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Feedback Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase in *Saccharomyces cerevisiae*

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In eukaryotic cells all isoprenoids are synthesized from a common precursor, mevalonate. The formation of mevalonate from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) is catalyzed by HMG-CoA reductase and is the first committed step in isoprenoid biosynthesis. In mammalian cells, synthesis of HMG-CoA reductase is subject to feedback regulation at multiple molecular levels. We examined the state of feedback regulation of the synthesis of the HMG-CoA reductase isozyme encoded by the yeast gene *HMG1* to examine the generality of this regulatory pattern. In yeast, synthesis of Hmg1p was subject to feedback regulation. This regulation of HMG-CoA reductase synthesis was independent of any change in the level of *HMG1* mRNA. Furthermore, regulation of Hmg1p synthesis was keyed to the level of a nonsterol product of the mevalonate pathway. Manipulations of endogenous levels of several isoprenoid intermediates, either pharmacologically or genetically, suggested that mevalonate levels may control the synthesis of Hmg1p through effects on translation.

INTRODUCTION

Eukaryotic cells utilize isoprenoids, a group of structurally related compounds derived from mevalonate, for a variety of cellular processes including sterol biosynthesis, respiration, protein glycosylation, and protein prenylation (Porter and Spurgeon, 1981). The biosynthesis of mevalonate from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) by the enzyme HMG-CoA reductase is the first committed step in isoprenoid biosynthesis (reviewed by Schroepfer, 1981). Moreover, hepatic HMG-CoA reductase has been shown to be the rate-limiting enzyme of sterol synthesis in cholesterol-fed rats (Siperstein and Fagan, 1966). These observations have led to an intense investigation of HMG-CoA reductase regulation.

In mammalian cells, sterol homeostasis requires a balance between endogenous sterol synthesis and receptor-mediated endocytosis of exogenous sterols in the form of low density lipoprotein (LDL) particles (reviewed by Brown and Goldstein, 1990). This homeostasis is achieved by the feedback regulation of a number of genes and proteins including HMG-CoA reductase by mevalonate-derived compounds. Early experiments demonstrated a repression of HMG-CoA reductase synthesis by exogenous LDL (reviewed by Brown and Goldstein, 1980). Although sterols are the bulk end product of mevalonate in the cell, a number of other essential compounds are derived from mevalonate. Therefore, even cells grown with high amounts of exogenous LDL retain some HMG-CoA reductase activity to supply the mevalonate necessary to make these essential nonsterol isoprenoids. Complete repression requires the addition of a small amount of mevalonate in addition to exogenous sterols. This observation suggests that nonsterol compound(s) derived from mevalonate, as well as sterols, are involved in the feedback regulation of HMG-CoA reductase.

The regulation of HMG-CoA reductase in mammalian cells occurs at multiple levels including transcription, translation, and protein stability (reviewed by Brown and Goldstein, 1990). Transcription of the structural genes encoding HMG-CoA synthase, HMG-CoA reductase, and the LDL-receptor are coordinately regulated by a sterol product (perhaps an oxysterol) acting through the sterol response elements present in the promoter regions of all three genes (reviewed by Brown and Goldstein, 1990). In contrast, an as yet unidentified nonsterol compound derived from mevalo-

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nate mediates the feedback regulation of HMG-CoA reductase translation (Nakanishi *et al.*, 1988). The stability of the HMG-CoA reductase protein also appears to be regulated by both sterol and nonsterol products (Peffley and Sinensky, 1985).

The pathway of isoprenoid biosynthesis in the budding yeast *Saccharomyces cerevisiae* is virtually identical to that of mammalian cells. Moreover, the functionally diverse collection of essential molecules derived from isoprenoids has been conserved from yeast to humans; the principle difference being that the primary sterol in yeast is ergosterol rather than cholesterol. This conservation of the pathway raises the interesting question of whether the complex feedback regulatory circuits controlling isoprenoid biosynthesis in mammalian cells are also present in yeast. Yeast grow in a wide range of ecological niches. Therefore, it seems reasonable that the relative demands for the various isoprenoids would vary under different physiological conditions and that the biosynthesis of these essential compounds be regulated. *S. cerevisiae* has two HMG-CoA reductase isozymes, Hmg1p and Hmg2p, encoded by two structural genes, *HMG1* and *HMG2* (Basson *et al.*, 1986). The expression of *HMG1* and *HMG2* has been shown to be regulated by oxygen availability (Thorsness *et al.*, 1989) and growth phase, i.e., logarithmic versus stationary (Casey *et al.*, 1992; Dimster-Denk and Hampton unpublished observations). In this study, we have examined the state of feedback regulation of Hmg1p, the major HMG-CoA reductase isozyme in aerobic cells. We demonstrated that Hmg1p synthesis is subject to feedback regulation. Furthermore, our experiments suggested that mevalonate itself is a nonsterol regulator of HMG-CoA reductase synthesis and indicated that the mode of regulation may be translation and not transcription.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP (400 Ci/mmol), 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA (52 mCi/mmol), [^{14}C]-mevalonate (60 mCi/mmol), [1-^{14}C] sodium acetate (57 mCi/mmol), [^{125}I]-protein A, ECL chemiluminescence immunodetection reagents, and the Multiprime DNA-labeling system were from Amersham (Arlington Heights, IL). O-nitrophenyl-β-*D*-galactopyranoside, *DL*-mevalonic acid lactone, and protease inhibitors were from Sigma (St. Louis, MO). Tran^{35}S-label (15.9 μCi/ml) was purchased from ICN (Costa Mesa, CA). Nitrocellulose was from Schleicher & Schuell (Keene, NH). Rabbit antisera to either a β-galactosidase-Hmg1p fusion antigen or to a TrpE-Hmg1p fusion antigen were provided by R. Hampton (University of California, Berkeley). These antisera recognized both isozymes of HMG-CoA reductase. Rabbit anti-Hmg1p specific antibodies against the carboxy-terminal 15 amino acids of Hmg1p were from R. Wright (Department of Zoology, University of Washington). Compactin and lovastatin were the gifts of A. Endo and A. Alberts (Merck, Rahway, NJ), respectively. Zaragozic acid was originally from J. Bergstrom (Merck) and provided by J. Watson (Department of Biochemistry and Biophysics, University of California, San Francisco [UCSF]).

Media and Genetic Methods

Yeast strains were grown at 30°C in yeast minimal medium (YM) containing either 2% glucose or galactose and sporulation medium prepared as described previously (Barnes *et al.*, 1984). Amino acid and base supplements were added, when needed, at the following concentrations: adenine, 30 μg/ml; methionine, histidine, and uracil, 20 μg/ml; lysine, 40 μg/ml from 200× stock solutions in dH_2O. Solid medium contained 2% agar (Difco, Detroit, MI). Yeast cells were transformed by the lithium acetate method (Ito *et al.*, 1983; Schiestl and Gietz, 1989). Stock solutions of lovastatin and compactin were prepared by hydrolysis of an 85 mg/ml solution in ethanol/0.2 M NaOH at 65°C for 40 min then diluted to a concentration of 25 mg/ml in tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.0) and adjusted to pH 8.0. A stock solution of 3 mM zaragozic acid was prepared in dH_2O. Unless stated otherwise, drug treatments were at a concentration of 40 μg/ml for ≥8 h. Mevalonic acid lactone was prepared as a 500 mg/ml stock solution in dH_2O, heated at 65°C for 1–4 h, filter sterilized, and then added either to liquid medium or top spread onto solid medium.

