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Regulation of Partitioned Sterol Biosynthesis in *Saccharomyces cerevisiae*

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Using yeast strains with null mutations in structural genes which encode δ-aminolevulinic acid synthetase (*HEM1*), isozymes of 3-hydroxy-3-methylglutaryl coenzyme A (*HMG1* and *HMG2*), squalene epoxidase (*ERG1*), and fatty acid Δ^9-desaturase (*OLE1*), we were able to determine the effect of hemes, sterols, and unsaturated fatty acids on both sterol production and the specific activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in *Saccharomyces cerevisiae*. We found that the HMGR isozymes direct essentially equal amounts of carbon to the biosynthesis of sterols under heme-competent conditions, despite a huge disparity (57-fold) in the specific activities of the reductases. Our results demonstrate that palmitoleic acid (16:1) acts as a rate-limiting positive regulator and that ergosterol acts as a potent inhibitor of sterol production in strains which possess only the HMGR1 isozyme (*HMG1 hmg2*). In strains which contain only the HMGR2 isozyme (*hmg1 HMG2*), sterol production was inhibited by oleic acid (18:1) and to a lesser degree by ergosterol. The specific activities of the two reductases (HMGR1 and HMGR2) were found to be differentially regulated by hemes but not by ergosterol, palmitoleic acid, or oleic acid. The disparate effects of unsaturated fatty acids and sterols on these strains lead us to consider the possibility of separate, compartmentalized isoprenoid pathways in *S. cerevisiae*.

The branched isoprenoid pathway (Fig. 1) provides a diverse class of biomolecules which serve key roles in protein synthesis (6), protein glycosylation (7), electron transport (12), cell cycle control (16), and the maintenance of membrane fluidity (4, 9). Consequently, drugs which inhibit various portions of this pathway have been effective in lowering serum cholesterol levels in humans (1), inhibiting fungal growth (10), and preventing Ras-mediated oncogenesis (16). A comprehensive understanding of isoprenoid biosynthesis is fundamental to the future exploitation of this pathway.

The yeast *Saccharomyces cerevisiae* has provided a valuable model system for examining the biosynthesis and function of sterols and other isoprenoids. The conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonic acid, a reaction catalyzed by HMG CoA reductase (HMGR), has long been considered to be the principal regulatory reaction in the synthesis of isopentenoids (15, 18). We previously showed that heme competency in the *S. cerevisiae* sterol auxotroph FY3 elicited a fivefold increase in HMGR activity which resulted in a marked accumulation of sterol precursors (8). Two structural genes (*HMG1* and *HMG2*) which encode isozymes of HMGR have been identified (3). The expression of *HMG1* is positively regulated in the presence of heme by an interaction with the *HAP1* (*CYP1*) transcriptional regulator, whereas heme competency represses *HMG2* expression by an unknown mechanism (23). (Abbreviated genotypes will be used in the text for clarity. Wild-type genes are indicated by capital letters [e.g., *HMG1*], and mutant alleles are designated by lowercase letters [e.g., *hmg1*].) In the study described here, we sought to ascertain the relationship between HMGR expression and sterol production and to determine what role heme or heme-related products play in the early stages of sterol biosynthesis.

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MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study are listed in Table 1. Standard techniques were used for mating, sporulation, and tetrad dissection (19). KC1011, KC1012, KC111, KC112B, KC11-Z2, and KC12-Z1 are segregants from crosses L814C × JRY2101, L814C × JRY2111, JRY1159 × FY14, JRY1160 × FY14, JRY2077 × JRY2101, and JRY2076 × JRY 2111, respectively.

Materials. Glucose, yeast nitrogen base, and Casamino Acids were from Difco (Detroit, Mich.). Succinic acid, δ-aminolevulinic acid (ALA), Tergitol Nonidet P-40, fatty acids, sterols, HMG CoA, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH (type I), and amino acids were from Sigma Chemical Co. (St. Louis, Mo.). [1-^{14}C]acetic acid (1.96 GBq/mmol) was from Amersham (Arlington Heights, Ill.). DL-3-[glutaryl-3-^{14}C]HMG CoA was from New England Nuclear (Wilmington, Del.). Ergosterol and lanosterol were purchased from Sigma and were purified by high-performance liquid chromatography prior to use (14). Solvents were from Fisher (Pittsburgh, Pa.).

