated and grown overnight at 30°C. The fresh cultures were then arrayed onto solid casamino acid medium at high density using an aspirate/dispense machine, the BioDot A/D3200 (Cartesian Technologies, Irvine CA). As stated, the drugs were added to the solid medium during preparation. Once arrayed, each plate was grown at 30°C, usually for 21 h.

The level of fluorescence expressed from each reporter gene fusion was determined using a Molecular Dynamics Fluorimager SI. AIS image analysis software (Imaging Research, Ontario CA) was used to quantitate the fluorescence of each colony in the images. Generally, the drug treatments were performed at several concentrations, with the analysis based upon the concentration producing the most informative expression profile (see below).

RESULTS AND DISCUSSION

The experiments presented here used a subset of the complete Genome Reporter Matrix™ (GRM) consisting of 864 independent yet isogenic yeast strains, each containing a different reporter gene fusion (the 864 GRM). The 864 reporter genes included genes known or implicated to be involved in isoprenoid metabolism as well as genes diagnostic of the overall physiological state of the cell. Specifically, with regard to isoprenoid metabolism, the partial genome matrix contained reporter gene fusions for 21 genes involved in the biosynthesis and storage of ergosterol, the major sterol product of the isoprenoid pathway in yeast. Reporter genes were also included for genes involved in the utilization of isoprenoid intermediates for the synthesis of coenzyme Q, hemeA, isopentyl tRNA modifications, and protein prenylation (**Fig. 1**). Additionally, reporter genes were included for genes involved in cellular processes that require isoprenoids for function, such as the mating pheromone response pathway and the ras oncoprotein.

Treatments with inhibitors of isoprenoid biosynthesis

The gene expression levels of the 864 GRM were determined in response to treatments of cells with eight differ-

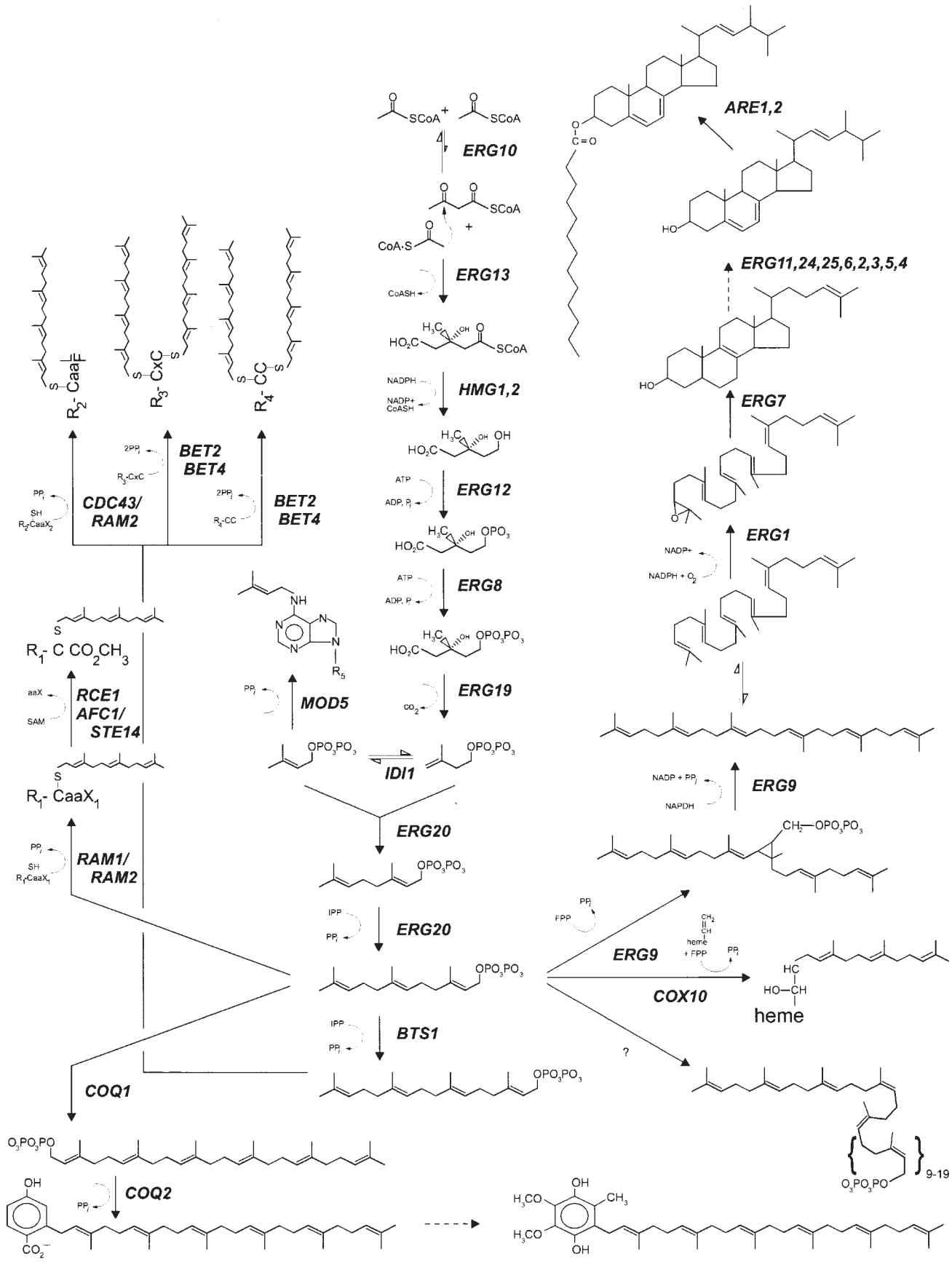

Fig. 1. Isoprenoid biosynthesis in the budding yeast, *Saccharomyces cerevisiae*. The structures of the intermediates and the gene names encoding the various enzymatic steps are shown. Farnesyl diphosphate, the product of the *ERG20* encoded step, is a major branch point in the pathway leading to the synthesis of sterols, hemeA, coenzymeQ , protein prenylation, and tRNA modification. As the biosynthesis of heme itself does not involve isoprenoids, the function of Hem14p is not shown. However, the farnesyl transferase activity (Cox10p) involved in forming hemeA is shown.

