The degradation rate of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-R), a key enzyme of the mevalonate pathway, is regulated through a feedback mechanism by the mevalonate pathway. To discover the intrinsic determinants involved in the regulated degradation of the yeast HMG-R isozyme Hmg2p, we replaced small regions of the Hmg2p transmembrane domain with the corresponding regions from the other, stable yeast HMG-R isozyme Hmg1p. When the first 26 amino acids of Hmg2p were replaced with the same region from Hmg1p, Hmg2p was stabilized. The stability of this mutant was not due to mislocalization, but rather to an inability to be recognized for degradation. When amino acid residues 27–54 of Hmg2p were replaced with those from Hmg1p, the mutant was still degraded, but its degradation rate was poorly regulated. The degradation of this mutant was still dependent on the first 26 amino acid residues and on the function of the HRD genes. These mutants showed altered ubiquitination levels that were well correlated with their degradative phenotypes. Neither determinant was sufficient to impart regulated degradation to Hmg1p. These studies provide evidence that there are sequence determinants in Hmg2p necessary for degradation and optimal regulation, and that independent processes may be involved in Hmg2p degradation and its regulation.

INTRODUCTION

Protein degradation is frequently used to control protein quality and quantity in living cells. This strategy for altering the level of a particular protein is used both in normal and pathological cellular processes. Examples include temporally controlled degradation of the cyclins (Glotzer et al., 1991), stimulated destruction of IκB (Scherer et al., 1995), endoplasmic reticulum (ER)-localized degradation of unfolded and mutant forms of cystic fibrosis transmembrane conductance regulator (Ward et al., 1995), human immunodeficiency virus-induced destruction of ER-resident CD4 molecules (Bour et al., 1995), human cyto-
tion of a protein are known (Hochstrasser and Varshavsky, 1990; Glotzer et al., 1991; Willey et al., 1994). However, the cellular degradation machinery can also degrade unfolded forms of normally stable proteins (Ward et al., 1995; Hiller et al., 1996; Schneider et al., 1996) which, at least in the correctly folded state, must by definition lack any small motifs directing rapid turnover. Furthermore, correctly folded proteins can be either degraded or stable depending on the physiological state of the cell. In these cases of regulated degradation, the stability of a protein is governed by structural or physiological conditions extrinsic from the primary structure of the protein.

How does the cell decide to degrade a protein in some circumstances but not others? One possibility is that transition from a stable state to a rapidly degraded state is accompanied by exposure of an intrinsic degradation motif to the degradation apparatus. For example, the degradation of ornithine decarboxylase (ODC) is first initiated by its binding to the protein antizyme (Li and Coffino, 1993). This binding of antizyme to ODC then targets ODC for degradation by the proteasome (Murakami et al., 1992). Specific regions in the C terminus of ODC have been shown to be essential for antizyme-dependent degradation of ODC (Li and Coffino, 1993). Perhaps this mechanism is also used for selective degradation of unfolded proteins, such that the misfolded conformation exposes degradation motifs that are normally buried in the properly folded protein.

A second possibility is that a degradation motif is always exposed, but some regulatory protein binds that motif and thus inhibits recognition by the degradation machinery. This mechanism appears to function in the control of $\mu$ transposase component MuA stability by a protective interaction with MuB (Levchenko et al., 1997). Similarly, the binding or attachment of a small molecule that interferes with recognition of a degradation motif could alter the stability of a protein. For example, the degradation of the C-jun oncprotein appears to be controlled by an inhibitory phosphorylation in the vicinity of the degradation-promoting $\delta$ domain (Fuchs et al., 1996).

Other variations of physiologically controlled protein degradation include regulation or modification of the cellular machinery that performs the degradation, as in the case of temporally controlled activation of the anaphase-promoting complex, required for the degradation of several cell cycle proteins (Hershko, 1997).

A particularly striking example of physiologically regulated protein degradation is provided by HMG-R, which catalyzes a key step in the mevalonate pathway, from which cholesterol and numerous other essential molecules are synthesized. The mevalonate pathway is controlled, in part, by regulated degradation of the HMG-R protein (Edwards et al., 1983; Nakanishi et al., 1988; Chun et al., 1990; Hampton and Rine, 1994). In general, when the production of mevalonate pathway products satisfies the cell’s needs, the degradation rate of the HMG-R protein is high and the steady-state level of the protein tends to be low. In contrast, when production of mevalonate pathway products is slowed, such as when HMG-R is inhibited with lovastatin, the degradation rate is slowed, and the steady-state level of HMG-R tends to increase. In this way, HMG-R degradation is regulated to meet changing cellular demands for products of the mevalonate pathway.