Plasmid Constructions

Plasmid pJR974, an integrating vector containing the *TRP1* gene as well as the *ERG8* gene under the control of the *GAL1* gene promoter, was constructed by cloning the 2.8-kilobase (kb) *Eco*RI fragment from pET77 (Tsay and Robinson, 1991) containing the *GAL1P-ERG8* fusion into the *Eco*RI site of the vector pRS304 (Sikorski and Hieter, 1989). The *GAL1P-ERG12(TRP1)* integrating vector, pJR975, was constructed by inserting the 3.2-kb *Pvu* II fragment from pGR44 harboring the *GAL1P-ERG12* fusion into the *Pvu* II site of pRS304.

Strains

All strains used in this study were derived from JRY527 (Wright *et al.*, 1988) and are isogenic except where indicated. The strains used in this study are described in Table 1.

To create a conditional allele of *ERG8* (phosphomevalonate kinase), an additional auxotrophic marker was created in JRY527 by disrupting the *TRP1* gene as described (Alani *et al.*, 1987). The resulting strain (JRY1323) contained a *trp1::hisG* allele. This strain was transformed with a plasmid containing the *ERG8* gene under the transcriptional control of the *GAL1* promoter (pJR974). This plasmid was linearized by cleavage in the *ERG8* gene by *Sph* I before transformation to target the integration to the *ERG8* locus creating a strain with a second *ERG8* gene under the control of the *GAL1* gene promoter, marked by *TRP1*, at the *ERG8* locus (JRY1325). To disrupt the endogenous *ERG8* gene, this strain was transformed with a 4.2-kb *Pvu* II/*Xba* I fragment from pET62 (Tsay and Robinson, 1991) containing an *erg8::HIS3* deletion/disruption allele. A His$^+$ Trp$^+$ transformant was picked that grew on galactose-containing medium but not on glucose-containing medium (JRY1326). The molecular structures of these strains were confirmed by genomic hybridization analysis. The strain with the conditional *ERG8* allele (JRY1326) was then mated to an isogenic α-strain containing an *HMG1-lacZ* reporter gene integrated at the *HMG1* locus (JRY3283). The resulting diploid (DAY85) was sporulated and an **a**-strain, containing both the conditional allele of *ERG8* and the *HMG1-lacZ* reporter, was identified (JRY3294). The conditional phosphomevalonate kinase activity was confirmed by enzyme assays.

The creation of a conditional mevalonate kinase strain was a bit more circuitous. An α-strain (JRY3278) was transformed with a plasmid containing the *ERG12* gene under the control of the *GAL1* promoter (pJR975) that had been cleaved in the *TRP1* gene by *Sna*BI. The resulting strain contained a second *ERG12* gene under *GAL1* promoter control, marked by *TRP1*, at the *TRP1* locus (DAY95). The endogenous *ERG12* gene was then disrupted in an isogenic **a**-strain (JRY1323), harboring the *GAL1-ERG12* fusion on a 2-μ plasmid marked by *URA3* (pGR44) to complement the disruption, by transforming with a 4.9-kb *Sma* I/*Eco*RI/*Sph* I fragment containing an *erg12::HIS3* allele (from pGR49) on galactose-containing medium. The resulting strain (DAY104) was not able to grow on galactose-containing medium supplemented with 5-fluoro-orotic acid (5-FOA) (Boeke *et al.*, 1984), indicating that it was unable to grow without the complementing plasmid (pGR44), and could not grow on glucose-containing medium at all.

Table 1. Strains used in this study

Strain	Genotype
JRY 527	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52*
JRY 1443[a]	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 HMG1-lacZ(URA3)*
JRY 1273	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 pBM746*
JRY 1274	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 pBM747*
JRY 1595	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 hmg2::HIS3*
JRY 1896	*MATα ade2-101 his3Δ200 lys2-801 met- ura3-52 HMG1-lacZ(URA3) trp1::hisG*
JRY 2085	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 pLGΔ312S*[b]
JRY 3278	*MATα ade2-101 his3Δ200 lys2-801 met- ura3-52 trp1::hisG*
JRY 3283	*MATα ade2-101 his3Δ200 lys2-801 met- ura3-52 HMG1-lacZ(URA3)*
JRY 3294	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 HMG1-lacZ(URA3) GAL1-ERG8(TRP1) erg8::HIS3*
JRY 3496	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 HMG1-lacZ(URA3) GAL1-ERG12(TRP1) erg12::HIS3*
JRY 4002[c]	*MATa ade2-101 his3Δ200 lys2-801 met- ura3-52 HMG1-lacZ(URA3) erg13::HIS3*

[a] The construction of the *HMG1-lacZ* reporter gene fusion and the creation of this strain is described in Thorsness *et al.* (1989).

[b] The construction of this plasmid is described in Guarente and Mason (1983).

[c] The *erg13::HIS3* deletion disruption allele used to make this strain is described in Schafer *et al.* (1989).

This strain containing the chromosomal disruption of *ERG12* (DAY104) was then mated to the strain with the conditional allele of *ERG12* (DAY95), and the resulting diploid (DAY110) was grown on 5-FOA containing medium to select for the cells that lost the complementing plasmid (pJR912) and then sporulated. A strain containing both the conditional allele of *ERG12* and the *ERG12* disruption was identified (DAY111) and mated to an α-strain containing the *HMG1-lacZ* reporter at the *HMG1* locus (JRY1896). Finally, this diploid (DAY116) was sporulated, and a strain was isolated that contained all three loci of interest: the conditional allele of *ERG12* at the *TRP1* locus, the *ERG12* disruption, and the *HMG1-lacZ* reporter (JRY3469). Again, the conditional mevalonate kinase activity was confirmed by enzyme assays.

β-Galactosidase Assays

The protocol for β-galactosidase assays was essentially that of Hagen and Sprague (1984) as modified from Miller (1972). The assays were performed in a volume of 1.5 ml in duplicate, and the units were normalized to cell density. In cells harboring the *lacZ* fusions on autonomous plasmids, assays were performed at least three times on several independent isolates. In strains containing the integrated *HMG1-lacZ* fusion, the assays were performed in at least three independent cultures and the standard deviations indicated.

HMG-CoA Reductase Enzyme Assays

Extracts were prepared from 50 ml of logarithmically growing cells as previously described (Thorsness *et al.*, 1989) with the exception that a 50 mM potassium phosphate buffer (pH 7.5) was used. The protein concentration of the extract was determined with a Bradford protein assay kit (Biorad, Richmond, CA). Extract aliquots were stored at −80°C. The reaction conditions for the HMG-CoA reductase assay were as described (Thorsness *et al.*, 1989) except that the mevalonate formed was separated from the substrate by applying the reaction to a Silica 60A thin-layer chromatography (TLC) plate (Whattman Int., Maidstone, United Kingdom) and running the plate in a benzene: acetone (1:1; vol:vol) solvent. The mevalonate was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Assays were performed at two different extract concentrations to insure the linearity of the reaction. Standard deviations were calculated from assays of three independent extracts.