TABLE 1. Treatments with inhibitors of isoprenoid biosynthesis

Experiment	Drug	Method of Treatment	Number of Genes at Least 2-Fold Induced	Number of Genes at Least 2-Fold Repressed	Total Number of Genes with 2-Fold Effect
1	L-659-699	0.8 μg/ml in 1% DMSO, 21 h growth	34 (3.9%)	71 (8.2%)	105 (12.2%)
2	Lovastatin	10 μg/ml in H_2O, 21 h growth	46 (5.3%)	36 (4.2%)	82 (9.5%)
3	Fluvastatin	8 μg/ml in H_2O, 21 h growth	58 (6.7%)	81 (9.4%)	139 (16.1%)
4	Atorvastatin	18 μg/ml in 0.36% MeOH 21 h growth	70 (8.1%)	77 (8.9%)	147 (17.0%)
5	Zaragozic Acid	6 μg/ml in 1% DMSO, 21 h growth	37 (4.3%)	33 (3.8%)	70 (8.1%)
6	Miconazole	0.08 μg/ml in 1% DMSO, 21 h growth	92 (10.6%)	121 (14.0%)	213 (24.6%)
7	Sulconazole	0.156 μg/ml in 1% DMSO, 21 h growth	161 (18.6%)	164 (19.0%)	325 (37.6%)
8	Fluconazole	4 μg/ml in 9 mg/ml NaCl in H_2O, 21 h growth	88 (10.2%)	96 (11.1%)	184 (21.3%)

GRM responses to representative treatments with inhibitors of isoprenoid biosynthesis. Cells were treated with eight drugs that target four different enzymatic steps: HMG-CoA synthase (L659-699), HMG-CoA reductase (lovastatin, fluvastatin, and atorvastatin), squalene synthase (zaragozic acid), and lanosterol 14α-demethylase (miconazole, sulconazole, and fluconazole). These experiments were conducted utilizing a Genome Reporter Matrix™ consisting of 864 yeast strains (864 GRM), each harboring a different reporter gene fusion. Fresh cultures of the 864 GRM were arrayed onto solid media plates containing either a drug (treated) or a no-drug solvent control (untreated). After 21 h of growth, the plates were assayed to determine the level of expression for each of the 864 GRM reporter gene fusions. For each of these representative treatments, the number of genes exhibiting at least a 2-fold change in expression, relative to untreated cells, are indicated.

ent inhibitors of the isoprenoid pathway. These eight drugs target four different enzymatic steps of the pathway. L659-699 inhibits HMG-CoA synthase, encoded by *ERG13* (11). Three of the drugs studied (lovastatin, fluvastatin, and atorvastatin) all inhibit HMG-CoA reductase, encoded by two genes *HMG1* and *HMG2* (12). Zaragozic acid inhibits squalene synthase, encoded by *ERG9* (13). Finally, three different azoles (miconazole, sulconazole, and fluconazole) inhibit lanosterol 14α-demethylase, encoded by *ERG11* (14).

The 864 GRM was treated with these eight inhibitors at varying concentrations and in varying solvents (**Table 1**). The concentration of each inhibitor was chosen to achieve a similar degree of growth inhibition. The number (and percentage) of genes among those represented in the 864 GRM exhibiting at least a 2-fold induction, or repression, of expression in response to each treatment is shown for a representative set of experiments (Table 1).

The specificity of the feedback regulation observed in this study was quite remarkable. For example, the HMG-CoA synthase inhibitor caused an induction of at least 2-fold of only 34 genes of the 864 GRM (Table 1). Of these 34 genes, 17 of them are known to be directly involved in ergosterol metabolism. Indeed, of these 17 genes induced by the inhibition of HMG-CoA synthase, 15 encode enzymes of the ergosterol biosynthetic pathway. The other two genes are involved in ergosterol storage (*ARE1*) and hemeA biosynthesis (*HEM14*). Many of the remaining 17 genes were of unknown function, and their involvement in ergosterol metabolism merits investigation. This block did not affect the genes that encode enzymes involved in the four branches off of the sterol pathway, with the exception of *HEM14*. Thus, the feedback regulation of the isoprenoid pathway primarily involved the central pathway leading to ergosterol biosynthesis, and not the various non-sterol branches of the pathway (Fig. 1).

Interestingly, for comparable degrees of growth inhibition, the azoles caused a larger percentage of genes to change expression levels than did other blocks of the isoprenoid pathway (Table 1). This wider response to azoles may represent a lower degree of specificity of these drugs for their target, or may reflect a broader response to the inhibition of lanosterol demethylase activity in the cell (see below). Nevertheless, all agents affected the expression of 8% or more of the genes in this subset of the genome. Thus, the gene expression profiles caused by enzyme inhibitors provided a rich signal by which to compare profiles.