Media. Strains were grown in defined medium containing 2% dextrose, 1% Casamino Acids, 0.67% yeast nitrogen base, and 50 μg each of methionine, uracil, and adenine per ml. The medium was buffered with 0.05 M succinic acid and adjusted to pH 5.5 with KOH pellets. ALA was added to the medium as indicated from an aqueous stock solution. Sterols and fatty acids were added to the medium from stock solutions made in Tergitol Nonidet P-40–ethanol (1:1, vol/vol).

MNL analysis. Cells were pregrown to early stationary phase in defined medium containing 200 μg of ALA per ml. Strains containing the *erg1*::*LEU2* mutation were grown on 10 μg of ergosterol per ml, and unless otherwise indicated, *ole1*::*LEU2* mutants were supplemented with 200 μg of oleic acid per ml. The cells were harvested, washed twice with defined medium, inoculated to a density of 2.5×10^5 cells per ml in 25 ml of test medium containing 0.1 μCi of [1-^{14}C]acetate per ml, and grown at 30°C with rotary shaking. Cells

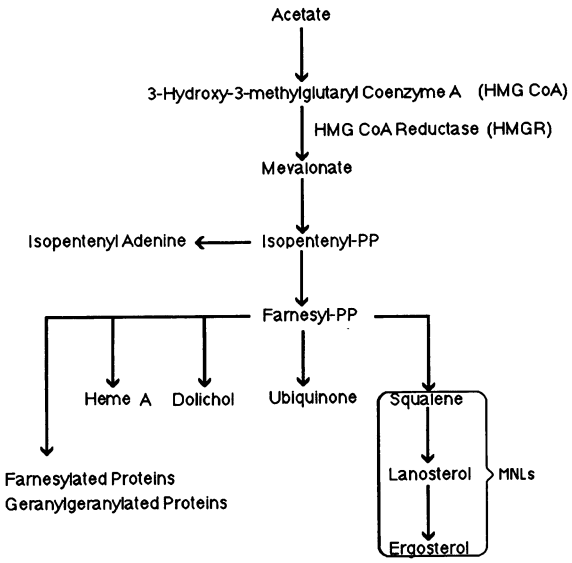

FIG. 1. Abbreviated isoprenoid pathway. Principal intermediates and products as well as MNLs are shown.

were harvested as described below, washed twice with distilled water, and resuspended in water to an optical density at 600 nm (OD_{600}) of approximately 1.00. Three samples of 10 ml each were then taken to be analyzed for acetate incorporation as follows. Cells were pelleted, resuspended in 0.1 N HCl, steamed for 30 min, and saponified in methanolic KOH (5). Nonsaponifiable lipids were extracted twice with hexane and once with hexane-diethyl ether (2:1, vol/vol). The extracts were combined and evaporated to dryness, and lipids were separated by thin-layer chromatography as described by Skipski et al. (20). The thin-layer chromatography plates were scanned for radioactivity by using a Bioscan 200 imaging system. Radioactive bands corresponding to squalene, lanosterol, and 14-demethyl sterols (ergosterol) were scraped into a 20-ml scintillation vial, and 10 ml of Aquasol LS cocktail (Amersham) was added. Radioactivity was determined by using a Beckman LS 5801 liquid scintillation system. The counts (disintegrations per minute) from these mevalonate-derived nonsaponifiable lipids (MNLs) were added together to determine total MNL radioactivity. MNL production is given as (total dpm in MNL/ml of sample)/OD_{600} of sample. All experiments were performed a minimum of three times; each sample within the experiment was analyzed in triplicate. The standard deviation for each triplicate was no larger than 10% for any sample. The mean values for repetitive experiments agreed within 25%.

