Identifying Mutations in Duplicated Functions in *Saccharomyces cerevisiae*: Recessive Mutations in HMG-CoA Reductase Genes

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ABSTRACT

The two yeast genes for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, *HMG1* and *HMG2*, each encode a functional isozyme. Although cells bearing null mutations in both genes are inviable, cells bearing a null mutation in either gene are viable. This paper describes a method of screening for recessive mutations in the *HMG1* gene, the gene encoding the majority of HMG-CoA reductase activity in the cell. This method should be applicable to the isolation of mutations in other cases of duplicated genes or duplicated functions in yeast. Four recessive point mutations were recovered in *HMG1*. These mutations exhibited intragenic complementation: one allele is in one complementation group and three alleles are in a second complementation group. Assays of HMG-CoA reductase activity indicated that the point mutations destroy most if not all of the activity encoded by *HMG1*. Intragenic complementation occurred with partial restoration of enzymatic activity. *HMG1* was mapped to the left arm of chromosome *XIII* near *SUP79*, and *HMG2* was mapped to the right arm of chromosome *XII* near *SST2*. A slight deleterious effect of a null mutation in either HMG-CoA reductase gene was detected by a co-cultivation experiment involving the wild-type strain and the two single mutants.

3-HYDROXY-3-METHYLGLUTARYL coenzyme A reductase (HMG-CoA reductase) is the rate-limiting enzyme of the sterol biosynthetic pathway. The products of this pathway have many diverse cellular roles including membrane structure, electron transport, glycoprotein biosynthesis, translation and DNA replication (reviewed in BROWN and GOLDSTEIN 1980). In mammalian cells, HMG-CoA reductase activity is regulated in a variety of ways. The level of HMG-CoA reductase activity is reduced by exogenously added sterols in the form of either serum cholesterol (BROWN, DANA and GOLDSTEIN 1973) or oxygenated sterols (TAYLOR *et al.* 1984). In addition, at least one unidentified nonsterol product of the pathway contributes to negative feedback regulation of HMG-CoA reductase activity (BROWN *et al.* 1978; COHEN, MASSOGLIA and GOSPODAROWICZ 1982; PANINI, SEXTON and RUDNEY 1984). Regulation of HMG-CoA reductase occurs at the level of mRNA coding for HMG-CoA reductase (LUSKEY *et al.* 1983; LISCUM *et al.* 1983b; CLARKE *et al.* 1983; CLARKE, FOGELMAN and EDWARDS 1985), the half-life of the protein (GIL *et al.* 1985; CHIN *et al.* 1985; EDWARDS, LAN and FOGELMAN 1983), covalent modification of the protein (BEG and BREWER 1982; GIBSON *et al.* 1982), and the level of allosteric effectors (ROITELMAN and SCHECHTER 1984; HARWOOD, BRANDT and RODWELL 1984). Other enzymes in the sterol biosynthetic pathway are regulated in a coordinated fashion with HMG-CoA reductase (BALASUBRAMANIAN, GOLDSTEIN and BROWN 1977; CHANG and LIMANEK 1980; GIL *et al.* 1986; SERVOUSE and KARST 1986).

In mammals, HMG-CoA reductase is an integral membrane protein of the endoplasmic reticulum (LISCUM *et al.* 1983a; BROWN and SIMONI 1984; EDWARDS *et al.* 1985) and may also be present in peroxisomes (KELLER, PAZIRANDEH and KRISANS 1986). Indirect evidence indicates that HMG-CoA reductase acts as a dimer (EDWARDS *et al.* 1985). However, since the intact protein has never been purified, the multimeric nature of HMG-CoA reductase remains unresolved. The yeast HMG-CoA reductase enzyme is probably also an integral membrane protein (M. E. BASSON, unpublished data; R. WRIGHT, unpublished data).

The mammalian genome apparently contains a single gene encoding HMG-CoA reductase (REYNOLDS *et al.* 1984). In contrast, yeast contains two genes, *HMG1* and *HMG2*, each encoding an isozyme of HMG-CoA reductase. Cells bearing a null allele of either *HMG1* or *HMG2* are viable. However, cells bearing null alleles of both *HMG1* and *HMG2* are inviable. Viability of the double mutant can be rescued with a plasmid that contains a copy of either *HMG1* or *HMG2* (BASSON, THORSNESS and RINE 1986). Regulation of HMG-CoA reductase activity in yeast in response to varying growth conditions or to mutational blocks in the sterol biosynthetic pathway has been described (QUAIN and HASLAM 1979; DOWNING, BURROWS and BARD 1980; SERVOUSE and KARST 1986).

Classical mutational analysis of the two yeast HMG-CoA reductase genes is cumbersome since mutants that lack one of the two isozymes have no obvious phenotypes and since it is difficult to supplement yeast

with mevalonate, the product of the HMG-CoA reductase reaction (PARKS 1978; SERVOUSE *et al.* 1984). In this paper, a novel method is described for isolating mutations in either member of a pair of duplicated genes. Using this method, four recessive point mutations were isolated in *HMG1*. Some pairs of *HMG1* heteroalleles exhibited intragenic complementation, and the effect of these mutations on the level of HMG-CoA reductase activity was characterized. In addition, the map positions of both *HMG1* and *HMG2* were determined. A subtle growth phenotype for these mutants was identified by measurement of the growth properties of *HMG1* and *HMG2* single mutants.

MATERIALS AND METHODS

Media: Yeast rich medium containing 2% glucose (YPD), yeast minimal medium containing 2% glucose (YM), and sporulation medium have been described (BARNES *et al.* 1984). *lys2* cells were selected by their ability to grow on solid α-aminoadipate medium (CHATTOO *et al.* 1979). *ura3* cells were selected by their ability to grow on solid 5-fluoroorotic acid (5-FOA) medium (BOEKE, LACROUTE and FINK 1984). Media were supplemented with amino acids and bases at 30 mg/liter as needed. Solid media contained 2% agar.