Neither the mechanism of HMG-R degradation nor the molecular signals that regulate this process are well understood. Unraveling the details of this regulatory loop should reveal both the molecules that mediate the degradation of HMG-R and the clinically important signaling pathway that couples the synthesis of cholesterol to HMG-R stability. HMG-R is a polytopic membrane protein that resides in the endoplasmic reticulum (ER), where the degradation of the protein is thought to occur (Chun et al., 1990; Hampton and Rine, 1994). Accordingly, understanding the degradation of HMG-R should also reveal principles of general significance to the degradation of a variety of membrane proteins. In particular, the sequence or structural requirements for the degradation of membrane proteins are poorly understood, even with respect to the compartment in which the degradation signal is measured.

To understand the molecular details of HMG-R degradation, we have launched a genetic analysis of the problem in *Saccharomyces cerevisiae*. We have previously shown that the yeast HMG-R isozyme Hmg2p undergoes regulated degradation in a manner similar to HMG-R degradation in mammals (Hampton and Rine, 1994; Hampton et al., 1996b; Hampton and Bhakta, 1997). Hmg2p appears to be degraded without exiting the ER, and the degradation rate is controlled by signals from the mevalonate pathway. The non-catalytic, membrane-anchoring N-terminal region is necessary and sufficient for regulated degradation (Hampton and Rine, 1994). More recently, we have implicated farnesyl pyrophosphate (FPP) as a likely source of a necessary signal for Hmg2p degradation (Hampton and Bhakta, 1997). Hmg2p degradation depends on several specific genes, known collectively as HRD genes (Hampton et al., 1996b). HRD2 (now also called *RPN1*) encodes the yeast homologue of the p97 component of 26S proteasome (DeMartino et al., 1994), indicating that the proteasome is involved in Hmg2p degradation.

Taken together, our studies reveal numerous similarities between HMG-R regulation in mammals and yeast. In both species, the N-terminal region is essential for regulated degradation, FPP serves as a
source of a positive signal, and the proteasome is implicated in the destruction of the protein. Furthermore, in both yeast and mammalian cells it appears that the regulation of HMG-R stability does not occur through an alteration of the degradation machinery. Rather, it seems that the susceptibility of HMG-R for degradation by this machinery is somehow altered by signals from the mevalonate pathway (Hampton et al. 1996b, and see below). Because of the similarities between yeast and mammalian HMG-R-regulated degradation, our studies are likely to reveal features of this process shared between these phyla.

Yeast expresses two isozymes of HMG-R, Hmg1p and Hmg2p, encoded by the HMG1 and HMG2 genes, respectively. The two proteins are ~50% identical in the N-terminal hydrophobic domain and ~93% identical in the C-terminal catalytic domain (Basson et al., 1988). Furthermore, the amino acid sequences of the two isozymes have nearly superimposable hydrophathy plots. The two are functionally similar in that each can synthesize adequate amounts of mevalonate (Basson et al., 1988), and both appear to be residents of the ER (Koning et al., 1996). In spite of these similarities, the dynamic behavior of the two proteins is strikingly different: Hmg1p is extremely stable, whereas Hmg2p is subject to regulated degradation (Hampton and Rine, 1994). Studies with fusions to reporter genes indicate that the isozone-specific differences in degradative behavior are imparted by the homologous, noncatalytic N-terminal regions (Hampton and Rine, 1994; Hampton et al., 1996a). Because these regions are homologous and structurally similar, reciprocal recombinants between the two proteins should allow mapping of stability determinants that differ between the two isoforms. A similar approach has been successfully employed to map the features of the N-terminal region that allow the Hmg1p-specific formation of the stacked nuclear structures known as karmellae (Parrish et al., 1995).

The data presented here revealed determinants of the Hmg2p sequence that were necessary for degradation or optimal regulation of Hmg2p degradation. These results indicated that degradation and its regulation may require independent recognition processes, each encoded by different genes. Neither determinant was sufficient to impart degradation or regulation to the stable Hmg1p molecule. Thus, regulated degradation of Hmg2p may require additional determinants in the Hmg2p transmembrane domain.