HMGR assay. HMGR was assayed essentially as described by Thorness et al. (23), with the following modifications. The assay mixture contained 50 mM Tris hydrochloride (pH 7.5), 5 mM dithiothreitol, 3 mM NADPH (type I), 300 μM DL-[^{14}C]HMG-CoA (1 μCi/μmol), 20 mM glucose-6-phosphate, and 0.1 U of glucose-6-phosphate dehydrogenase. The reaction mixture was incubated for 8 min at 37°C after the addition of 200 μg of protein from the cell extract. Each sample was assayed a minimum of four times before the mean HMGR activity was determined. The standard deviation was not larger than 20% for any sample.

β-Galactosidase assay. Cultures were grown as described above, and β-galactosidase activity was assayed as described by Miller (11). All experiments were performed in triplicate. The standard deviation was not larger than 10% for any given experiment.

RESULTS

HMGR activity and MNL production vary over the culture cycle. JRY2111 (*hem1 HMG1 hmg2*) and JRY2101 (*hem1 hmg1 HMG2*) were grown on ALA at 100 and 0.5 μg/ml, respectively, to allow for optimal expression of each isozyme (23). Maximal stimulation of HMGR1 (JRY2111) specific activity was observed during logarithmic growth, while HMGR2 (JRY2101) specific activity was optimal during stationary phase (Fig. 2). MNL accumulation over the culture cycle corresponded to reductase activity in both JRY2111 and JRY2101 (Fig. 2). On the basis of these data, we chose to harvest cells after 30 h when examining *HMG1* and after 50 h when examining *HMG2*.

Palmitoleic acid and ALA stimulate *HMG1*-mediated MNL production. To determine the effects of lipid components on MNL production, JRY2111 (*hem1 HMG1 hmg2*) was grown in the presence of various concentrations of either ALA or palmitoleic (16:1), oleic (18:1), palmitic (16:0), or stearic (18:0) acid. MNL production was measured after 30 h of growth. Supplementation with either ALA or palmitoleic acid effected a four- to fivefold increase in the amount of carbon devoted to sterol biosynthesis in comparison with nonsupplemented cells (Fig. 3). Palmitic, oleic, and stearic acids elicited no such response.

TABLE 1. Strains used

Strain	Genotype	Source
JRY1159	a *his3 lys2 ade2 met ura3 hmg2::HIS3*	J. Rine (23)
JRY1160	a *his3 lys2 ade2 met ura3 hmg1::LYS2*	J. Rine (23)
JRY2101	α *his3 ade2 met ura3 hmg1::LYS2 hem1::LEU2*	J. Rine (23)
JRY2111	α *his3 ade2 met ura3 hmg2::HIS3 hem1::LEU2*	J. Rine (23)
JRY2076	a *ade1 his4 ura3 leu2 HMG1-lacZ*	J. Rine (23)
JRY2077	a *ade1 his4 ura3 leu2 HMG2-lacZ*	J. Rine (23)
FY14	α *ade2 hem1 erg1::URA3 leu2*	R. Lorenz
L814C	a *leu2 ura3 his4 ole1::LEU2*	C. Martin (22)
KC1011	α *hem1::LEU2 hmg1::LYS2 ole1::LEU2 ura3 his ade2*	This study
KC1012	α *hem1::LEU2 hmg2::HIS3 ole1::LEU2 ura3 his ade2*	This study
KC11-Z2	a *hem1::LEU2 hmg1::LYS2 HMG2-lacZ ade*	This study
KC12-Z1	a *hem1::LEU2 hmg2::HIS3 HMG1-lacZ ade*	This study
KC111	α *hem1 ade2 erg1::URA3 hmg2::HIS3*	This study
KC112B	α *hem1 ade2 erg1::URA3 hmg1::LYS2*	This study