Materials: 5-FOA was obtained from P. L. Biochemicals or from Specialty Chemicals, Inc. (Gainesville, FL). Enzymes were purchased from commercial sources and used according to suppliers' specifications. All other chemicals were from Sigma (St. Louis, MO).

Strains: The complete genotypes of all yeast strains are described in Table 1. The relevant characteristics of all plasmids are described below. *Escherichia coli* strain DH1 was used for the propagation of all plasmids (HANAHAN 1983).

Genetic techniques: Mating, sporulation induction, tetrad analysis, and mating type determination have been described (MORTIMER and HAWTHORNE 1969). Yeast cells were mutagenized with ethylmethanesulfonate (EMS) as described (SHERMAN, FINK and HICKS 1983) except that the amount of EMS was doubled to 100 μl for the second round of mutagenesis. Yeast spheroplasts were transformed by a modification of the procedure of HINNEN, HICKS and FINK (1978). *In vitro* plasmid constructions were performed as described (MANIATIS, FRITSCH and SAMBROOK 1982). A null allele of *RAD52* containing the *LEU2* gene inserted within the coding sequences was provided by D. SCHILD (personal communication), and is designated *rad52::LEU2*. Null alleles of *HMG1* and *HMG2*, designated *hmg1::LYS2* and *hmg2::HIS3*, respectively, have been described (BASSON, THORSNESS and RINE 1986). In some meiotic mapping experiments, the *HMG1* locus was marked by *URA3* by integration of pJR5923 at *HMG1* (see below). This integration disrupts *HMG1* function (M. THORSNESS, personal communication), and the resulting locus was designated *hmg1::URA3*. The segregation of *SUP79um*, a UGA suppressor, was monitored by suppression of the umber alleles *lys1-1um* and *his5-2um*. The segregation of *SUF7*, a frameshift suppressor, was monitored by suppression of the frameshift allele *his4-713*. Random spore analysis was used to establish the map order of *HMG1*, *SUP79* and *RAD52* in a three-factor cross (see RESULTS). In this cross, *HMG1* was marked by *URA3* and *RAD52* was marked by *LEU2*. *SUP79um hmg1::URA3* spores were selected on medium lacking histidine and uracil. The spore clones were screened for mating proficiency to distinguish haploid spore clones from non-sporulated diploid cells, and then tested for leucine dependence to examine segregation of the *rad52::LEU2* allele.

Plasmid constructions: The plasmids pJR401 and pJR402 both contain the *HMG2*, *LYS2* and *URA3* genes. Both plasmids are stably inherited during mitotic growth. pJR401 contains the origin of replication from the endogenous 2-μm plasmid and thus is maintained at multiple copies per cell (JAYARAM, LI and BROACH 1983). pJR402 contains *ARS1* and *CEN4*, which allow its stable inheritance at one or two copies per cell (STINCHCOMB, MANN and DAVIS 1982). pJR401 was constructed as follows: the intact *LYS2* gene was removed from pDA6200 as a 4.6-kilobase (kb) *Eco*RI-*Hin*dIII fragment (BARNES and THORNER 1986) and this fragment was inserted into the polylinker of pSEY8 (EMR *et al.* 1986) at the *Eco*RI and *Hin*dIII sites, generating pJR369. The intact *HMG2* gene was removed from pJR322 (BASSON, THORSNESS and RINE 1986) as a 4.9-kb *Eco*RI fragment and was inserted into the unique *Eco*RI site in pJR369, generating pJR401. pJR402 was constructed as follows: a 4.6-kb *Eco*RI-*Hin*dIII fragment containing the *LYS2* gene (see above) was inserted into the polylinker of pSEYC58, a centromere-containing plasmid (EMR *et al.* 1986) at the *Eco*RI and *Hin*dIII sites, generating pJR370. A 4.9-kb *Eco*RI fragment containing the *HMG2* gene (see above) was inserted into the unique *Eco*RI site in pJR370, generating pJR402. The structures of pJR401 and pJR402 are shown in Figure 1. pJR5923 was constructed by inserting a 1.57-kb *Sph*I-*Eco*RI fragment containing *HMG1* coding sequences from pJR59 (BASSON, THORSNESS and RINE 1986) into the *Sph*I and *Eco*RI sites of the integrating vector YIp5, which contains the *URA3* gene as its selectable marker (STRUHL *et al.* 1979). This plasmid was linearized at the unique *Xba*I site in the *HMG1* coding sequences and integrated at the *HMG1* locus by transformation of *ura3* cells with selection for uracil independence. A gel-transfer hybridization experiment confirmed that integration had occurred at the *HMG1* locus as expected.

Preparation of cell extracts: A modification of the procedure of QUAIN and HASLAM (1979) was used to prepare cell extracts. Cell cultures (50 ml) were grown in YPD medium at 30° with vigorous shaking to an OD_{600} of 2–4. The cells were pelleted and washed twice with 25 ml of 50 mM potassium phosphate (KPO_4) buffer, pH 7.0. The cells were then resuspended in 300 μl of lysis buffer (100 mM KPO_4, pH 7.0, 1 mM Na_2EDTA, pH 8.0, 5 mM dithiothreitol (DTT)) in a glass test tube. Glass beads (0.5 mm, Biospec Products, Bartlesville, OK) were added to reach the meniscus, and the cells were broken by vortexing at high speed for five 1-min intervals separated by periods of cooling on ice. An additional 300 μl of lysis buffer was added to assist in recovery of the extract. The extract was transferred to microfuge tubes, brought to 0.5% (w/v) Nonidet P-40 (NP40), and incubated on ice for 1 hr. The extract was centrifuged at 1500 × *g* for 2 min at 4°. The supernatant fraction was then centrifuged at 8000 × *g* for 10 min at 4°. The supernatant fraction was then frozen in liquid nitrogen and stored at −20°.