MATERIALS AND METHODS

Materials and Reagents

All restriction enzymes, Vent DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA) and used according to manufacturer’s instructions. Chemical reagents were obtained from Sigma Chemical (St. Louis, MO), Lovastatin, zaragozic acid (ZA), and L659,699 were generously donated by Merck (Rahway, NJ). ECL immunodetection reagents were from Amer sham (Arlington Heights, IL). DNA for yeast transformation was prepared from 25 ml bacterial (DH5α) cultures using the Plasmid Midi Kit from Qiagen (Chatsworth, CA). The anti-myc 9E10 antibody was used as a cell culture supernatant obtained by growing the 9E10 hybridoma (ATCC CRL 1729) in RPMI 1640 culture medium (Life Technologies, Grand Island, NY) with 10% FCS. The anti-hemagglutinin (HA) 12CA5 antibody was an ascites fluid obtained from Babco (Berkeley, CA). Affinity-purified goat anti-mouse HRP-conjugated anti-serum was obtained from Sigma Chemical.

Recombinant DNA and Molecular Cloning

PCR was performed using Vent DNA polymerase in 100 μl reaction volumes (1× Thermopol buffer, 200 μM dNTPs, 1 μg template, 1 μM primers, and 0.5 μl Vent DNA polymerase). PCR was carried out with a dwell at 94°C for 5 min, followed by 15 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and then a final dwell at 72°C for 7 min. The plasmid pRH144–2 (YIp, URA3; Hampton and Rine, 1994), which expressed the Hmg22 coding region from a GAPDH promoter, was altered by removal of a PstI site at the 5’ end of the promoter so that the remaining PstI site was unique and directly flanking the Hmg22 start codon and was subsequently named pRH386. Portions of the Hmg22 coding region engineered to contain replacements of the original sequence with the homologous region of Hmg1 were synthesized by PCR and cloned into pRH386 to yield pRH409, pRH418, and pRH410. pRH409 had the first 26 codons of Hmg22 replaced with those from Hmg1, pRH418 had Hmg22 codons 27–54 so replaced, and pRH410 had Hmg22 codons 1–54 so replaced. The splicing by overlap extension method, or “SOEing” (Horton et al., 1989), was used to synthesize the chimeric portions of each plasmid, which were then cloned into the original Hmg22 coding region in pRH386 between the PstI site and the AltI site in the Hmg22 coding region. A list of primers used in the PCR reactions is available upon request. All resulting plasmids were tested for their ability to complement mevalonate auxotrophy of yeast lacking any wild-type HMG-R genes, and the regions produced by SOEing PCR were sequenced to verify correct amplification.

The chimeric regions of Hmg22 in each of the above plasmids were next subcloned into pRH423, which was identical to pRH386 except that the sequence of a single myc epitope tag was inserted between codons 618 and 619 (Hampton and Bhakta, 1997). 4kb Ncol/Sphl fragments of pRH409, pRH410, and pRH418 were substituted for their analogous regions in pRH423, resulting in plasmids pRH408, pRH409, and pRH502. These plasmids expressed myc epitope-tagged, chimeric proteins 2–11–26, 2–11–54, and 2–127–54, respectively (Figure 1 and Table 1).

pRH469 (YIp, URA3) was analogous to pRH386 but contained the Hmg22::GFP fusion gene with the S65T bright mutation (Hampton et al., 1996b), pRH469 was used to subclone Hmg22::GFP coding regions with chimeric transmembrane regions for optical analysis of the degradation of each chimeric protein. The 4.7-kilobase (kb) NsiI/Sphl fragments from pRH409, pRH410, and pRH418 were cloned into the 3.9-kb, green fluorescent protein (GFP)-containing fragment of pRH469 to yield pRH477, pRH503, and pRH506, respectively. These plasmids expressed 2–11–26-GFP, 2–11–54-GFP, and 2–127–54-GFP, respectively. Plasmid pRH555, which expressed the 2–1212–524 protein, was expressed as follows: The segment of Hmg22 that encompasses codons 1–212 was used to replace the homologous region of HMG1 in pRH105–25 (YIp, URA3), which expressed the HMG1 coding region from the GAPDH promoter, by a combination of PCR amplification and subcloning to make pRH419. pRH419 expressed a coding region with the first 212 codons of Hmg22 (up to the T61111 site) followed by the remainder of HMG1. pRH419 was then modified by site-directed mutagenesis so that a BsoHII site was incorporated within codons 523 and 524. A BsoHII site was also placed in
pRH423 at codons 522 and 523 in the HMG2 coding region. pRH555 (which expressed the 2–121–524 with a myc tag in the linker region) was produced by cloning the PstI/BssHII fragment containing the 2–121–524 coding region into the PstI/BssHII-modified pRH423 that contained the remainder of the HMG2 coding region (524-stop) including the myc tag. pRH561 (which expressed the 2–1,524 chimera) was cloned from pRH555 by replacement of the region between PstI and Tth111I that contained HMG2 codons 1–212 with a PstI/Tth111I fragment that contained codons 1–213 of HMG1.