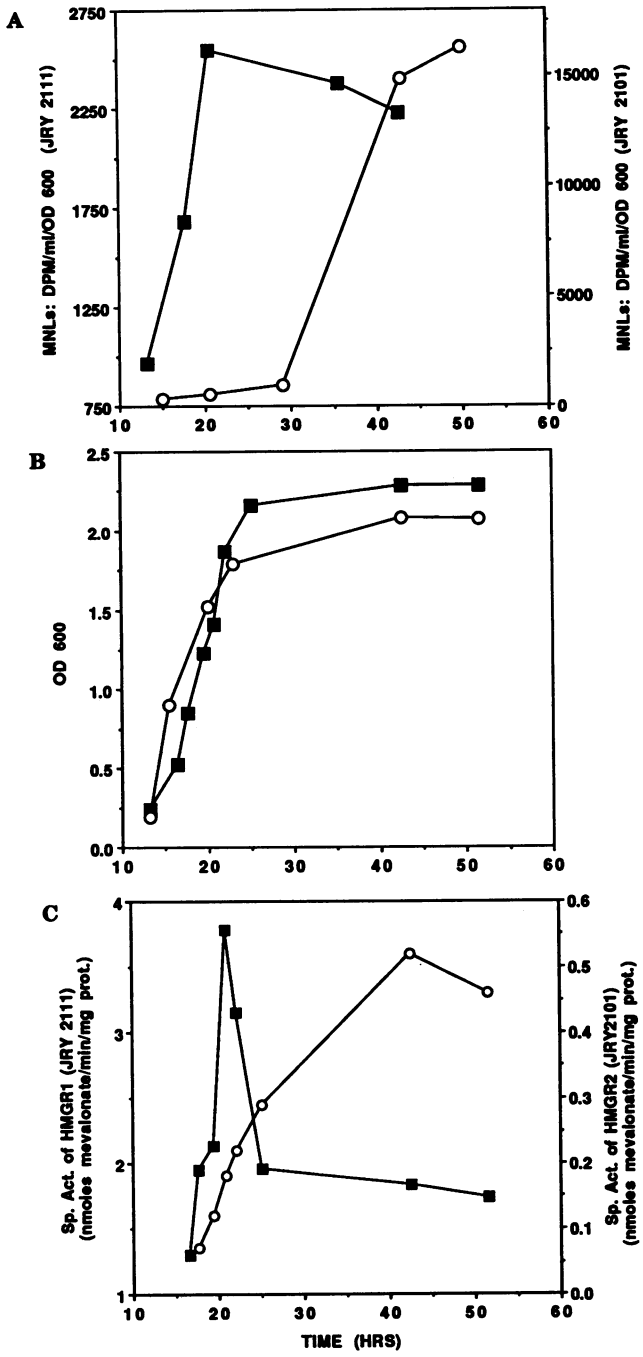

FIG. 2. MNL production (A) and HMGR activity (C) over the culture cycle (B) in JRY2101 (○) and JRY2111 (■). MNL production and HMGR activity were determined as described in the text.

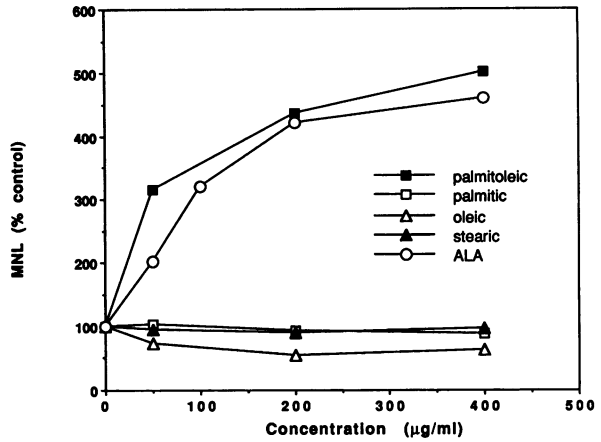

FIG. 3. MNL production in JRY2111 grown on different fatty acids. Cells were grown on fatty acids, as indicated, and MNLs were quantitated after 30 h. Results are given as a percentage of the value for an unsupplemented (no fatty acids or ALA) control culture.

Palmitoleic acid stimulates MNL production but not HMGR1 specific activity. Production of palmitoleic acid is contingent on the heme (cytochrome b_5)-requiring *OLE1* gene product, fatty acid Δ^9-desaturase (22). Consequently, palmitoleic acid is produced by the addition of ALA to *hem1* mutants. We therefore sought to determine whether the previously reported effects of ALA (23) and our current observations with palmitoleic acid were consistent.