Mevalonate Kinase and Phosphomevalonate Kinase Assays

The mevalonate kinase and phosphomevalonate kinase coupled assay was modified from Porter (1985). The reaction conditions were as follows: 100 mM potassium phosphate buffer (pH 7.4), 8 mM $MgCl_2$, 1 mM dithiothreitol, 15 mM ATP, and 2.4 mM [^{14}C]-mevalonate (0.095 μCi/μl) in a total volume of 20 μl. The reaction was started by the addition of 20 μg crude extract and incubated at 37°C for 3 min. The mix was boiled for 3 min to terminate the reaction and precipitate the proteins followed by pelleting in a microfuge for 1 min. Mevalonate phosphate and mevalonate diphosphate were separated on a Silica 60A TLC plate with a 1-propanol:NH_3 (25% aqueous solution):H_2O (6:3:1) solvent (Beyer *et al.*, 1985) and visualized by autoradiography or by PhosporImager for quantitation.

Analysis of Yeast mRNA

For the isolation of yeast mRNA, 100-ml cultures were grown in supplemented YM medium and harvested during logarithmic growth. Total RNA was isolated as described previously (VanArsdell *et al.*, 1987). Poly(A) RNA was isolated by oligo(dT)-cellulose chromatography essentially by the method of Aviv and Leder (1972) with minor modifications. Poly(A) RNA samples were separated on a 1.2% agarose/formaldehyde gel and analyzed by gel-transfer-hybridization experiments (Maniatis *et al.*, 1982). Probes were radiolabeled utilizing the Multiprime DNA labeling system (Amersham) and hybridized at 42°C for ≥12 h. The most stringent wash to remove nonspecific hybridization was at 42°C in 1× SSPE, 0.1% sodium dodecyl sulfate (SDS).

A 22 nucleotide primer (5′-CAGTCCCTTGAATAGCGGCGGC-3′) complementary to nucleotides +3 to +24 of the coding strand of *HMG1* was used to map the 5′ end of the *HMG1* transcript by primer extension analysis. Twenty-five or fifty μg of total RNA was coprecipitated with 0.1 pmol of ^{32}P-labeled primer and extended as described by Jones *et al.* (1985). The reaction products were analyzed by fractionation on 5% acrylamide/8.3 M urea sequencing gels followed by autoradiography.

For S1 nuclease-protection analysis of *HMG1* mRNA, ^{32}P-labeled, single-stranded hybridization probes for the *HMG1* mRNA were prepared from plasmid pJR389. This plasmid contains a 1014-base pair (bp) fragment of the *HMG1* gene that extends from nucleotide −907 in the upstream region of *HMG1* to nucleotide +107 of the coding sequence in the vector pEMBL18. Single-stranded pJR389 DNA was prepared, mixed with universal primer DNA (complementary to the sequence adjacent to nucleotide +107 of the *HMG1* insert), incubated for 10 min at 70°C, and cooled slowly to room temperature. DNA synthesis directed from the universal primer was carried out in a 20-μl reaction that contained 10 mM Tris-HCl (pH 7.5), 70 mM KPO_4 (pH 7.5), 10 mM $MgCl_2$, 10 mM β-ME, 10 μg/ml bovine serum albumin, 8% glycerol, 100 μM dNTPs, 50 μCi α-[^{32}P]-dCTP (400 Ci/

mmol), and 2.5 U of the Klenow fragment of *Escherichia coli* DNA polymerase I. After a 2-h incubation at 14°C the reaction was stopped by ethanol precipitation. The nucleic acid pellet was washed with 70% ethanol, dissolved, and digested with *Eco*RI (site adjacent to nucleotide −907 of the *HMG1* insert). The reaction was then adjusted to 0.2 N NaOH to denature the DNA and subjected to electrophoresis on a 5% polyacrylamide gel. The single-stranded, ^{32}P-labeled fragment complementary to the *HMG1* coding sequence was isolated from the gel by electroelution. The probe (10 000–30 000 cpm) was mixed with 20 or 40 μg total yeast RNA or with 20 μg carrier tRNA, and S1 nuclease-protection experiments were performed as described by Gilman and Chamberlain (1983). The protected fragments were identified by fractionation on 6% polyacrylamide/8.3 M urea sequencing gels followed by autoradiography.

Analysis of Yeast Protein

For steady-state protein analysis whole cell lysates were prepared from 5 A_{600} U of log phase cultures and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as described (Hampton and Rine, 1994). The proteins were then transferred onto nitrocellulose electrophoretically in transfer buffer (6.6 mM Tris, 50 mM glycine, 0.01% SDS, 6.7% methanol) at 150 mA at 4°C for 1 h (Burnette, 1981). The front nitrocellulose blot was incubated in TBS-TM (5 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01% Tween-20, 2% Carnation [Andronicos, Berkeley, CA] nonfat dry milk) shaking for 30 min at room temperature. The primary antibody was either an anti-peptide Hmg1p-specific antibody against the carboxy-terminal 15 amino acids made by R. Wright (Department of Zoology, University of Washington) diluted 1:200 in TBS-TM (Figure 2A) or an antiserum against a β-galactosidase-Hmg1p fusion antigen diluted 1:1000 in TBS-TM (Figure 2B, inset). The diluted primary antibody was incubated with the blot shaking at 4°C for 12 to 15 h. The blot was then washed (4 times for 5 min) with TBS-TM and incubated with 10 μCi ^{125}I-Protein A (>30 μCi/μg) (ICN) in TBS-TM, shaking at room temperature for 45 min. The blot was washed in TBS for 10 minutes, TBS-T for 10 min, and finally 10 minutes in TBS. After allowing the blot to air dry for 15 min, it was evaluated by autoradiography. Alternatively, the Amersham ECL chemiluminescence detection system was used with horseradish peroxidase–conjugated donkey anti-rabbit IgG, as per the manufacturer's instructions, to visualize the HMG-CoA reductase protein levels. The second back blot was stained with india ink in TBS-T to evaluate the total protein load.

Relative rates of protein synthesis were determined by the method of R. Hampton (Hampton and Rine, 1994) as modified from Sengstag *et al.* (1990). Logarithmically growing cells either treated with lovastatin or left untreated were pulsed for 10 min with Tran^{35}S-label. Incorporation of label was stopped by the addition of unlabeled methionine and cysteine (final concentration 50 mM each) and immediately placed on ice. Lysates were then prepared. Under these conditions lovastatin and/or zaragozic acid treatment caused a significant reduction in the amount of label incorporated into protein. To account for this difference, a volume of the lysate was counted on a scintillation counter to normalize the lysates. The normalized lysates (i.e., the lysates were adjusted by dilution such that a given volume contained equal counts) were incubated with 10 μl anti-TrpE-Hmg1p antisera and rotated end-over-end at 4°C for 12 to 15 h. The remainder of the immunoprecipitation and SDS-PAGE was as described (Hampton and Rine, 1994). Relative band intensities were quantitated by serial dilution and/or utilizing the PhosphorImager.