The results presented here focus on reporters for 50 genes involved in isoprenoid metabolism (**Table 2**). Specific changes in the expression of these 50 genes in response to the inhibitor treatments are shown (**Table 3**). The responses are expressed as the fold change relative to the solvent control. No change is indicated by a fold change of 1.0. Induced expression is indicated by a positive value while repressed expression has a negative value. The ratios shown are an average of at least two experiments, except miconazole and sulconazole which were single experiments. Graphic representations of these data are also presented (**Fig. 2**, **Fig. 3**, and **Fig. 4**).

Feedback regulation of ergosterol biosynthesis genes

In general, a block at all four steps of ergosterol biosynthesis examined caused a compensatory induction of the expression of genes involved in ergosterol biosynthesis (Fig. 2). The expression of a subset of genes encoding enzymes of sterol biosynthesis (*ERG12*, *ERG8*, *ERG19*, *ERG9*,

TABLE 2. Translational reporter gene fusion

Plasmid	Promoter Size	Gene	Gene Product Function
pACA1	1027	*ERG10*	Acetoacetyl-CoA thiolase
pACA2	1165	*ERG13*	HMG-CoA synthase
pACA3	1596	*HMG1*	HMG-CoA reductase, isozyme 1
pACA4	1378	*HMG2*	HMG-CoA reductase, isozyme 2
pACA5	714	*ERG12*	Mevalonate kinase
pACA6	1277	*ERG8*	Phosphomevalonate kinase
pACA7	1420	*ERG19*	Mevalonate diphosphate decarboxylase
pACA8	891	*IDI1*	Isopentenyl-diphosphate δ-isomerase
pACA9	1240	*ERG20*	Farnesyl pyrophosphate synthase
pACA10	975	*ERG9*	Squalene synthase
pACA11	1600	*ERG1*	Squalene monooxygenase
pACA12	1512	*ERG7*	Lanosterol synthase
pACA13	1239	*ERG11*	Cytochrome P450, lanosterol 14α-demethylase
pACA14	530	*ERG24*	C-4 sterol reductase
pACA15	891	*ERG6*	S-adenosylmethionine δ-24-sterol-C-methyltransferase
pACA16	752	*ERG2*	C-8 sterol isomerase
pACA17	811	*ERG3*	C-5 sterol desaturase
pACA18	1397	*ERG5*	Cytochrome P450, sterol C-22 desaturase
pACA19	596	*ERG4*	Sterol C-24 reductase
pACA40	808	*ARE1*	Acyl-CoA:sterol acyltransferase
pACA41	1000	*ARE2*	Acyl-CoA:sterol acyltransferase
pACA20	1516	*COQ1*	Hexaprenyl pyrophosphate synthase
pACA21	819	*COQ2*	Para-hydroxybenzoate polyprenyltransferase
pACA22	966	*COQ3*	3-Demethylubiquinone-9 3-methyltransferase
pACA23	619	*COQ6*	Monooxygenase, required for coenzymeQ synthesis
pACA26	600	*COQ7*	Required for coenzymeQ synthesis
pACA28	1120	*COX10*	Farnesyl transferase, required for heme A synthesis
pACA29	1290	*MOD5*	tRNA isopentenyltransferase
pACA502	1211	*HEM14*	Protoporphyringen oxidase
pACA30	509	*RAM1*	Farnesyl transferase, α subunit
pACA31	536	*RAM2*	Farnesyl (geranylgeranyl, type I) transferase, β subunit
pACA33	1590	*RCE1*	CAAX protease
pACA32	1026	*AFC1*	CAAX protease
pACA34	1211	*STE14*	Farnesyl cystein:carboxyl methyltransferase
pACA35	1333	*BTS1*	Geranylgeranyl diphosphate synthase
pACA36	985	*CDC43*	Geranylgeranyltransferase type I, α subunit
pACA37	1047	*BET2*	Geranylgeranyltransferase type II β subunit
pACA38	711	*BET4*	Geranylgeranyltransferase type II α subunit
pACA64	1211	*MFA1*	Mating pheromone **a**-factor
pACA128	1208	*MFA2*	Mating pheromone **a**-factor
pACA84	692	*STE2*	α-factor receptor
pACA357	611	*STE18*	Guanine nucleotide-binding protein, γ subunit of the pheromone pathway
pACA1753	1211	*BEM1*	Component of the mating pathway, involved in polarized cell growth
pACA184	1211	*FAR1*	Involved in cell cycle arrest for mating
pACA1600	1211	*FIG1*	Factor induced gene, required for efficient mating
pACA56	1607	*FUS1*	MAP kinase, involved in the mating pheromone signal transduction pathway
pACA1865	581	*KAR4*	Regulatory protein required for pheromone induction of karyogamy genes
pACA844	1211	*FAR3*	Involved in pheromone mediated cell cycle arrest
pACA66	987	*RAS1*	GTP-binding protein, involved in regulation of the cAMP pathway
pACA131	1013	*RAS2*	GTP-binding protein, involved in regulation of the cAMP pathway

Fifty translational reporter gene fusions examined. The plasmid name, approximate size of the promoter included, the gene name, and the function of the gene are listed for each reporter fusion. The sizes of the promoter fragments present in the reporter gene fusion are approximate due to the addition of restriction sites engineered into the ends of the PCR primers for purposes of cloning. The first few codons of each gene (up to eight) are also included in the fusion.