We constructed strain KC1012 (*hem1 ole1 HMG1 hmg2*) by crossing L814C (*ole1*) and JRY2111 (*hem1 HMG1 hmg2*). This segregant, which is unable to synthesize unsaturated fatty acids (UFAs), was inoculated into the test medium supplemented with either ALA (200 μg/ml) or palmitoleic acid (400 μg/ml). An unsupplemented culture was used as the control. The cultures were divided into two 25-ml halves. [1-^{14}C]acetate (0.1 μCi/ml) was added to one half for quantitation of MNLs; the sister culture was used to determine the specific activity of HMGR1. As shown in Table 2, experiment A, after 30 h of growth, ALA (heme) alone increased HMGR1 specific activity 49-fold, but there was only a marginal (37%) increase in total MNLs. Cells grown on only palmitoleic acid did not have elevated reductase activity but contained almost sixfold more radiolabeled acetate incorporated into MNLs than did nonsupplemented cells.

To determine the effect of palmitoleic acid on expression of the *HMG1* gene, we crossed JRY2111 (*hem1 HMG1 hmg2*) with JRY2076 (*HMG1-lacZ*) to obtain segregant KC12-Z1 (*hem1 HMG1 hmg2 HMG1-lacZ*). Palmitoleic acid supplementation did not alter the level of *HMG1* expression in KC12-Z1, as measured by β-galactosidase production, in comparison with an unsupplemented control culture (12.6 versus 13.0 Miller units, respectively).

These experiments demonstrate that palmitoleic acid does not increase MNL production by increasing the expression of *HMG1* or the specific activity of HMGR1. Furthermore, palmitoleic acid is shown to facilitate the conversion of mevalonate to MNL at wild-type (ALA-supplemented) levels, even though HMGR1 activity (and presumably mevalonate production) is fully repressed. This finding suggests that less than 2% (compare specific activities in Table 2) of the mevalonate produced under heme-competent conditions is actually needed for sterol biosynthesis.

Ergosterol inhibits HMGR1-mediated MNL production. Another consequence of heme competency in yeast cells, in addition to the initiation of UFA biosynthesis, is the biosynthesis of the major sterol end product, ergosterol. We therefore sought to determine whether sterol production would feedback inhibit HMGR1-mediated MNL production. Strain KC112B (*hem1 erg1 HMG1 hmg2*) was a segregant from a cross between FY14 (*hem1 erg1*) and JRY2111 (*hem1*

TABLE 2. Effects of ALA and UFAs on HMGR activity and MNL production[a]

Expt	Strain	Medium supplementation (μg/ml)		HMGR sp act (nmol/min/mg of protein)	MNL production (dpm/ml/OD_{600})
		ALA	UFA		
A	JRY2111 (*hem1 HMG1 hmg2*)	0	0	0.050	721
		200	0	2.31	2,615
	KC1012 (*hem1 ole1 HMG1 hmg2*)	0	0	0.052	487
		200	0	2.46	667
		0	400 (16:1)	0.048	2,859
		0	400 (18:1)	0.055	433
B	JRY2101 (*hem1 hmg1 HMG2*)	0	0	0.48	15,991
		200	0	0.07	3,039
		0	400 (16:1)	0.57	14,080
		0	400 (18:1)	0.50	3,360
	KC1011 (*hem1 ole1 hmg1 HMG2*)	0	0	0.52	15,454
		200	0	0.05	2,793

[a] Strains were supplemented with ALA, palmitoleic acid (16:1), or oleic acid (18:1), as indicated. HMGR activity and MNL production were quantitated after 30 h for JRY2111 and KC1012 and after 50 h for JRY2101 and KC1011.

HMG1 hmg2). This strain is incapable of producing sterols as a result of the mutation in *ERG1* (encoding squalene epoxidase) and is therefore totally dependent on sterol supplementation in the medium. Ergosterol supplementation resulted in a 98% decrease in MNLs produced from HMGR1 in comparison with cells grown on lanosterol, although the specific activity of HMGR1 was not significantly affected (Table 3).