Acetate Labeling of Cells

Measurements of sterol synthesis were determined by the incorporation of [1^{14}-C] acetate into ergosterol as described (Parks *et al.*, 1985; Hampton and Rine, 1994). As a control, the incorporation of acetate into the fatty acid pool was also determined in the following manner: after the sterols were extracted with hexanes, the lysates were acidified with concentrated HCl, and the fatty acids were extracted twice with 1 ml of hexanes. The samples were then evaporated under N_2 gas, resuspended in a small volume of hexanes, and applied to a Silica 60A TLC plate. The plate was resolved in petroleum ether:diethyl ether:acetic acid (95:5:1), dried, and subjected to PhosphorImaging and autoradiography.

RESULTS

Feedback Regulation of HMG-CoA Reductase

The gene *HMG1* encodes ~80% of the HMG-CoA reductase in logarithmically growing aerobic yeast cells (Basson *et al.*, 1986). Because Hmg1p is the major isozyme in aerobic cells, we examined whether the synthesis of this isozyme was under feedback control, akin to the mammalian enzyme. Cells were starved for mevalonate to reduce the level of any potential feedback regulator in one of two ways: pharmacologically, by treatment with a competitive inhibitor of HMG-CoA reductase (lovastatin or compactin), or genetically, by disrupting the gene encoding HMG-CoA synthase (*ERG13*) thereby creating a mevalonate auxotroph (Figure 1). In studies with inhibitors, we used concentrations that resulted in slow growth that was the physiological equivalent of supplementing mevalonate auxotrophs with low (5 mg/ml) concentrations of exogenous me-

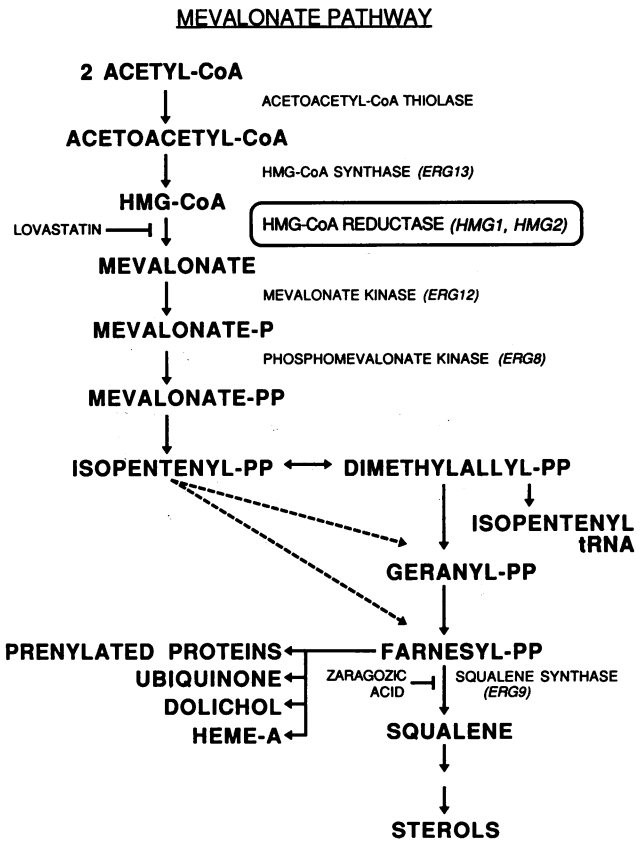


Figure 1. Isoprenoid biosynthesis in yeast cells. The enzymes and corresponding yeast genes discussed in the text are indicated, as is the site of action for the competitive inhibitors lovastatin and zaragozic acid.

valonic acid. In addition, flux through the pathway could be controlled in the mevalonate auxotroph by the amount of exogenous mevalonic acid supplementation.

To determine whether yeast HMG-CoA reductase was subject to feedback regulation, we examined the effect of altered flux through the mevalonate pathway on Hmg1p levels. Cells grown in the presence of 40 μg/ml lovastatin had roughly fourfold more Hmg1p than untreated cells (Figure 2A). Determinations of HMG-CoA reductase activity in lovastatin-treated cells are problematic because of the necessity to dialyze the extracts to remove the lovastatin. Creating mevalonate-starved cells genetically has the advantage of facile determinations of both HMG-CoA reductase enzyme levels as well as protein levels. Mevalonate auxotrophs supplemented with low amounts of exogenous mevalonate (5 mg/ml) had approximately fourfold higher levels of HMG-CoA reductase enzyme activity (Figure 2B) as well as induced protein levels (Figure 2B inset) that correlated with the induction observed in lovastatin-treated cells. Increased amounts of exogenous mevalonate were partially able to reverse the induced activity and protein level (Figure 2, B and inset). These results demonstrated a clear induction of Hmg1p levels in response to mevalonate starvation and established the existence of a feedback regulatory circuit involving Hmg1p and one or more intermediate of the mevalonate pathway. The partial reversal of Hmg1p increases by exogenous mevalonate was consistent with the inefficient utilization of mevalonate by mevalonate auxotrophs, as evidenced by an increase in the cell division time under these conditions.

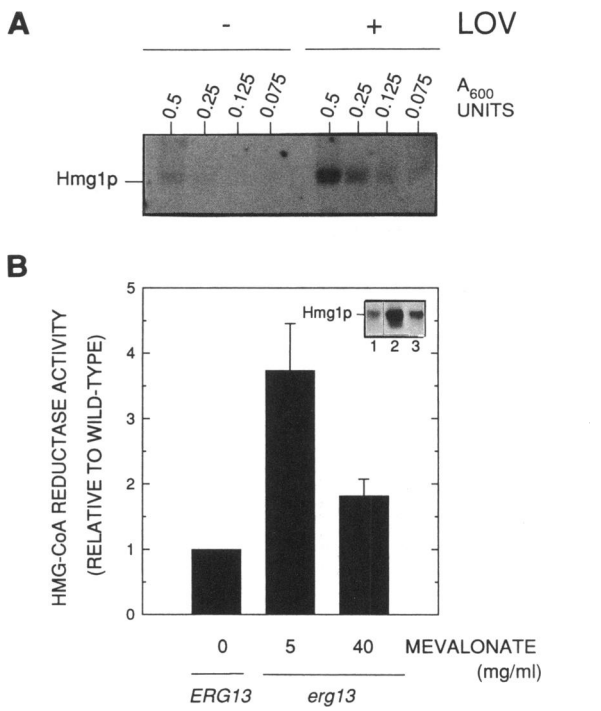


Figure 2. Effect of mevalonate starvation on HMG-CoA reductase levels. (A) Immunoblot of whole cell lysates from logarithmically grown JRY1443 cultures treated with 40 μg/ml lovastatin (LOV) or untreated with an anti-Hmg1p-specific antibody. (B) HMG-CoA reductase enzyme activity in logarithmically grown wild-type (JRY1443) and mevalonate auxotrophs (JRY4002) supplemented with either low (5 mg/ml) or high (40 mg/ml) concentrations of exogenous mevalonate. (Inset) Representative immunoblot of the crude cell extracts used for HMG-CoA reductase activity measurements, utilizing an anti-β-galactosidase-Hmg1p antibody that recognized both isozymes, Hmg1p and Hmg2p.