HMG-CoA reductase activity assays: Activity assays were modified from the procedure of QUAIN and HASLAM (1979). Activity in cell extracts was assayed in 100 mM KPO_4, pH 7.0, 0.5% (w/v) NP40, 5 mM DTT and 150 μM NADPH. HMG-CoA was added to a final concentration of 150 μM to start the reaction. The assay was carried out in 400 μl at room temperature. Activity was monitored as the change in absorbance at 340 nm over time using a Spectronic 2000 dual beam spectrophotometer. Protein determinations were by the method of BRADFORD (1976) using bovine serum albumin as the standard.

Orthogonal field alternating gel electrophoresis:

TABLE 1

Strain List

Strain[a]	Genotype	Strain[a]	Genotype
JRY527	**a** *ura3-52 his3Δ200 lys2-801 ade2-101 met*	JRY1404	*α hmg1-4 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 tyr1* pJR401
JRY528	*α ura3-52 his3Δ200 lys2-801 ade2-101 tyr1*	JRY1408	diploid formed by mating JRY1370 to JRY1231
JRY980	**a** *HMG1 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met tyr1*	JRY1409	diploid formed by mating JRY1370 to JRY1124
JRY986	*α HMG1 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met tyr1*	JRY1412	*α hmg1-5 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met* pJR401
JRY1124	**a** *HMG1 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101*	JRY1413	**a** *hmg1-5 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 tyr1* pJR401
JRY1125	*α HMG1 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101*	JRY1415	diploid formed by mating JRY1391 to JRY1404
JRY1145	*α hmg1::LYS2 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met* pJR401	JRY1416	diploid formed by mating JRY1257 to JRY1412
JRY1159	**a** *hmg1::LYS2 HMG2 ura3-52 his3Δ200 lys2-801 ade2-101 met*	JRY1417	diploid formed by mating JRY1389 to JRY1413
JRY1160	**a** *HMG1 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met*	JRY1419	diploid formed by mating JRY1391 to JRY1412
JRY1196	JRY980 + pJR401 (*HMG2 LYS2 URA3 2μ*)	JRY1422	diploid formed by mating JRY1257 to JRY1404
JRY1197	JRY980 + pJR402 (*HMG2 LYS2 URA3 CEN4 ARS1*)	JRY1468	**a** *hmg1-2 HMG2 ura3-52 his3Δ200 lys2-801 ade2-101*
JRY1198	JRY986 + pJR401 (*HMG2 LYS2 URA3 2μ*)	JRY1469	*α hmg1-3 HMG2 ura3-52 his3Δ200 lys2-801 ade2-101*
JRY1228	**a** *hmg1-2 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met tyr1* pJR401	JRY1470	*α hmg1-4 HMG2 ura3-52 his3Δ200 lys2-801 ade2-101*
JRY1229	diploid formed by mating JRY1228 to JRY1125	JRY1564	**a** *hmg1::URA3 rad52::LEU2 ura3-52 leu2 his5-2um lys1-1um ade2 ade6 met2 trp1*
JRY1231	**a** *hmg1::LYS2 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met tyr1* pJR401	JRY1565	*α SUP79 ura3-52 leu2 his5-2um lys1-1um ade2 met2*
JRY1257	**a** *hmg1-2 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met* pJR401	JRY1568	**a** *SUF7 ura3-52 leu2 his4-713 lys2-20 met2-1*
JRY1258	*α hmg1-2 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 tyr1* pJR401	JRY1569	*α hmg1::URA3 rad52::LEU2 ura3-52 leu2 his4-713 ade2 lys2-20*
JRY1368	*α hmg1-3 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met tyr1* pJR401	JRY1649	*α hmg2::HIS3 sir3-8[u] his3 ura3 trp1 lys2 leu2*
JRY1370	*α hmg1-5 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met tyr1* pJR401	JRY1650	**a** *SST2::URA3 ura3-52 his3Δ1 ade2-1* alias RK512-5B
JRY1387	diploid formed by mating JRY1368 to JRY1231	JRY1656	*α his3 ura4 arg4 ade aro lys*
JRY1388	diploid formed by mating JRY1368 to JRY1124	JRY1657	**a** *hmg2::HIS3 his3 ade2 met lys*
JRY1389	*α hmg1-3 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met* pJR401	JRY1711	JRY1393 without pJR401
JRY1391	**a** *hmg1-3 hmg2::HIS3 ura3-52 his3Δ200 lys2-801 ade2-101 met tyr1* pJR401	JRY1712	JRY1415 without pJR401
JRY1393	diploid formed by mating JRY1258 to JRY1391	JRY1714	JRY1419 without pJR401
		JRY1715	*α hmg1-5 HMG2 ura3-52 his3Δ200 lys2-801 ade2-101 met*

[a] JRY1650 was from W. COURSCHESNE. All other strains are from the lab collection or were constructed during the course of this work.

Pulsed-field gels were prepared and run by a modification of the procedures of SCHWARTZ and CANTOR (1984) and CARLE and OLSEN (1984) as described (O'REAR and RINE 1986). For mapping *HMG1*, the hybridization probe was a 2.5-kb *Eco*RI fragment from pJR59. For mapping *HMG2*, the hybridization probe was a 4.9-kb *Eco*RI fragment from pJR322 (BASSON, THORSNESS and RINE 1986).

RESULTS

Rationale for mutant isolation: *HMG1* encodes the majority of the HMG-CoA reductase activity in the cell (BASSON, THORSNESS and RINE 1986) and was chosen as the gene with which to begin isolation of

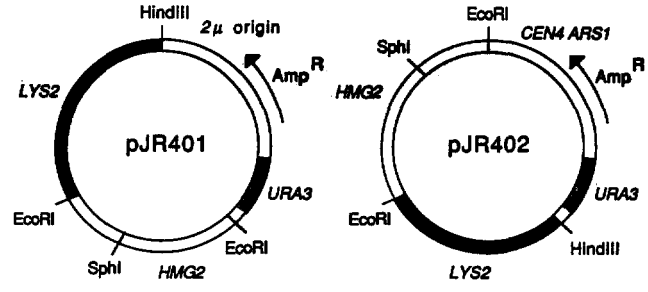

FIGURE 1.—Plasmid maps of pJR401 and pJR402 are shown approximately to scale. pSEY8 and pSEYC58 (EMR *et al.* 1986) were the parental vectors used to construct pJR401 and pJR402, respectively.

point mutations. These mutations will serve as a starting point for the goal of understanding the regulation of the sterol biosynthetic pathway in yeast. Classical approaches to the isolation of recessive point mutations in *HMG1* are limited in two respects. First, recessive mutations would have no easily detectable phenotype since *HMG1* function can be replaced by *HMG2*. Second, there have been no reports of uptake by yeast of exogenously added intermediates in this pathway. Thus *hmg1 hmg2* double mutants have no permissive growth media.