Oleic acid and ALA inhibit HMGR2-mediated MNL production. The ability of heme to inhibit the expression of *HMG2* has previously been reported (23). We sought to determine whether UFAs were involved in the regulation of *HMG2* expression or HMGR2-mediated MNL production. JRY2101 (*hem1 hmg1 HMG2*) was grown on defined medium, harvested, and reinoculated to unsupplemented medium or medium containing ALA, palmitoleic acid, or oleic acid. After 50 h of growth, both ALA and oleic acid supplementation caused a fivefold decrease in HMGR2-mediated MNL production (Table 2, experiment B). The addition of ALA inhibited the specific activity of HMGR2, whereas oleic acid had no effect on reductase activity.

The level of *HMG2* expression was virtually unaltered in KC11-Z2 (*hem1 hmg1 HMG2 HMG2-lacZ*) grown on medium supplemented with oleic acid in comparison with an unsupplemented control culture (72.0 versus 68.1 Miller units, respectively).

When ALA was added to the medium of KC1011 (*hem1 hmg1 HMG2 ole1*), both the specific activity of the reductase and MNL production were significantly decreased (Table 2, experiment B), demonstrating that the inhibitory effects of ALA (heme) were separate and independent of those observed with oleic acid.

Ergosterol slightly decreases HMGR2-mediated MNL production. Strain KC111 (*hem1 erg1 hmg1 HMG2*) was used to examine the effects of sterols on MNL production. Cells grown on ergosterol had 33% less radioactivity incorporated into MNLs than did cells grown on lanosterol (Table 3).

Comparison of MNL production and isozyme activities in JRY2111 and JRY2101. During our study, it became apparent that isozyme activity was not necessarily indicative of MNL production. To investigate this observation further, JRY2111 and JRY2101 were grown in parallel cultures, with and without the addition of ALA (200 μg/ml). Cells were harvested after 50 h, and isozyme activities and MNL production were determined. The results (Table 4) demonstrate that under conditions of heme competency (ALA supplemented), the in vitro specific activity of HMGR was 57-fold higher in JRY2111 (HMGR1) than in JRY2101 (HMGR2), although levels of MNL production were essentially equal. Under heme-depleted conditions, the specific activity of the reductase in JRY2101 (HMGR2) was 10-fold higher than in JRY2111 (HMGR1), and JRY2101 had 24-fold more [^{14}C]acetate incorporated into MNLs. The heme-competent cells more closely resembled wild-type cells grown under aerobic conditions.

DISCUSSION

By measuring radiolabeled acetate incorporation into MNLs of JRY2111 (*hem1 HMG1 hmg2*), we demonstrated that both ALA and palmitoleic acid caused roughly a fivefold increase in the amount of carbon directed for sterol production (Fig. 3). The ability of palmitoleic acid (16:1) to stimulate MNL production in the absence of ALA was structur-

TABLE 3. Effect of sterols on MNL production and the specific activity of HMGR[a]

Strain	Ergosterol (μg/ml)	Lanosterol (μg/ml)	HMGR sp act (nmol/min/mg of protein)	MNL production (dpm/ml/OD_{600})
KC112B (*hem1 erg1 HMG1 hmg2*)	20	0	0.047	846
	0	20	0.052	53,435
KC111 (*hem1 erg1 hmg1 HMG2*)	20	0	0.493	11,460
	0	20	0.538	16,972

[a] KC112B and KC111 were harvested after 30 and 50 h of growth, respectively. In addition to being supplemented with the sterols listed, both strains were supplemented with 400 μg of palmitoleic acid per ml to allow for optimal MNL production (see Table 2).

TABLE 4. HMGR activity and MNL production in JRY2101 and JRY2111 after 50 h of growth

Strain	ALA (μg/ml)	HMGR sp act (nmol/min/mg of protein)	MNL production (dpm/ml/OD_{600})
JRY2111 (*hem1 HMG1 hmg2*)	0	0.052	749
	200	2.730	3,390
JRY2101 (*hem1 hmg1 HMG2*)	0	0.499	18,032
	200	0.048	3,453

ally specific in that oleic (18:1), stearic (18:0), and palmitic (16:0) acids had no effect.