Analysis of HMG1 Transcription

Feedback regulation of HMG-CoA reductase in mammalian cells occurs at multiple levels including transcription, translation, and protein stability (Nakanishi *et al.*, 1988). To determine if the feedback regulation of Hmg1p levels was because of an effect on transcription of *HMG1*, we examined *HMG1* mRNA in untreated and in mevalonate-starved cells. mRNA blot hybridization analysis revealed no increase in the steady-state *HMG1* mRNA levels upon treatment of cells with compactin under the same conditions that resulted in a fourfold increase of Hmg1p levels (Figure 3A). In fact, there appeared to be a slight but reproducible decrease in the *HMG1* mRNA level in mevalonate-starved cells. However, this slight decrease in mRNA levels was observed with a number of unrelated mRNAs including *MATa1*, *GAL10*, and *TRP1* (Figure 3A for *MATa1*) and therefore appeared not to reflect specific regulation of *HMG1* expression. There was also no increase in the steady-state *HMG1* message in mevalonate auxotrophs supplemented with low levels of mevalonate.

RNA blot hybridization measurements do not exclude possible qualitative differences in the mRNA species made under different conditions. In a higher resolution study, transcriptional start sites for the *HMG1* mRNA were mapped by primer extension (Figure 3B) and S1 hybridization analysis (Figure 3C) of total RNA. Both methods of analysis indicated that there were multiple transcriptional start sites for *HMG1*, clustered in regions 220 and 80 bp upstream of the initiator ATG codon. Rather than an increase, there was an overall decrease in *HMG1* mRNA of less than twofold, as evidenced from the serial dilutions. In addition, there was no change in the relative abundance of the *HMG1* transcription start sites upon mevalonate starvation. These results demonstrated the absence of feedback repression of *HMG1* transcription and suggested a posttranscriptional mechanism for the feedback regulation of *HMG1* expression.

Feedback Regulation of an HMG1-lacZ Reporter Gene Construct

Studies of posttranscriptional regulation that rely exclusively upon measures of the protein can be complicated by multiple levels of regulation, including effects on synthesis or on stability. Therefore, we constructed

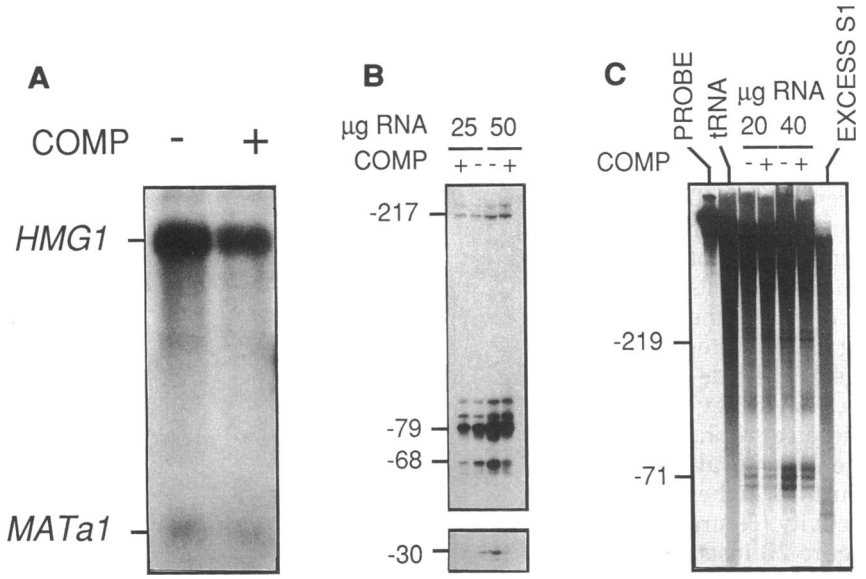


Figure 3. Analysis of steady-state Hmg1p mRNA in wild-type and mevalonate-starved cells. (A) Poly(A) RNA from JRY527 with 40 μg/ml compactin (COMP) or untreated, blotted onto nitrocellulose and probed with a 2.46-kb *Eco*RI fragment of pJR302 containing *HMG1* and a 3-kb *Bam*HI fragment from pJR438 containing the *MATa1* gene. (B) Primer extension analysis of total RNA from JRY527 with 40 μg/ml compactin or untreated. (C) S1 protection analysis of total RNA from JRY527 with 40 μg/ml compactin or untreated.

a reporter gene consisting of the *HMG1* promoter and 5′ untranslated leader fused to the bacterial *lacZ* gene at the initiator methionine. This reporter gene encoded a β-galactosidase protein devoid of any amino acids from Hmg1p except the initiator methionine. Therefore, changes in β-galactosidase activity should reflect transcriptional and/or translational regulation of *HMG1*, but should not reflect any posttranslational effects upon Hmg1p. This reporter gene was integrated into the yeast genome at the *HMG1* locus (Figure 4A) to minimize any context or copy-number effects. Cells harboring this reporter gene exhibited a two- to fivefold induction of β-galactosidase activity when starved for mevalonate (Figures 4, B and C, and Figure 7). In contrast, expression of *lacZ* from the *GAL1*, *GAL10*, or *CYC1* gene promoters was not induced upon mevalonate starvation (Figure 4B). The induction of β-galactosidase synthesis from the fusion gene suggested that the feedback regulation of *HMG1* involved de novo synthesis. Taken together with the RNA analysis that demonstrated the induction of Hmg1p synthesis with no change in *HMG1* transcription, these results suggested that the mechanism for the feedback regulation of *HMG1* expression was translation.

Translation Rate of HMG1 mRNA

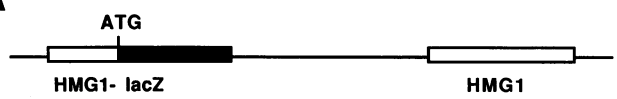
The translation rate of *HMG1* mRNA was measured directly in wild-type and mevalonate-starved cells by pulse-labeling cells with ^{35}S-methionine and -cysteine, followed immediately by exhaustive immunoprecipitation of HMG-CoA reductase. Because the strain used in these experiments contained a disrupted allele of *HMG2* (JRY1595), these measurements of HMG-CoA reductase translation were specific to Hmg1p. Treatment of cells with lovastatin caused a time- and dosage-dependent decrease in the incorporation of ^{35}S into total protein. To compensate for this effect, equal counts of the cell lysates were immunoprecipitated from mevalonate-starved and nonstarved cells. Under these conditions, there was an approximately twofold increase in Hmg1p synthesis rate after 3 h of lovastatin treatment (Figure 5). The magnitude of this induction did not appear to increase with the length of lovastatin treatment from 3 h to 21.5 h. However, because of the drastic inhibition of protein synthesis with longer lovastatin treatments, immunoprecipitation of equal counts of the cell lysates relative to untreated cells was problematic, and rigorous quantitation of the level of induction at these late times was not possible.