and *ERG2*) were strongly induced by all eight drugs. These genes encode enzymes that act both early in the pathway (prior to the synthesis of farnesyl diphosphate) and late in the pathway. In principle, this regulation could be due to the depletion of an intermediate(s) downstream of a given block, or the accumulation of an intermediate(s) upstream of the block. The blocks imposed at various steps of the pathway would be expected to accumulate different upstream intermediate(s). In contrast, each block would deplete some common late intermediate(s) or end product(s). Therefore, the simplest interpretation was that the regulatory response of these genes was keyed to the depletion of a common intermediate(s). The latest block examined was that of lanosterol demethylase, so by this interpretation the regulation was keyed to a pathway product(s) downstream of lanosterol. Also, *ERG9*

TABLE 3. Specific changes in gene expression in response to treatments with inhibitors of isoprenoid biosynthesis

Gene	L659–699	Lovastatin	Fluvastatin	Atorvastatin	Zaragozic Acid	Miconazole	Sulconazole	Fluconazole
				Ergosterol Biosynthesis				
ERG10	2.2	3.2	4.7	3.5	2.5	−1.3	−3.0	−1.3
ERG13	−1.2	1.3	1.1	1.0	1.3	1.0	n/a	−1.1
HMG1	3.7	2.3	1.8	1.8	3.2	2.7	1.5	1.3
HMG2	−1.3	−1.1	−1.3	−1.5	−1.3	−1.3	−1.3	−1.2
ERG12	3.9	3.0	5.8	3.9	2.9	3.0	2.2	2.9
ERG8	7.8	5.0	5.0	6.4	5.5	9.0	11.0	4.3
ERG19	5.5	3.9	2.7	5.2	3.9	3.0	2.2	3.5
IDI1	1.2	1.3	1.5	1.2	1.8	−4.1	−3.0	−1.2
ERG20	2.1	2.4	2.9	2.1	1.6	2.0	1.2	−1.3
ERG9	4.3	2.0	2.3	2.3	2.0	4.5	11.0	2.3
ERG1	3.0	2.0	3.3	1.5	1.6	1.8	5.5	−1.3
ERG7	2.5	1.8	1.6	n/a	n/a	2.5	4.1	−1.2
ERG11	2.9	2.0	2.7	2.5	1.4	1.5	3.7	−1.8
ERG24	2.9	1.2	1.4	1.3	1.3	2.5	3.3	1.1
ERG6	1.9	1.7	1.9	2.0	1.6	1.3	2.7	−1.6
ERG2	14.9	3.9	4.5	7.8	6.7	12.2	14.9	3.7
ERG3	−1.3	1.5	1.5	1.0	1.1	−2.5	−2.0	−2.1
ERG5	2.6	1.6	2.0	1.3	1.3	1.3	2.5	−2.3
ERG4	1.9	1.6	1.6	1.3	1.3	1.5	2.7	−3.3
ARE1	3.3	1.5	1.4	2.5	1.9	6.0	4.1	1.5
ARE2	1.3	1.4	1.7	1.5	1.4	−1.8	−2.2	1.6
				Heme Biosynthesis				
COX10	−1.3	−1.3	−1.1	−1.3	−1.4	1.1	1.5	1.0
HEM14	4.1	2.7	5.5	3.5	2.3	4.1	2.0	2.2
				Protein Prenylation				
RAM1	−1.2	1.3	−1.2	−1.3	1.0	1.0	1.0	−1.2
RAM2	1.0	1.3	−1.2	1.2	1.1	1.2	1.5	−1.3
RCE1	1.1	−1.2	−1.4	−1.3	−1.1	1.5	2.2	−1.1
AFC1	1.1	−1.3	−1.4	−1.3	−1.4	−3.3	1.8	−1.3
STE14	−1.3	−1.4	1.1	−1.5	−1.8	1.5	2.0	−1.1
BTS1	−1.1	−1.1	1.1	−1.2	−1.1	2.0	2.2	1.1
CDC43	−1.3	−1.2	1.1	−1.3	−1.3	−1.1	−1.2	−1.2
BET2	−1.1	−1.1	1.1	1.3	−1.1	−1.1	−1.3	1.1
BET4	1.0	−1.2	1.1	1.1	−1.2	1.8	1.0	1.0
				tRNA Modification				
MOD5	1.7	1.1	1.2	1.1	−1.2	1.0	−1.2	−2.5
				CoenzymeQ Biosynthesis				
COQ1	−1.5	−1.7	−1.7	−2.1	−1.3	2.5	5.0	1.4
COQ2	1.3	1.2	1.3	1.7	1.0	1.5	2.2	−1.1
COQ3	−1.1	1.1	1.1	1.0	1.2	1.0	1.6	−1.1
COQ6	−1.5	−1.3	−1.7	1.0	−1.2	1.2	2.0	1.1
COQ7	−1.7	1.2	−1.2	−1.1	1.1	1.1	1.3	−1.8
				Mating Response Pathway				
MFA1	−2.2	−1.7	−2.2	−2.5	−1.9	−6.7	−2.2	−1.6
MFA2	−1.6	−1.5	−1.9	−1.7	−2.5	−1.2	−1.1	−3.0
STE2	−1.5	−1.7	−1.1	−1.6	−2.6	−1.1	−1.5	−3.2
STE18	1.2	−1.3	−1.8	−1.4	1.2	−1.8	−2.0	−1.2
BEM1	1.6	2.0	2.2	2.2	2.3	−5.5	−4.1	1.5
FAR1	−2.3	−1.5	−1.5	−1.7	−1.7	1.6	1.0	−1.2
FIG1	−10.0	−2.5	−4.1	−2.5	−5.0	−5.0	−5.5	−2.5
FUS1	−8.6	−3.0	−5.0	−5.0	−5.0	18.2	−3.3	−1.4
KAR4	−2.0	−1.4	−2.0	−1.3	−1.4	−1.2	1.6	−1.1
FAR3	−1.5	1.0	−1.4	−1.2	−1.1	1.1	−1.3	−2.2
				Ras				
RAS1	−3.0	−2.5	−2.9	−2.7	−3.2	1.6	1.6	−1.1
RAS2	−1.2	−1.1	−1.7	−1.5	−1.2	1.3	1.1	1.0