To circumvent these limitations, a screen for mutations in *HMG1* was developed. These experiments made use of an *HMG1* strain containing a disrupted chromosomal copy of the *HMG2* gene (*hmg2::HIS3*) but containing an intact copy of the *HMG2* gene on an autonomous plasmid (pJR401 or pJR402). These plasmids are stably propagated during mitotic growth such that, after ten generations of growth in nonselective medium, greater than 85% of the cells contain at least one copy of the plasmid. However, by requiring cells to grow on medium that prevented the growth of those cells in the population that contained the plasmid, the screening procedure used in this study exploited the small fraction of cells in the population that had lost the plasmid. Specifically, the *LYS2* and *URA3* genes contained on pJR401 and pJR402 confer a severe growth disadvantage to cells grown on medium containing α-aminoadipate and medium containing 5-FOA, respectively. If a single colony is grown without selection for the plasmid and then replica-plated onto medium that selected against either *LYS2* or *URA3* (negative selection medium), strong growth is observed due to those cells in the colony that have lost the plasmid. However, if a mutation in *HMG1* occurred, the *HMG2* gene on the plasmid becomes essential for growth. Thus a single colony grown from a cell that had sustained a mutation in *HMG1* would exhibit no growth when replica-plated onto the negative selection medium.

To isolate mutants, strains JRY1196, JRY1197, and JRY1198 were mutagenized, plated onto YPD medium, and grown into single colonies. The colonies were then replica-plated onto negative selection medium. Those colonies that failed to grow were considered putative *hmg1* mutants.

Isolation of mutants: Mutagenesis was performed in two rounds. In the first round, three independent cultures of both JRY1196 and JRY1197 were lightly mutagenized such that 92% of the cells survived mutagenesis. In the second round, four independent cultures of JRY1198 were mutagenized such that 7% of the cells survived. Mutagenized cells were plated onto YPD solid medium at a density of 100 viable cells per plate and grown at 34°. Colonies were replica-plated onto α-aminoadipate plates to select against the *LYS2* marker on pJR401 or pJR402. Potential *hmg1* mutants were identified by their inability to grow at 34° on the α-aminoadipate plate and were picked from the YPD plate. Forty-eight candidates were recovered from 10,000 colonies screened from the first round, and 126 candidates were recovered from 2500 colonies screened from the second round. To confirm their inability to grow without the plasmid, the 174 candidates were rescreened by streaking onto 5-FOA plates to select against the *URA3* marker on pJR401 or pJR402. Twenty-nine candidates were unable to grow on the 5-FOA plates and were studied further. The remaining candidates that passed the *LYS2* negative screen but failed the *URA3* negative screen were discarded. None of the 29 candidates displayed a temperature-sensitive requirement for pJR401 or pJR402.

Cells in which the plasmid integrated into a chromosome would appear to be *hmg1* mutants in both screens since the *LYS2* and *URA3* markers would be lost during mitotic growth at a very low frequency. To test for plasmid integration, each of the 29 candidates was mated to an *HMG1 hmg2::HIS3* strain (JRY1124 or JRY1125) and the resulting diploids were tested for their ability to survive on medium containing 5-FOA. *hmg1* mutations are predicted to be recessive to the wild-type *HMG1* gene in the diploid, thus eliminating the requirement for the *HMG2* gene and allowing loss of the plasmid-borne *URA3* marker. In contrast, integrated plasmids would remain stable in diploids and prevent loss of the *URA3* marker. Two of the 29 diploids were unable to grow on the negative selection medium and were not studied further. It should be noted that a dominant *HMG1* mutation would be indistinguishable from an integrated plasmid by this test.

Identification of *hmg1* mutants: The 27 remaining candidates were each mated to a strain of the genotype *hmg1::LYS2 hmg2::HIS3* containing *HMG2* on pJR401 (JRY1231 and JRY1145) and mutations in *HMG1* were identified by their inability to complement the null allele *hmg1::LYS2*. If the candidate carried a mutation in *HMG1*, the resulting diploid would require the plasmid-borne *HMG2* gene and would be unable to grow on medium that selected against the plasmid. The diploids were streaked onto 5-FOA plates to determine whether the mutations complemented *hmg1::LYS2*. Four of the 27 diploids were unable to grow on 5-FOA plates, indicating that these four diploids contained mutations that could not complement *hmg1::LYS2*. These mutations were therefore presumptive *hmg1* mutations.

Evidence that the phenotype of these *hmg1* mutants was due to a single nuclear mutation came from crosses between three of the mutants and a strain of the genotype *HMG1 hmg2::HIS3*. The resulting diploids were subjected to tetrad analysis to determine whether the inability to grow without the *HMG2*-

TABLE 2

The 2+:2− segregation of *hmg1* mutations

Diploid	Genotype[a]	No. of tetrads analyzed	No. of tetrads with 2+:2− viability on 5-FOA plates
JRY1229	*hmg1-2/HMG1*	22	22
JRY1388	*hmg1-3/HMG1*	21	21
JRY1409	*hmg1-5/HMG1*	10	10

[a] All three strains were *hmg2:HIS3/hmg2::HIS3*.

containing plasmid segregated in a 2+:2− fashion (Table 2). Analysis of these crosses was limited to those tetrads in which all four spores inherited pJR401 and hence were viable on YPD medium. All segregants from each of the three diploids were streaked onto 5-FOA plates. In each tetrad, the ability to grow on 5-FOA medium and hence without *HMG2* segregated in a 2+:2− manner as expected for a single mutation in the *HMG1* gene. The *hmg1-4* mutation could not be analyzed due to extremely poor spore viability in this cross.