Because larger inductions were observed with experiments on steady-state Hmg1p measurements after longer lovastatin treatments (Figure 2, A and B), we considered the possibility that the induction of Hmg1p under these conditions reflected a combination of two or more regulatory effects. To address this possibility, the steady-state level of Hmg1p was measured from the same cultures used for the measurements of Hmg1p translation. In these experiments the level of induction of Hmg1p was quantitatively identical to the induction of the Hmg1p translation rate. Moreover, half-life measurements of Hmg1p indicated that Hmg1p is very stable in untreated cells (Hampton and Rine, 1994), so an increase in protein stability would not be expected to contribute to an increase in steady-state protein levels under these conditions. Therefore, the magnitude of induction could be entirely accounted for by increased synthesis.

Identification of the Signal for Feedback Regulation

The induction of Hmg1p synthesis in response to decreased flux through the mevalonate pathway indicated that the level of one or more intermediates acts as a

A



B

STRAIN		β-GAL ACTIVITY	
		COMPACTIN	
		-	+
JRY 1443	HMG1-lacZ	4.1	12.6
JRY 1273	GAL10-lacZ	101.1	49.9
JRY 1274	GAL1-lacZ	191.0	97.8
JRY 2085	CYC1-lacZ	121.4	135.2

C

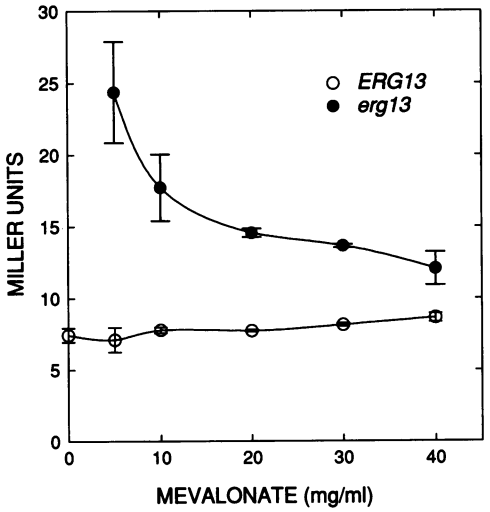


Figure 4. Expression of an *HMG1-lacZ* reporter. (A) Map of the HMG1-lacZ fusion. (B) β-galactosidase expression from *lacZ* promoter fusions in cells treated with 40 μg/ml compactin (COMP) or untreated. (C) β-galactosidase expression in wild-type (JRY1443) and mevalonate auxotroph (JRY4002) cells with increasing amounts of exogenous mevalonate supplementation.

signal to control Hmg1p synthesis. In an attempt to identify the signal involved in the feedback regulation of Hmg1p, we created additional blocks in the mevalonate pathway at various points either by genetic lesion or the employment of drugs. The rationale behind this approach was that blocks in the mevalonate pathway upstream of the signal molecule would cause a depletion of the signal resulting in an induction of Hmg1p synthesis, whereas a block in the pathway downstream of the signal molecule would fail to cause such a depletion and therefore would also fail to cause an induction. Sequential blocks at the earliest points in the mevalonate pathway such as acetoacetyl-CoA thiolase (Hiser and Dimster-Denk unpublished observations), HMG-CoA synthase (Figures 2B and 4C), and HMG-CoA reductase (Figures 2A and 4B) all caused an induction of Hmg1p synthesis. These data suggested that the intermediate monitored by the cell for the feedback regulation of Hmg1p synthesis was downstream of HMG-CoA in the mevalonate pathway. Furthermore, these observations indicated that the regulation of Hmg1p synthesis was a negative feedback loop and therefore the rationale outlined above for the identification of the signal was reasonable. We utilized the *HMG1-lacZ* reporter gene to examine the effect of blocks in the pathway downstream of HMG-CoA reductase for their potential effect on HMG-CoA reductase induction.

Because the translational regulation of mammalian HMG-CoA reductase is mediated by a nonsterol compound (Nakanishi *et al.*, 1988), we examined the effect of a late block in the mevalonate pathway to determine if the signal regulating Hmg1p synthesis in yeast was a sterol or nonsterol compound. Sterol synthesis was inhibited in cells by treatment with zaragozic acid, a competitive inhibitor of squalene synthase (Bergstrom *et al.*, 1993) (Figure 1). This block depletes the cells of squalene, the immediate precursor of sterols, without inhibiting early isoprenoid biosynthesis. Parallel cultures of a strain containing the *HMG1-lacZ* reporter gene (JRY1443) were treated with either lovastatin, zaragozic acid, both drugs, or left untreated. Interestingly, zaragozic acid treatment of cells caused a time- and dosage-dependent inhibition of protein synthesis reminiscent of lovastatin treatments. At a point when the drug treatments had inhibited >95% of the incorporation of radiolabeled acetate into sterols (Figure 6 inset), *HMG1-lacZ* expression was measured by β-galactosidase assays. As expected, lovastatin treatment caused an induction of *HMG1-lacZ* expression. In contrast, zaragozic acid treatment alone did not induce *HMG1-lacZ* expression. These cells were still capable of induction, as evidenced by an equivalent level of induction with lovastatin

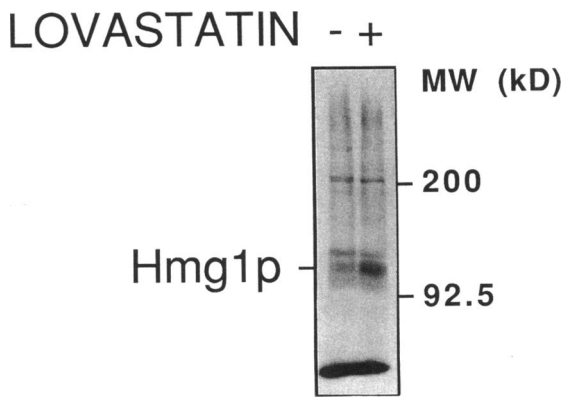


Figure 5. Induced synthesis of Hmg1p in mevalonate-starved cells. Logarithmically grown *HMG1 hmg2* cells (JRY1595) were treated with 40 μg/ml lovastatin or untreated, as indicated, for 3 h. The cells were then incubated for 10 min with Tran^{35}S-label™, whole cell lysates were prepared, and Hmg1p was immunoprecipitated from normalized lysates with an anti-TrpE-Hmg1p antibody.

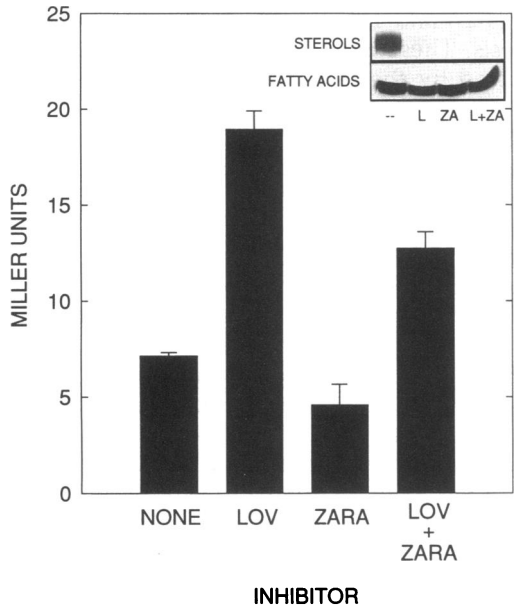


Figure 6. Induction of *HMG1-lacZ* reporter gene expression in a wild-type strain (JRY1443) by lovastatin but not by zaragozic acid. Parallel, logarithmically growing cultures were treated with either lovastatin (40 μg/ml), zaragozic acid (40 μg/ml), both, or neither. After 8 h β-galactosidase activity assays were performed. (Inset) Acetate labeling of a duplicate set of cultures. [1-^{14}C] sodium acetate was added to each culture along with the inhibitors. After 8 h, the sterols and fatty acids were extracted and resolved by TLC. Under these conditions, both drugs were very effective in specifically blocking sterol synthesis without inhibiting other modes of acetate metabolism.

compared to cells not treated with zaragozic acid (Figure 6). These data suggested that the intermediate involved in the feedback regulation of Hmg1p synthesis was upstream of squalene and therefore was a nonsterol compound derived from mevalonate.