Specific changes in levels of gene expression in response to treatments with inhibitors of isoprenoid biosynthesis. Specific treatments are listed in the top row. Subsets of the genes comprising the 864 GRM are organized by functional groupings. Responses are expressed as the fold change relative to the solvent control (i.e., no change is a fold change of 1.0). Those genes that exhibited an induction of expression in response to a treatment have a positive numerical value, while those that were repressed have a negative value. Responses shown are an average of multiple experiments.

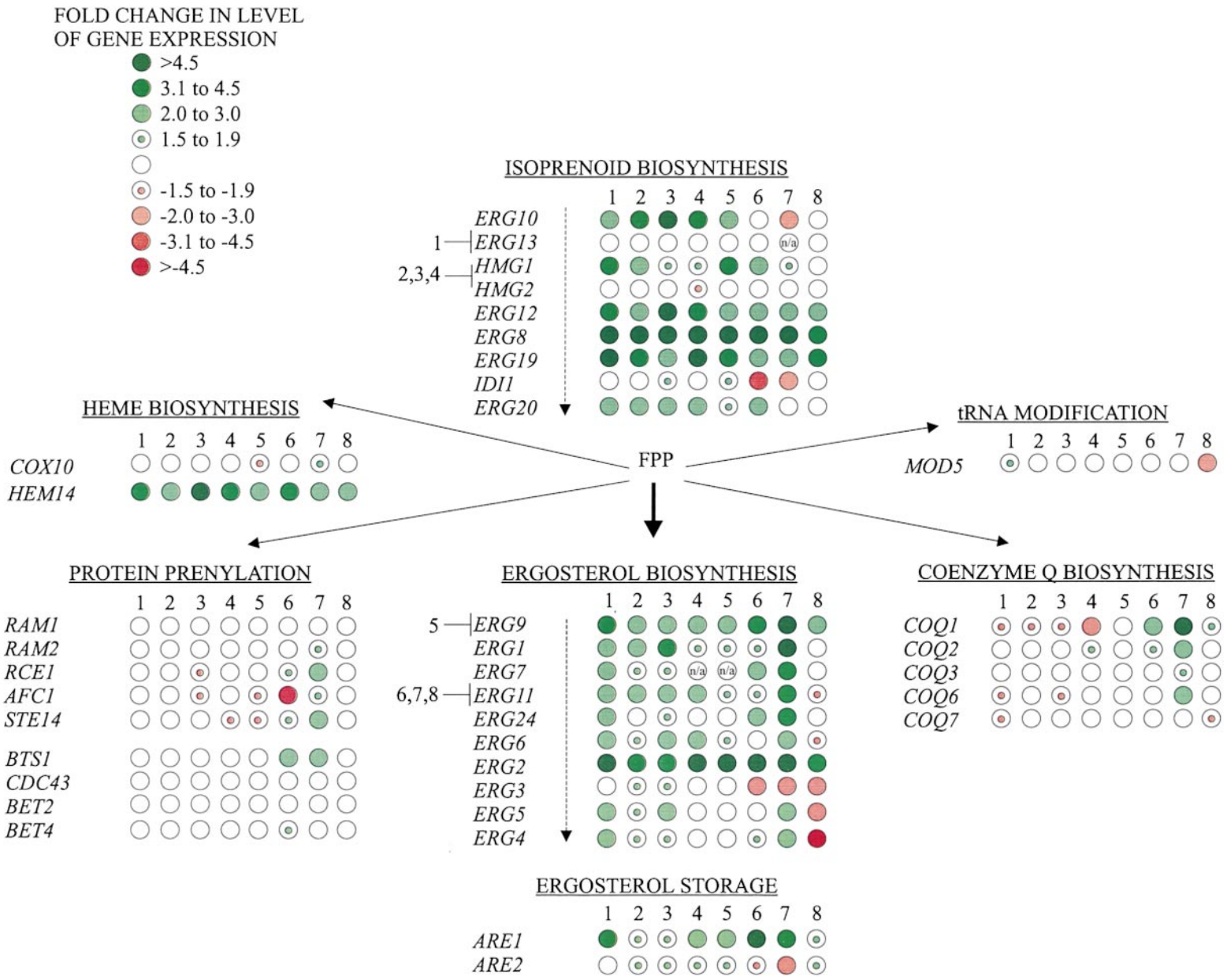

Fig. 2. Changes in the expression of genes involved in isoprenoid metabolism caused by inhibition of isoprenoid biosynthesis. Cells were treated with eight drugs: L659-699 (1), lovastatin (2), fluvastatin (3), atorvastatin (4), zaragozic acid (5), miconazole (6), sulconazole (7), and fluconazole (8). The points along the isoprenoid biosynthetic pathway where each of these drugs blocks the pathway are indicated (bars). The genes are organized into functional groups, and those of the isoprenoid and ergosterol biosynthesis groups are ordered with respect to the pathway (dashed arrows). Genes that exhibited less than a 2-fold change in expression are either not colored, or have a smaller colored dot to demonstrate the trends of slight changes (greater than 1.5-fold but less than 2-fold). Genes that exhibited at least a 2-fold change in expression are color coded according to the gradation shown (green for genes induced and red for genes repressed).