Allelism tests: To determine whether the putative *hmg1* mutations as defined by complementation tests were truly alleles of *HMG1*, crosses were performed between two putative *hmg1* mutants and an *hmg1::LYS2* strain and the frequency of recombinants bearing a wild-type *HMG1* gene was measured. If the mutations were not *HMG1* alleles, *HMG1* recombinants would arise at a high frequency. As in the previous section, analysis of the tetrads from these diploids, JRY1387 and JRY1408, was limited to tetrads in which all four spores were viable and thus inherited the plasmid. As shown in Table 3, all segregants from these two diploids failed to grow on 5-FOA medium, indicating that all segregants contained an *hmg1* mutation. Therefore, the mutation that caused the inability of cells to grow in the absence of *HMG2* mapped close to and presumably within the *HMG1* gene.

Five additional diploids heteroallelic for two *hmg1* point mutations were tested for *HMG1* recombinants. Among a total of 56 complete tetrads from these diploids, only one 5-FOA resistant segregant was recovered (Table 3). We presume that this segregant arose either from gene conversion or intragenic recombination between *hmg1-2* and *hmg1-4*. Thus all of the putative *hmg1* mutations were alleles of the *HMG1* locus.

Intragenic complementation: As mentioned above, all four *hmg1* mutations failed to complement the null allele *hmg1::LYS2*. Since mammalian HMG-CoA reductase is thought to contain two or more structural domains (LISCUM *et al.* 1985) and since the predicted structure of the yeast enzyme is similar to the mammalian enzyme (M. E. BASSON, unpublished data), the ability of *HMG1* alleles to exhibit intragenic complementation was examined. All possible pairwise combinations of *hmg1* alleles were combined in diploids and tested for their ability to complement. In these experiments, haploids of opposite mating types, each containing the *hmg1* allele of interest, *hmg2::HIS3* and pJR401, were mated to produce diploids able to grow (due to the *HMG2* gene carried on pJR401) whether or not the *hmg1* alleles complemented. Complementation between the *hmg1* alleles was tested by streaking these diploids onto 5-FOA medium and determining whether the diploids could grow without the plasmid (complementation) or not (no complementation). The results presented in Table 4 demonstrate the existence of two complementation groups within the *HMG1* gene.

TABLE 3

Allelism tests of *hmg1* mutations

Strain	Genotype[a]	Diploid growth on 5-FOA	No. of tetrads	No. of *HMG1* recombinants
JRY1387	*hmg1-3/hmg1::LYS2*	−	14	0
JRY1408	*hmg1-5/hmg1::LYS2*	−	10	0
JRY1422	*hmg1-2/hmg1-4*	−	16	1
JRY1416	*hmg1-2/hmg1-5*	−	10	0
JRY1393	*hmg1-2/hmg1-3*	+	5	0
JRY1415	*hmg1-3/hmg1-4*	+	5	0
JRY1417	*hmg1-3/hmg1-5*	+	20	0

[a] All strains were *hmg2::HIS3/hmg2::HIS3*.

It seemed unlikely that mitotic recombination between *hmg1* point mutations had reconstituted a wild-type *HMG1* gene in the diploids heteroallelic for *hmg1-3* and any of the other three *hmg1* point mutations. Nevertheless, this possibility was tested directly by analyzing tetrads from diploids JRY1393, JRY1415 and JRY1417 which contained complementing *hmg1* heteroalleles. Diploid cells that still contained pJR401 were sporulated and segregants were tested for their

TABLE 4

***HMG1* complementation groups[a]**

Allele	Allele					
	hmg1-2	*hmg1-3*	*hmg1-4*	*hmg1-5*	*HMG1*	*hmg1::LYS2*
hmg1-2	−	+	−	−	+	−
hmg1-3	+	−	+	+	+	−
hmg1-4	−	+	−	−	+	−
hmg1-5	−	+	−	−	+	−
HMG1	+	+	+	+	+	+
hmg1::LYS2	−	−	−	−	+	−

[a] *hmg1 hmg2::HIS3* strains containing pJR401 were mated in all pairwise combinations. The *hmg1::LYS2 hmg2::HIS3* strains used were JRY1145 and JRY1231. The *HMG1 hmg2::HIS3* strains used were JRY1124 and JRY1125. Growth of diploids on 5-FOA medium is represented by (+), indicating complementation. No growth of diploids on 5-FOA medium is represented by (−), indicating lack of complementation. See text for details.

TABLE 5

Specific activity of HMG-CoA reductase in extracts from *hmg1* mutants

Strain	Genotype	Extract[a]	Specific activity[b] Exp. 1	Exp. 2
JRY527	*HMG1 HMG2*	A	5.83	4.56
		B	6.01	4.60
JRY528	*HMG1 HMG2*	A	4.91	
		B	4.35	
JRY1159	*hmg1::LYS2 HMG2*	A	1.31	
		B	1.04	
JRY1468	*hmg1-2 HMG2*	A	1.31	
		B	1.66	
JRY1469	*hmg1-3 HMG2*	A	1.34	
		B	1.40	
JRY1470	*hmg1-4 HMG2*	A	1.40	
		B	1.58	
JRY1715	*hmg1-5 HMG2*	A	1.43	
		B	1.33	
JRY1711	*hmg1-2 hmg2::HIS3*	A	0.45	
	hmg1-3 hmg2::HIS3	B	0.44	
JRY1712	*hmg1-4 hmg2::HIS3*	A		0.50
	hmg1-3 hmg2::HIS3	B	0.54	0.46
JRY1714	*hmg1-5 hmg2::HIS3*	A	1.18	
	hmg1-3 hmg2::HIS3	B	1.12	

[a] Extracts A and B were prepared from two cultures grown from independent single colonies. The extracts for experiments 1 and 2 were prepared from separate sets of parallel cultures. Each of the extracts was assayed using three different amounts of extract. The activity was linear with dilution.