Additional blocks in the mevalonate pathway were constructed to further define the identity of the nonsterol intermediate responsible for the regulation of Hmg1p. To construct a conditional allele of the structural gene for mevalonate kinase (*ERG12*), the endogenous mevalonate kinase gene was disrupted and complemented by a copy of the mevalonate kinase gene expressed from the *GAL1* gene promoter (Figure 7A) in cells grown on galactose-containing medium. Mevalonate kinase activity was shut off in this strain by shifting to medium containing glucose, and the effect on Hmg1p synthesis was monitored by analysis of the expression from the *HMG1-lacZ* reporter gene. Under these conditions, an induction of *HMG1-lacZ* expression was never observed up to the point when mevalonate kinase activity was undetected and cell division ceased (Figure 7B). Measurements of radiolabeled acetate into sterol pools confirmed that flux through the pathway was being inhibited; 44 h after shifting to glucose-containing medium, flux was reduced to ~10% of wild-type. Moreover, these cells retained responsiveness to flux through the mevalonate pathway. Treatment of cells with lovastatin 24 h after shifting to glucose-containing medium caused an induction of *HMG1-lacZ* expression 12 h later that was partially reversed by exogenous mevalonate. These results implied that cells monitored the level of mevalonate itself to set the appropriate level of Hmg1p synthesis.

To test further the possibility that mevalonate levels regulated synthesis of Hmg1p, a third block in the pathway was explored. The creation of a conditional allele of phosphomevalonate kinase, the next step in the mevalonate pathway (Figure 1), employed a similar strategy as that used for *ERG12*. The endogenous phosphomevalonate kinase structural gene (*ERG8*) was disrupted and replaced by a copy of *ERG8* under the control of the *GAL1* promoter (Figure 8A). As before, the phosphomevalonate kinase activity was shut off by growing the cells on medium containing glucose, and the effect on Hmg1p synthesis was monitored by measuring β-galactosidase activity expressed from the *HMG1-lacZ* reporter gene. Interestingly, *HMG1-lacZ* expression was induced upon a depletion of phosphomevalonate kinase activity (Figure 8B). At face value, this result was surprising because, if mevalonate levels set the level of Hmg1p, there was no reason to expect that mevalonate levels would decrease in a cell blocked at phosphomevalonate kinase. Rather, a simple explanation would be that mevalonate and another intermediate made distal to phosphomevalonate each contribute to feedback control of Hmg1p. However, this hypothesis in its simplest form is unlikely to be correct because the induction caused by the depletion of phosphomevalonate kinase could be partially reversed with exogenous mevalonate (Figure 8C), indicating that the regulatory axis still functioned when phosphomevalonate kinase activity was depleted. Some possible implications of these results are discussed below.

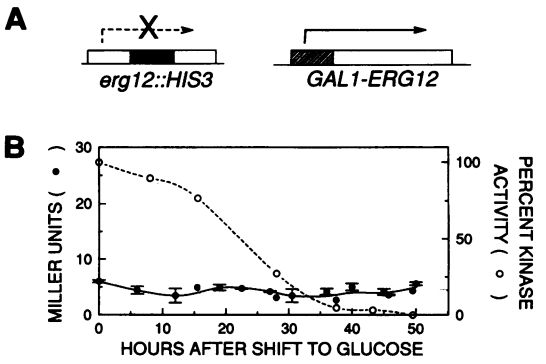


Figure 7. Effect of conditional mevalonate kinase expression upon *HMG1-lacZ* reporter gene expression. (A) Schematic map of the *GAL1-ERG12* fusion. (B) Time course of mevalonate kinase activity (---), expressed as a percentage of the activity of cells grown in galactose-containing medium, and β-galactosidase activity (—) in JRY3469 after a shift to glucose-supplemented medium. The cultures were periodically diluted into fresh medium to maintain continuous growth.

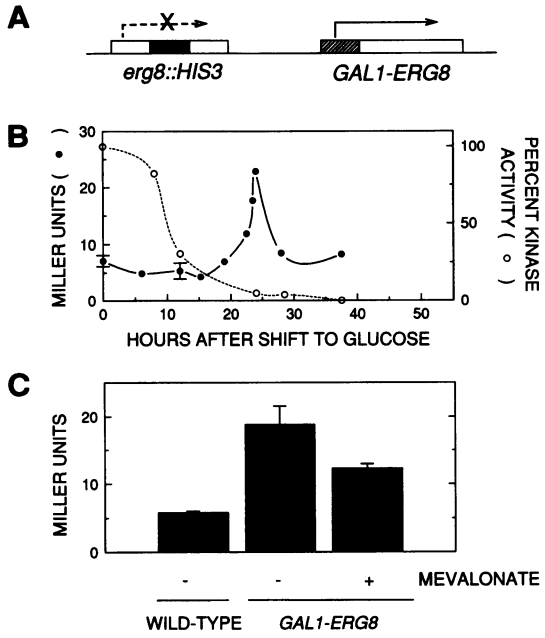


Figure 8. Effect of conditional phosphomevalonate kinase expression upon *HMG1-lacZ* reporter gene expression. (A) Schematic map of the *GAL1-ERG8* fusion. (B) Time course of phosphomevalonate kinase activity (---), expressed as a percentage of the activity of cells grown in galactose-containing medium, and β-galactosidase activity (—) in JRY3294 after a shift to glucose-containing medium. The cultures were periodically diluted into fresh medium to maintain continuous growth. (C) Partial rescue of the induced *HMG1-lacZ* expression. Forty mg/ml of exogenous mevalonate was either added to medium, or not, at the time that the *GAL1-ERG8* strain (JRY3294) was shifted to glucose-containing medium. β-galactosidase activity assays were performed on the cells after 24 h of continuous growth.

DISCUSSION

Mammalian HMG-CoA reductase is subject to feedback regulation by nonsterol and sterol products derived from mevalonate. The nonsterol product(s) appears to be involved in the regulation of HMG-CoA reductase translation and protein stability (Peffley and Sinensky, 1985; Nakanishi *et al.*, 1988; reviewed by Brown and Goldstein, 1990), whereas the sterol product(s) is responsible for the coordinate regulation of the transcription of a number of genes encoding isoprenoid biosynthetic enzymes and may play a role in regulating HMG-CoA reductase protein stability (Nakanishi *et al.*, 1988; reviewed by Brown and Goldstein, 1990). This study demonstrated the existence of feedback regulation of Hmg1p synthesis in yeast and provided information on the molecular mechanism of the regulation and the signals involved.