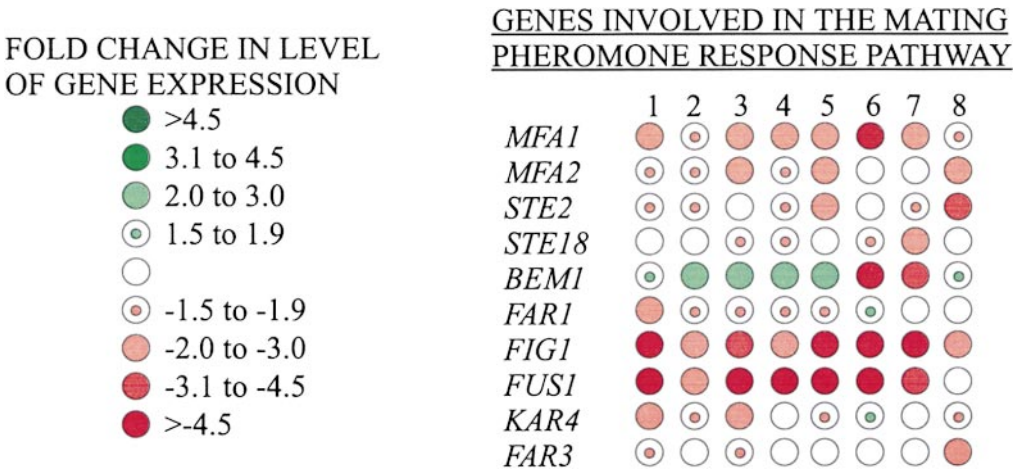

Fig. 3. Changes in the expression of genes involved in the mating pheromone response pathway caused by inhibition of isoprenoid biosynthesis. Cells were treated with eight drugs: L659-699 (1), lovastatin (2), fluvastatin (3), atorvastatin (4), zaragozic acid (5), miconazole (6), sulconazole (7), and fluconazole (8). Genes that exhibited less than a 2-fold change in expression are not colored (or have a smaller colored dot to indicate slight effects). Genes that exhibited at least a 2-fold change in expression are color coded according to the gradation shown.

mRNA is known to be induced in cells treated with lovastatin (15), indicating that the regulatory mechanism at work here includes changes in the mRNA levels.

Several other genes of this pathway (including *ERG10*, *HMG1*, *ERG20*, *ERG1*, *ERG11*, and *ERG6*) were induced by some, but not all, blocks to ergosterol biosynthesis (Fig. 2). Among these genes, some are known to respond to feedback regulation by the isoprenoid pathway. The regulated expression of *ERG10* observed in this study was consistent with a previous report demonstrating that *ERG10* is transcriptionally repressed by a late intermediate(s) or product(s) of the pathway (16). One of the two HMG-CoA reductase isozymes, Hmg1p, is translationally repressed by a non-sterol product of the pathway (8). In the experiments shown here, the *HMG1* reporter gene was induced by an inhibitor of squalene synthase and an inhibitor of lanosterol demethylase, suggesting that *HMG1* responded to the levels of a sterol product of the pathway (Fig. 2). These disparate observations may be due to some procedural differences in the execution of the experiments (see below).

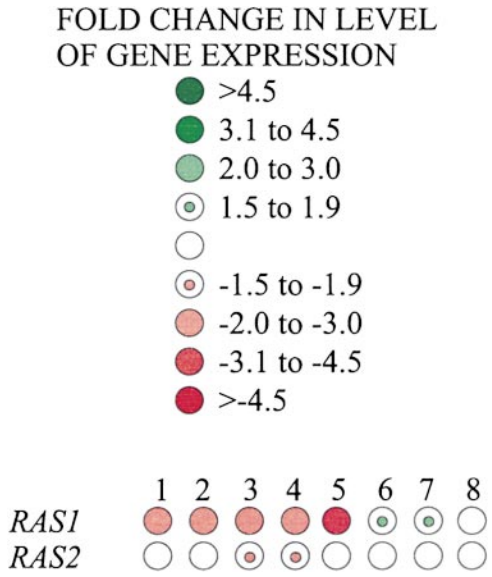

Fig. 4. Differential regulation of Ras gene expression caused by inhibition of isoprenoid biosynthesis. Cells were treated with eight drugs: L659-699 (1), lovastatin (2), fluvastatin (3), atorvastatin (4), zaragozic acid (5), miconazole (6), sulconazole (7), and fluconazole (8). *RAS2* exhibited less than 2-fold changes in expression in response to the treatments and the changes are therefore not colored (or have a smaller colored dot to indicate slight effects). *RAS1* on the other hand, exhibited at least a 2-fold change in expression in response to some of the treatments. The responses are color coded according to the gradation shown.