[b] Activity is expressed as nmol of NADPH oxidized·min^{-1}·(mg of protein)$^{-1}$.

ability to grow on 5-FOA plates (Table 3). None of the segregants from a total of 30 tetrads was *HMG1*. Thus, the growth of the diploids in the absence of pJR401 was due to intragenic complementation and not to recombination between point mutations.

HMG-CoA reductase activity assays of extracts from *hmg1* strains: The growth of diploids containing complementing mutant alleles of *HMG1* was dramatically slower than that of wild-type strains, suggesting that complementation did not completely restore the wild-type level of enzymatic activity. To determine how much activity was restored by intragenic complementation, the specific activity of HMG-CoA reductase in extracts prepared from closely related strains containing *hmg1* mutations either singly or in complementing pairs was determined (Table 5). These data indicate that point mutations in *HMG1* decreased the amount of HMG-CoA reductase activity to approximately the same extent as the *hmg1::LYS2* null allele. Furthermore, the *hmg1-2/hmg1-3* and *hmg1-3/hmg1-4* diploids had significantly less activity than any *hmg1 HMG2* strain, which has only 17% of the activity found in an *HMG1 hmg2* strain (BASSON, THORSNESS and RINE 1986). Therefore the complementing alleles did not completely restore the wild-type level of enzyme activity. The *hmg1-3/hmg1-5* allele combination restored significantly greater activity than the other two allele combinations.

Identification of *cpa* mutations: Nine of the 23 remaining mutants that had passed both the *LYS2* and the *URA3* negative selection tests had an unexpected phenotype. They had passed these tests not because a mutation had rendered the *HMG2* gene on the plasmid essential, but rather because a mutation had occurred that caused the strain to be unable to grow on minimal medium containing uracil. Thus, these mutants were not in any way defective in HMG-CoA reductase, but had been recovered simply because both the α-aminoadipate screen and the 5-FOA screen required the addition of uracil to the medium to allow the growth of cells lacking the plasmid.

Although these nine mutants were unable to grow on minimal medium containing uracil, they could grow on YPD medium even when the YPD medium was supplemented with uracil. Therefore some component of YPD medium was relieving the uracil-dependent growth inhibition. An explanation for this phenotype was suggested by the phenotypes of mutants defective in *CPA1* and *CPA2*, genes encoding the two subunits of the carbamoylphosphate synthetase of the arginine biosynthetic pathway (LACROUTE *et al.* 1965). Under normal growth conditions, enough carbamoylphosphate is produced by the carbomoylphosphate synthetase of the uracil biosynthetic pathway to supply both the arginine and the uracil needs of the cell. However, in the presence of exogenous uracil, the carbamoylphosphate synthetase of the uracil pathway is inhibited, causing *cpa* mutants to be starved for arginine. Thus in minimal medium containing uracil, mutations in the *CPA1* and *CPA2* genes cause an arginine requirement. The nine mutants unable to grow on minimal medium containing uracil were found to be able to grow on minimal medium containing both arginine and uracil. Complementation tests were performed between these nine mutants and known *cpa1* and *cpa2* mutants. The results of these complementation tests revealed that two of these mutations were in the *CPA1* gene and seven were in the *CPA2* gene (Table 6).

Other mutants: When mated to an *HMG1 hmg2::HIS3* strain, 12 of the remaining mutants yielded tetrads in which all four segregants were viable even in the absence of the plasmid. These mutants were not studied further. The remaining two mutants contained a single mutation that caused two spores in each tetrad to be unable to germinate regardless of the presence or absence of the plasmid. The nature of the germination defect was not investigated.

Genetic mapping of *HMG1* and *HMG2*: An *HMG1* restriction fragment with little sequence homology to

TABLE 6

Complementation groups of *cpa* mutants[a]

Strain	Strain								
	α-104	α-115	α-116	α-117	α-118	α-120	α-121	α-*cpa1-2*	α-*cpa2-2*
a-102	+	+	−	+	+	+	+	−	+
a-104	−	−	+	−	−	−	−	+	−
a-105	−	−	+	−	−	−	−	+	−
a-115	−	−	+	−	−	−	−	+	−
a-117	−	−	+	−	−	−	−	+	−
a-118	−	−	+	−	−	−	−	+	−
a-120	−	−	+	−	−	−	−	+	−
a-121	−	−	+	−	−	−	−	+	−
a-*cpa1-2 cpa2-1*	−	−	−	−	−	−	−	−	−
a-*cpa2-1*	−	−	+	−	−	−	−	+	−
a-CPA1 CPA2	+	+	+	+	+	+	+	+	+

[a] Mutants 102 through 121 were derived from JRY1198, an α strain. Each mutant was mated to JRY1124 to obtain a haploid strain of the opposite mating type that contained the mutation. **a** mutant strains were mated to α mutant strains and the resulting diploids were streaked onto minimal medium containing uracil. Growth on this medium indicated complementation and is represented by (+). No growth indicated lack of complementation and is represented by (−).

HMG2 was radiolabeled and hybridized to a nitrocellulose filter replica of a pulsed-field gel containing yeast chromosomes separated into bands by electrophoresis. A single hybridizing band was observed corresponding to a doublet containing chromosomes *XIII* and *XVI*. A cross between JRY1568 and JRY1569 established that *HMG1* was on the left arm of chromosome *XIII*, 7.1 cM from *SUF7*. A cross between JRY1564 and JRY1565 established close linkage of *HMG1* to *SUP79* (1.7 cM). To determine the gene order, random spore analysis was performed on this last cross (see MATERIALS AND METHODS). Among 63 recombinants for *SUP79* and *hmg1::URA3*, 50 were *RAD52* (coupled with *SUP79* in JRY1565), and 13 were *rad52*. These data place *HMG1* distal to *SUP79* on the left arm of chromosome *XIII*. The map distances for the relevant markers in these crosses are summarized in Table 7.