Mevalonate-starved yeast cells contained induced levels of Hmg1p and correspondingly induced levels of enzyme activity. This induction was not accompanied by an increase in the Hmg1p steady-state mRNA level or in any changes in transcription start site selection. In contrast to mammalian HMG-CoA reductase, there was no evidence for feedback regulation of *HMG1* transcription under these conditions. There was, however, evidence for feedback regulation of Hmg1p mRNA translation. Specifically, measurements of the incorporation of ^{35}S-methionine, -cysteine upon mevalonate starvation revealed an induction of Hmg1p synthesis with no changes in mRNA levels. This result implied that the synthesis of Hmg1p was regulated by a translational control mechanism. Because the *HMG1-lacZ* reporter gene was similarly regulated, it would appear that translational control was mediated by the 5′ untranslated region of the Hmg1p mRNA. A construct expressing only the *HMG1* coding region from the glyceraldehyde-phosphate dehydrogenase promoter does not exhibit induced steady-state levels of Hmg1p in response to lovastatin treatment (Hampton and Rine, 1994). Moreover, because the level of induced enzyme activity could be accounted for entirely by the increased translation rate of the Hmg1p mRNA, there was no evidence for any other level of feedback regulation of Hmg1p. Thus the regulation of Hmg1p synthesis employed a subset of the mechanisms operating on the mammalian enzyme, which should make subsequent analysis of this one mechanism in yeast more tractable.

Comparisons of the effect of various blocks at different points of the mevalonate pathway upon the expression of an *HMG1-lacZ* reporter gene offered some clues as to the identity of the mevalonate-derived intermediate responsible for regulating Hmg1p synthesis. It appeared that Hmg1p synthesis was keyed to the abundance of a nonsterol compound. Treatment of cells with zaragozic acid, an inhibitor of squalene synthase, did not cause an induction of reporter gene expression akin to that caused by lovastatin. This result was convenient because it demonstrated the specificity of Hmg1p feedback regulation. One possible explanation for the apparent induction of Hmg1p translation relative to overall protein synthesis was that the translation of Hmg1p mRNA simply was not inhibited to the same degree, and therefore there was no specific regulation of Hmg1p synthesis in response to mevalonate starvation. However, because zaragozic acid caused a similar inhibition of general protein synthesis without an induction of *HMG1-lacZ* expression, feedback regulation of Hmg1p synthesis appeared to be a specific regulatory response to the depletion of a nonsterol, mevalonate-derived compound. In contrast, the general inhibition of translation appeared to be a response to sterol (or squalene) depletion and may reflect a sterol regulatory axis on overall translation rates.

In an effort to define further the nonsterol regulator of Hmg1p synthesis, strains containing conditional alleles of the structural genes encoding mevalonate kinase (*ERG12*) and phosphomevalonate kinase (*ERG8*) were constructed to examine the effects of additional blocks in early steps of the mevalonate pathway on *HMG1-lacZ* reporter gene expression. Loss of mevalonate kinase activity, resulting in the depletion of all known meval-

onate-derived compounds, did not cause an induction of *HMG1-lacZ* expression. The absence of induction was even noted in cells lacking detectable mevalonate kinase activity that had ceased to divide. Treatment of these cells with lovastatin under conditions of reduced mevalonate kinase activity still caused an induction of *HMG1-lacZ* expression that could be reversed with exogenous mevalonate. These observations demonstrated that the signaling axis was still intact in cells blocked in mevalonate kinase activity and strongly suggested that mevalonate itself was the nonsterol product responsible for the feedback regulation of Hmg1p translation. The results presented here are consistent with previously published results that implicated mevalonate pools as a determinant of HMG-CoA reductase activity (Servouse and Karst, 1986).

In contrast, depletion of mevalonate diphosphate and distal intermediates caused by a loss of phosphomevalonate kinase activity (*ERG8*) caused the induction of β-galactosidase activity expressed from the *HMG1-lacZ* reporter. One interpretation of this result is that the nonsterol product responsible for the feedback regulation of Hmg1p synthesis was distal to phosphomevalonate. However, this interpretation does not explain the ability to reverse the induction with exogenous mevalonate that would not be chased into later intermediates in the pathway. In addition, it conflicts with the results of the mevalonate kinase block. Some models involving two nonsterol regulators of Hmg1p synthesis, mevalonate and an intermediate derived from mevalonate diphosphate, both of which are involved in signaling the regulation of Hmg1p synthesis, could explain the data. In the case of the mevalonate kinase block, the depletion of the downstream signal is compensated for by the accumulation of mevalonate, resulting in no induction of *HMG1-lacZ*. If this explanation is correct, the phosphomevalonate kinase block must result in the depletion of the downstream signal without a compensatory buildup of mevalonate pools, leading to an induction of *HMG1-lacZ* expression. A simpler model is that mevalonate is the only regulator of Hmg1p synthesis. By this model, phosphomevalonate would accumulate in the phosphomevalonate kinase mutant and interfere with the mevalonate signaling, perhaps by competing with mevalonate for binding to a regulatory site on a key regulatory protein, resulting in the induction of the *HMG1-lacZ* reporter. The data presented here do not distinguish between these two models. In either case, the data suggested that mevalonate was a signal for the feedback regulation of Hmg1p synthesis, although it may not be the only one.

The mechanism by which mevalonate might regulate Hmg1p translation is unclear. There are no known products derived from mevalonate other than phosphomevalonate. However, there is some indication that mevalonate and/or the phosphomevalonates are involved in regulating cell proliferation (Cuthbert and Lipsky, 1990, 1991). Both lines of evidence imply a mechanism that monitors the mevalonate pool size.

Aerobically growing yeast cells take up very little, if any, isoprenoids from their surroundings. All isoprenoids are supplied by endogenous synthesis. Therefore, it is intriguing that at least one aspect of the feedback regulatory axis of HMG-CoA reductase characteristic of mammalian cells appears to exist in yeast. Because aerobic yeast do not take up isoprenoids, it is unlikely that this regulation is involved in balancing endogenous sterol biosynthesis with exogenous supply, as it is in mammalian cells. The existence of feedback regulation of HMG-CoA reductase in yeast may be an indication of the differing sterol and/or nonsterol demands of the cells under different physiological states.

Yeast encode two isozymes of HMG-CoA reductase with almost identical catalytic domains (Basson *et al.*, 1986). Therefore, the existence of two HMG-CoA reductase isozymes may not reflect differences in their catalytic activities. More likely, the existence of two genes reflects the cell's need to regulate that activity differentially. Indeed, Hmg1p is expressed during logarithmic, aerobic growth, whereas Hmg2p is induced as cells enter stationary phase and during anaerobic conditions (Thorsness *et al.*, 1989; Casey *et al.*, 1992). One interesting possibility is that most of the mechanisms of feedback regulation characteristic of mammalian HMG-CoA reductase exist in yeast, but that yeast cells have segregated the different mechanisms to different HMG-CoA reductase isozymes, or perhaps even to other genes of the mevalonate pathway.

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