Three genes (*ERG10*, *IDI1*, and *ERG3*) exhibited decreased expression in response to the treatment of cells with azoles, but not other blocks to the isoprenoid pathway (Fig. 2). It is probable that the inhibition of lanosterol demethylase by the azoles caused a buildup of some upstream intermediate (e.g., lanosterol) that is depleted by the inhibition of squalene synthase by zaragozic acid (and the two other upstream blocks). If the intracellular concentration of this intermediate were being monitored to set the expression levels of these genes, it would be consistent with the observation that treatments with azoles cause a repression of expression while zaragozic acid causes an induction. However, it has been reported that ketoconazole, an azole not examined in this study, causes increased *ERG3* mRNA levels (17). Moreover, mutations in several genes that act late in the ergosterol pathway, including *ERG24* and *ERG5*, also cause an increase in *ERG3* mRNA levels. These observations indicate that ergosterol availability regulates *ERG3* expression levels (17). Thus, treatments with azoles would be expected to deplete ergosterol and thereby induce *ERG3* expression, as has been reported with ketoconazole. These disparate results may reflect a

different biological activity of the three azoles examined here that is not shared with ketoconazole, or different treatment protocols that utilize chronic versus acute exposure regimens. Previous yeast experiments utilized higher concentrations of drugs for shorter amounts of time, thereby eliciting a more acute response. In contrast, the experiments described here used a chronic exposure to lower concentrations of drug. Considering the dynamic regulation of this pathway, it is conceivable that the cells treated with azoles in our experiments are approaching a new homeostasis reflected by the repression of some pathway genes that had been induced during the earlier acute phase of the regulatory response. This notion is consistent with the expectation that mutations of late ergosterol pathway genes, such as an *ERG5* null allele, would more closely mimic an acute drug treatment as induced expression of pathway genes would not increase ergosterol biosynthesis, and a new homeostasis would not be reached.

Mechanism of lanosterol demethylase inhibitors

Azole inhibitors of lanosterol demethylase are the most widely used antifungal compounds in human medicine. Thus, there is a strong interest in determining just how these inhibitors achieve their therapeutic effect. Several observations suggest that the biological activity of fluconazole may be subtly different from that of miconazole and sulconazole. This difference was particularly evident in the regulation of the genes *ERG5* and *ERG4*. Sulconazole, and to some extent miconazole, induced *ERG5* and *ERG4* expression, whereas fluconazole repressed their expression (Table 3). The two acyl-CoA:sterol acyltransferase genes, *ARE1* and *ARE2*, also responded differently to the two classes of azoles. Both miconazole and sulconazole induced *ARE1* expression and repressed *ARE2* expression (Table 3). This opposing effect of a treatment on the two isozymes of an enzymatic activity has been seen before in the yeast sterol pathway (2, 4). In contrast, fluconazole treatment induced both *ARE1* and *ARE2* (Table 3).

Considering the breadth of the effect elicited by the azoles compared to other inhibitors of the isoprenoid pathway (Table 1), perhaps only a portion of these responses reflect inhibition of lanosterol demethylase activity per se. If this idea is correct, then part of the expression response profile of each azole reflects indirect bioactivities of that azole. Thus, it may be possible to inhibit the lanosterol demethylase enzyme with greater specificity than that of the azoles presently available. Preliminary experiments examining the GRM expression profile of an *ERG11* mutant strain that encodes a conditional lanosterol demethylase allele suggest that the azoles have bioactivities in addition to the inhibition of lanosterol demethylase (data not shown).

Mating pheromone response pathway

The mating pheromone response pathway is one of five MAP kinase signaling pathways in yeast. In the case of the mating pathway, the binding of pheromone to its cell surface receptor triggers the signaling cascade, and ultimately results in the induction of the expression of a number of genes required for mating. There is a biochemical connection between isoprenoids and the yeast mating pheromone response pathway. Two components of the pathway are subject to post-translational protein prenylation (18). Yeast **a**-factor, encoded by the genes *MFA1* and *MFA2*, and the γ subunit of the heterotrimeric G protein, Ste18p, are both modified by a farnesyl diphosphate moiety. Because this modification is required for **a**-factor function, inhibition of farnesyl diphosphate biosynthesis results in *MAT**a*** cell-type specific sterility (19, 20). The Ste18p protein, utilized by both haploid cell types, also requires prenylation for full activity (21). Thus, inhibition of isoprenoid biosynthesis, and in particular farnesyl diphosphate, would be expected to affect induced signaling via the mating pathway and consequently limit the level of induction of the pheromone responsive genes. Presumably the same effect upon basal signaling would occur, and therefore the uninduced levels of expression of these genes would be lower.

The expression of *MFA1*, which is itself pheromone inducible, is known to be reduced in mutants with a defective farnesyl transferase (22). This regulation is evident at the level of mRNA, and presumably reflects the decreased prenylation and hence decreased function of Ste18p. These experiments were conducted in the absence of mating pheromone and thus involve effects upon the basal level of pathway signaling and gene expression. The regulation of *MFA1* by isoprenoid availability was recapitulated by the *MFA1* reporter gene in the experiments described here (Fig. 3). Consistent with the notion of decreased basal pathway signaling, the expression levels of *FUS1* and *FIG1*, two other pheromone inducible genes, also decreased in response to blocks in isoprenoid biosynthesis (Fig. 3).