Hybridization analysis of a radiolabeled *HMG2* restriction fragment to a nylon filter replica of a pulsed-field gel indicated that *HMG2* resides on chromosome *XII*. *HMG2* was mapped to the right arm of chromosome *XII* by a cross between JRY1656 and JRY1657, establishing linkage of *HMG2* to *URA4*. A three-factor cross between JRY1649 and JRY1650 indicated that *HMG2* was tightly linked to *SST2* (1.0 cM) and lay between *SIR3* and *SST2*. The segregation of *SIR3* in the four recombinant segregants for *HMG2* and *SST2* was consistent with this gene order; namely, the two *HMG2 SST2* segregants were both *SIR3*, and the two *hmg2::HIS3 SST2::URA3* segregants were both *sir3-8*. The map distances for the relevant markers in these crosses are summarized in Table 7.

Growth properties of *hmg1* and *hmg2* mutants: *hmg1* and *hmg2* single mutants have an increased sensitivity to compactin, a competitive inhibitor of

TABLE 7

Summary of map distances

Interval	PD	T	NPD	Distance (cM)
HMG1				
SUP79-HMG1[a]	58	2	0	1.7
SUF7-HMG1[b]	42	7	0	7.1
SUP79-RAD52[a]	26	30	1	32
SUF7-RAD52[b]	18	30	0	31
HMG2				
URA4-HMG2[c]	22	21	0	24
SIR3-HMG2[d]	90	13	0	6.3
SIR3-SST2[d]	85	15	0	7.5
SST2-HMG2[d]	98	2	0	1.0

SUP79 *SUF7* *RAD52* *CENXIII*
HMG1

CENXII *URA4* (*SIR3* *SST2*)
HMG2

[a] JRY1564 × JRY1565.
[b] JRY1568 × JRY1569.
[c] JRY1656 × JRY1657.
[d] JRY1649 × JRY1650.

HMG-CoA reductase (BASSON, THORSNESS and RINE 1986). Observation of *hmg1* and *hmg2* single mutants on standard minimal or rich medium containing 2% glucose or rich medium containing 2% glycerol revealed no obvious growth phenotype. Since 16 generations of growth is sufficient for a single cell to form a full-sized colony, subtle effects on growth rate might be missed during observation on this time scale. To provide a more sensitive measure of the relative growth properties of the mutants in comparison to a wild-type strain, equal numbers of cells from a trio of strains with the genotypes *HMG1 HMG2*, *hmg1::LYS2 HMG2*, and *HMG1 hmg2::HIS3* (JRY527, JRY1159,

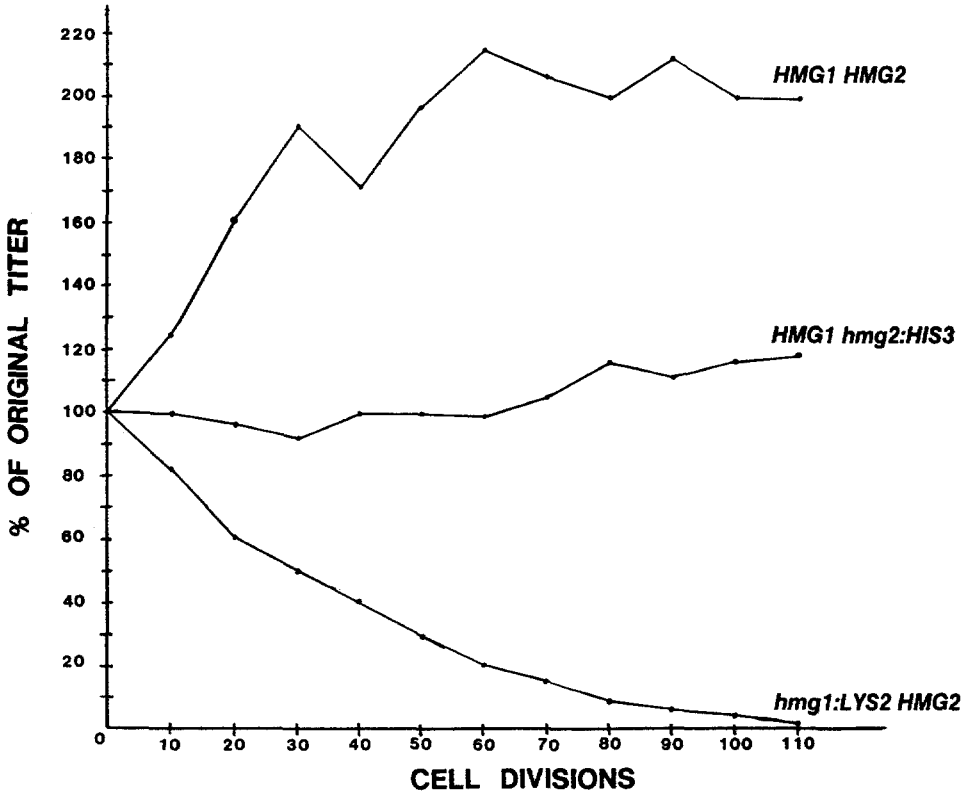

FIGURE 2.—A co-cultivation experiment measured the relative growth of JRY527 (*HMG1 HMG2*), JRY1159 (*hmg1::LYS2 HMG2*), and JRY1160 (*HMG1 hmg2::HIS3*). Each curve represents the average values for each strain from two independent co-cultivation experiments. The fraction of the total population contributed by a given strain divided by the fraction of the population contributed by that strain at the beginning of the experiment, multiplied by 100, is plotted *vs.* the number of generations of growth of the culture. See the text for details.

and JRY1160, respectively, isogenic except at *HMG1* and *HMG2*) were inoculated into YPD liquid medium supplemented with lysine and histidine. The three strains were co-cultivated for 12 days with daily 1:1000 dilutions into fresh supplemented YPD medium. The fraction of the total culture made up of each of the three strains was determined by plating diluted aliquots of the culture onto YPD plates, growing the cells into colonies, and replica-plating the colonies onto appropriately supplemented minimal medium plates. The results from two independent trials were almost identical and are shown in Figure 2. The *HMG1 HMG2* strain had the highest fitness under these conditions. The *hmg1* strain was at a significant growth disadvantage and the *hmg2* strain was of intermediate fitness. Thus the relative growth of these three strains in YPD medium parallels their relative sensitivity to compactin (BASSON, THORSNESS and RINE 1986). It should be noted that the relative fitness of these three strains in the co-cultivation experiment may reflect differences in any of their growth characteristics, including their doubling times, their lag periods for recovery from stationary phase, or their viability in stationary phase.