To examine the effects of heme and UFAs independently, we introduced an insertionally inactivated *OLE1* gene (*ole1::LEU2*) into a strain containing null mutations in *HEM1* and *HMG2*. The resulting strain was incapable of producing UFAs even under heme-competent (ALA-supplemented) conditions. The data in Table 2 demonstrate that heme mediated a 47-fold increase in HMGR1 activity in the absence of UFAs. ALA supplementation alone, however, did not invoke a significant increase in HMGR1-mediated MNL production. Conversely, palmitoleic acid did not elevate *HMG1* expression or HMGR1 activity yet elicited a sixfold increase in MNL production. These data indicate that palmitoleic acid biosynthesis, not increased HMGR1 activity, is the primary contributor in the heme-mediated stimulation of MNL production. These results demonstrate that the effects that we have observed are unique and separate from the previously reported effects of heme or heme-derived products on *HMG1* (23).

Both ALA (heme) and oleic acid repressed HMGR2-mediated MNL production in JRY2101 (*hem1 hmg1 HMG2*) (Table 2, experiment B). The addition of ALA significantly reduced the specific activity of HMGR2, while addition of oleic acid did not. ALA supplementation also caused a reduction in both the specific activity of HMGR2 and MNL production in KC1011 (*hem1 ole1 hmg1*), which is unable to synthesize UFAs even during heme-competent growth. These data lead us to conclude that the effects of ALA and oleic acid are independent, such that heme reduces MNL production at the level of *HMG2* expression, regardless of UFA availability. Oleic acid represses MNL production by an unknown mechanism which apparently does not involve the repression of *HMG2* expression or HMGR2 specific activity. There have been conflicting reports on the ability of ergosterol to feedback inhibit sterol biosynthesis in *S. cerevisiae* (2, 5, 13, 17, 21). In light of our new data on the differential effects of heme and UFAs on MNL production, it now appears that the discrepancies probably arose because of the differences in conditions under which the experiments were performed. Our current work demonstrates that ergosterol is an effective inhibitor of HMGR1-mediated sterol biosynthesis and only moderately affects MNL production, when mevalonate is formed by HMGR2 (Table 3). Although the site of action of ergosterol is not presently known, it does not appear to be at the level of HMGR.

Throughout the course of our investigation, it became apparent that the specific activity of the reductase was not necessarily indicative of total MNL production. Therefore, reductase specific activities and levels of MNL production in JRY2111 and JRY2101 were compared (Table 4). The most striking results were observed when we compared the two strains under conditions of heme competency (ALA supplemented). Although the specific activity of the reductase was 57-fold higher in JRY2111 than in JRY2101 (the difference is even greater if total activity over the culture cycle is taken into account), equal amounts of radiolabeled acetate were found in the MNLs. We speculate that this finding could be a reflection of partitioning in the *HMG1* pathway, which may be directing the carbon flow to other isopentenoids. These data also corroborate our assertion that only a relatively small percentage of the mevalonate produced by HMGR is needed to satisfy fully the sterol requirements of the cells.

If the mevalonate which originates from the HMG isozymes were part of a common (single) isopentenoid biosynthetic pathway, then one might expect equal subsequent metabolism of mevalonate to MNLs. This is clearly not the case. When mevalonate is formed via HMGR1, its metabolism to MNLs is limited by palmitoleic acid availability, unaffected by oleic acid, and strongly inhibited by ergosterol. Conversely, HMGR2-mediated MNL production is unaffected by palmitoleic acid, inhibited by oleic acid, and only moderately decreased in the presence of ergosterol. In light of the fact that the regulatory interactions that we have observed are independent of the expression of the *HMG* genes or activity of the reductase isozymes but are directly related to the source of mevalonate, we anticipate that the pathways headed by these two isozymes are not only separate but also physiologically compartmentalized such that they are unable to share enzymes. Just as the research described here would not have been possible without the genetic separation of the two HMGR isozymes, future work on the pathways may be inconclusive until mutants that allow the further genetic separation of the pathways are identified. Research toward that end is in progress in our laboratory.

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