However, not all the effects upon the expression of mating pheromone pathway target genes could be explained by reduced farnesylation of the Ste18p. For example, late blocks in the pathway repressed the expression levels of all three genes, *MFA1*, *FUS1*, and *FIG1* (Fig. 3). These late blocks would not be predicted to deplete farnesyl diphosphate pools, and thus would have no effect on prenylation of Ste18p per se. Although we cannot as yet pinpoint the basis of these responses, these late blocks are expected to fundamentally alter the composition of some of the major structural lipids of the plasma membrane. Thus, because the pheromone receptor itself as well as its cognate G-protein are intimately associated with the plasma membrane, it is not surprising that these late blocks would also have an impact, albeit indirect, on expression of the targets of the mating pheromone pathway. This is reminiscent of the sterol "sensing" enzymes such as the sterol regulatory element binding protein (SREBP) cleavage activating protein (SCAP) that appear to respond to the sterol content of the endoplasmic reticulum membrane (23). In any event, the notion that there are two modes of regulation, both isoprenoid dependent and one farnesylation dependent, is supported by the observation that a mevalonate auxotroph has lower levels of *MFA1* mRNA than a mutant defective in the farnesyltransferase, although both are repressed with respect to wild-type (22).

The expression of another mating response gene,

BEM1, was induced by blocks up to and including squalene synthase, but was repressed by blocks of lanosterol demethylase (Fig. 3). Thus, blocking squalene synthase and blocking lanosterol demethylase were not equivalent with respect to the mating response pathway, specifically *BEM1* espression. The nature of this difference was puzzling. Yet, these data revealed the power of broad-based gene expression profiling to avoid over simplistic interpretations based upon the analysis of too few genes and conditions.

RAS1 versus RAS2

The yeast *S. cerevisiae* has two isozymes of the Ras oncoprotein, encoded by the genes *RAS1* and *RAS2* (24, 25). In yeast as in humans, the Ras protein is prenylated by farnesyl diphosphate (19, 20). This modification is important for membrane localization, and therefore the function of the Ras protein. Both *RAS1* and *RAS2* mRNA levels have previously been shown to be repressed in response to limited isoprenoid biosynthesis (22). The 864 GRM utilized in this study allowed us to examine the expression levels of *RAS1* and *RAS2* in response to the various blocks in the isoprenoid pathway.

RAS1 expression was strongly repressed by all of the blocks up to and including squalene synthase (Fig. 4). Because inhibition of squalene synthase caused decreased *RAS1-GFP* expression, the regulation of *RAS1* expression presumably did not reflect the level of function of a farnesylated regulatory protein (or Ras1p itself) as inhibition of this enzyme would not result in a depletion of FPP. This is consistent with previous experiments demonstrating that reduced isoprenoid biosynthesis causes reduced *RAS1* mRNA levels, and that loss of farnesyltransferase activity (through mutation) fails to elicit this response (22). This last observation was also consistent with the notion that the *RAS1* regulatory response to the squalene synthase inhibitor was not due to its ability to inhibit the farnesyl transferase activity. Thus, *RAS1* mRNA levels are regulated in an isoprenoid-dependent, yet farnesylation-independent manner. Although other modes of regulation cannot be ruled out, it would seem that transcription is the most likely candidate.

RAS2 expression levels remained essentially unchanged in all of the treatments examined (Fig. 4). Although previous studies revealed that *RAS2* mRNA levels were lowered in response to limited isoprenoid biosynthesis in a farnesylation-independent fashion, this effect reflected altered mRNA stability and was mediated by sequences within the *RAS2* coding region (22). These sequences were not included in the *RAS2-GFP* reporter used in this study. Thus, the *RAS2* reporter would not be expected to reflect this regulation. The data reported here combined with earlier studies revealed that both *RAS1* and *RAS2* mRNA levels were reduced in response to blocks in the isoprenoid biosynthetic pathway. However, the shared regulation was mediated by different mechanisms. This is reminiscent of the *HMG1* and *HMG2* isozymes. Both HMG-CoA reductase isozymes are subject to feedback regulation by the isoprenoid pathway, but by distinct mechanisms (8, 26). At present, we do not have a clear picture of whether the response of Ras gene expression to blocks in the isoprenoid pathway reflects a crude response of growth-regulating genes to blocks in the synthesis of an essential component, or whether there is a more specific regulatory response at work. Certainly the lack of a response of *RAS1* and *RAS2* to late blocks implies the potential for a specific regulatory axis.

Concluding remarks

The application of broad-based gene expression profiling to the regulation of the isoprenoid pathway and associated pathways provided a much richer picture of which steps are regulated and which steps are not. Several simple pictures emerged. For example, we found little evidence that blocks in isoprenoid biosynthesis affected the expression of genes encoding enzymes that prenylate proteins or tRNA. Similarly, coenzyme Q biosynthesis is largely unaffected at the level of gene expression by these blocks.

The most noteworthy regulatory effects were in opposite directions. Specifically, blocks in the isoprenoid pathway repressed expression of a large number of the genes in the mating pheromone response pathway. In contrast, the enzymes catalyzing the central isoprenoid pathway for the synthesis of sterols presented a mosaic of some genes that were induced and others that were relatively unaffected. The study identified *ERG12*, *ERG8*, *ERG19*, *ERG9*, and *ERG2* as the most consistently induced genes in the pathway, and HMG-CoA synthase (*ERG13*) and one of the isozymes of HMG-CoA reductase (*HMG2*) as relatively nonresponsive members of the pathway.

Perhaps the most surprising results came from analysis of the various azole inhibitors, which had remarkably different effects. The differences in the GRM expression profiles caused by the various azoles suggested that they may have additional bioactivities other than the inhibition of lanosterol demethylase.

We thank J. Bergstrom (Merck) for providing L659-699 and zaragozic acid. Thanks to both Miranda Yee and Millicent Yee for their sequencing efforts. We also thank the staff at Acacia Biosciences and the members of the Rine laboratory at UC Berkeley who provided much support and many words of encouragement.

Manuscript received 26 October 1998 and in revised form 22 January 1999.

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