DISCUSSION

We have devised a screen to allow the recovery of recessive mutations in *HMG1*, which encodes one of the two isozymes of HMG-CoA reductase in *Saccharomyces cerevisiae*. Using this screen, four independent alleles of *HMG1* were recovered. HMG-CoA reductase activity assays confirmed that there is little if any activity provided by these *hmg1* alleles. Analysis of all possible pairwise combinations of *hmg1* heteroalleles indicated that these mutations represent two complementation groups. Each point mutation is defective in only one of the two complementation groups and the null allele is defective in both. The presumptive *HMG1* gene convertant from JRY1422 (Table 3) suggests that there are at least two mutable sites in one of the complementation groups.

Models to explain how intragenic complementation might occur in *HMG1* are of two general classes. The reaction catalyzed by HMG-CoA reductase is a two step reduction. In the first step, HMG-CoA is reduced to mevaldate with the oxidation of one molecule of NADPH, and in the second step mevaldate is reduced to mevalonate with the oxidation of a second molecule of NADPH. Mevaldate need not be enzyme-bound for the second reaction to occur (QURESHI *et al.* 1976). In principle, intragenic complementation could occur if each mutant were defective in one half-reaction. According to this model, one *hmg1* allele would produce an enzyme that catalyzes the first half-reaction forming mevaldate from HMG-CoA, and its complementing allele would produce an enzyme that catalyzes the second half-reaction forming mevalonate from mevaldate. In the simplest case, mutants defective only in the second half-reaction would still contain an HMG-CoA-dependent NADPH oxidation activity. However, the HMG-CoA-dependent NADPH oxida-

tion activity measured in extracts from each of the *hmg1 HMG2* mutants was not significantly greater than that provided by *HMG2* alone. Alternatively, intragenic complementation could occur due to formation of a multimer containing both mutant polypeptides. According to this model, multimers formed entirely from a subunit encoded by a single mutant allele would be inactive, whereas multimers formed from a mixture of subunits encoded by a pair of complementing alleles would be active. Although it is easy to imagine a gene in which some alleles would have this property, it would be surprising for all alleles to fit neatly into complementation groups. The isolation of a larger number of *hmg1* alleles and assay of mevaldate reduction in extracts from *hmg1* mutants will be required to determine the mechanism of intragenic complementation. In at least one case, the yeast *TRP5* gene encoding tryptophan synthase, both mechanisms of intragenic complementation have been observed (DUNTZE and MANNEY 1968).

The map positions of *HMG1* and *HMG2* were determined. *HMG1* is distal to *SUP79* on the left arm of chromosome *XIII* and *HMG2* is on the right arm of chromosome *XII* closely linked to *SST2*. Other genes for enzymes of the sterol biosynthetic pathway have not yet been mapped.

A co-cultivation experiment demonstrated that cells with only one copy of a functional HMG-CoA reductase gene are at a competitive disadvantage with respect to wild-type cells. The *hmg1* null mutant performed more poorly than the *hmg2* null mutant, as expected from the relative levels of HMG-CoA reductase activity present in extracts from these mutants (BASSON, THORSNESS and RINE 1986). These three strains are not strictly isogenic since the *HMG1* and *HMG2* null alleles were constructed by insertion of marker genes, which allowed each of the three strains to be identified by its nutritional requirements. The differences in growth observed among these strains is not likely to be due to their different nutritional requirements for two reasons. First, to prevent the cells from suffering from any nutritional limitation, the cells were grown in rich medium that was supplemented with excess histidine and lysine. Secondly, the strain that grew best (JRY527) had more nutritional requirements than either of the other two strains.

The relative growth characteristics of these three strains may not be simply a function of their residual HMG-CoA reductase activity. Even though the *hmg2* mutant has a slight growth defect compared to wild-type cells, there is no significant difference between the activity measured in extracts from these two strains (BASSON, THORSNESS and RINE 1986). The NH_2-terminal halves of the two HMG-CoA reductase isozymes differ considerably in primary sequence (M. E. BASSON, unpublished data). This portion of both isozymes contains seven potential membrane-spanning domains and is probably not required for the catalytic activity of the enzyme, but may have other cellular functions (LISCUM *et al.* 1985; GIL *et al.* 1985; R. WRIGHT, unpublished data). Null mutations of *HMG1* and *HMG2* would thus eliminate not only a fraction of the total HMG-CoA reductase activity but also these as yet undefined functions.

The screen described here makes use of a cloned gene to isolate mutations in a gene encoding a duplicated function. In this case the cloned gene was *HMG2* and the gene encoding the duplicated function was *HMG1*. A reversal of the screen described in this paper, using *HMG1* on a plasmid in cells containing a null allele of *HMG1*, should allow recovery of *hmg2* point mutations. Since many other duplicated functions in yeast have been identified, we note that this screen may be readily modified to isolate mutations in any set of duplicated functions. The unexpected background of *cpa* mutants can be avoided by including arginine in the media.

An additional feature of this screening procedure is that it should allow the recovery of mutations in any gene whose product has a positive regulatory role specific for the duplicated gene or the protein encoded by it. A useful feature of the screen is that it does not impose any bias towards the level upon which the positive regulator must act. In contrast, the use of *lacZ* gene fusions restricts the mutations recovered to those genes that function at the level of synthesis. Plasmid-loss screens may thus provide an effective method for identifying positive regulators that act either at the level of synthesis or at the level of function.

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