**Strains and Media**

Escherichia coli DH5α strains were grown at 37°C in LB + amp (100 μg/ml). Yeast strains were grown at 30°C in minimal medium supplemented with glucose and the appropriate amino acids, as described previously (Hampton and Rine, 1994). The LiOAc method was used to transform yeast with plasmid DNA (Ito et al., 1983).

Mevalonate auxotrophic yeast strain RHY468 (a his3Δ200 leu2–801 ade2–101 ura3–52 met2 hmg1::LYS2 hmg2::HIS3), alias JRY1593 (Bason et al., 1988), was used as a parent strain for all plasmids expressing enzymatically active HMG-R recombinants. Each integrating plasmid was introduced into the recipient strain by targeted insertion at the BamHI site of the HMG2 genomic locus, and yeast transformants were subsequently selected for mevalonate prototrophy. pRH498, pRH499, pRH502, pRH555, and pRH561 were transformed into RHY468 to yield RHY536, RHY537, RHY539, pRH662 and RHY636, respectively (see Table 1 and Figure 1). In all cases, the recombinant plasmids were able to restore mevalonate prototrophy, with the engineered HMG-R protein as the sole source of HMG-R activity in each strain.

All fusions with the GFP reporter gene were transformed into strain RHY519 (a his3Δ200 leu2–801 ade2–101 ura3–52::HMG2cd met2 hmg1::LYS2 hmg2::HIS3). RHY519 expressed a soluble, enzymatically active Hmg2p catalytic domain as the sole source of HMG-R activity. Thus, all GFP-derived optical reporter proteins were expressed in strains with identical mevalonate pathway activity. Strain RHY519 was transformed with pRH477, pRH503, and pRH506 to yield RHY528, RHY545, and RHY548 respectively. The strains expressed the fluorescent chimeric proteins 2–1,26-GFP, 2–1,54-GFP, and 2–1,27–54-GFP, respectively. GFP fusion plasmids were introduced into RHY519 by targeted insertion at the StuI site of the ura3–52 allele, and yeast transformants were subsequently selected for Ura⁺ prototrophy.

**Figure 1.** Cartoon of the chimeric proteins in this study. The ovals represent either the appropriate HMG-R catalytic region (which provides HMG-R enzymatic activity), or the GFP optical reporter protein, as indicated in text. Thick lines represent sequence from Hmg1p; thin lines represent sequence from Hmg2p. The box labeled “M” indicates the presence of the myc epitope tag in the poorly conserved linker region.

pRH423 at codons 522 and 523 in the HMG2 coding region. pRH555 (which expressed the 2–121–524 with a myc tag in the linker region) was produced by cloning the PstI/BssHII fragment containing the 2–121–524 coding region into the PstI/BssHII-modified pRH423 that contained the remainder of the HMG2 coding region (524-stop) including the myc tag. pRH561 (which expressed the 2–1,524 chimera) was cloned from pRH555 by replacement of the region between PstI and Tth111I that contained HMG2 codons 1–212 with a PstI/Tth111I fragment that contained codons 1–213 of HMG1.
washed glass beads were added, and the mixture was vortexed at pRH555 2-1 212-524 mM PMSF, 100 

samples with lovastatin added to one of the samples to a final and vortexed briefly. Each culture was separated into three 2-ml 

Vol. 9, September 1998 2615 

mide was added to each culture to a final concentration of 50 

(OD600) of 0.3. Cells were pelleted by centrifugation and resus-
formed (Hampton and Rine, 1994), except that the lysis buffer was 

Lysis of cells for immunoblotting was performed as described (Hampton and Rine, 1994). Briefly, cells were pelleted by centrifugation and then lysed for immunoblotting. The steady-state assays were performed as follows: yeast strains were grown in 25 ml of supplemented minimal medium to an approximate OD600 of 0.1. Each culture was separated into three 8-ml samples with drugs added to the indicated concentrations. The samples were incubated at 30°C for 3 h and then lysed for immunoblotting.

Cellular lysates were immunoblotted as described (Hampton and Rine, 1994). Briefly, 15-μl samples were resolved on 8% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with the 9E10 anti-myc antibody. In some circumstances, relative levels of immunoreactivity were compared between samples by assessing the exposure times required to give similar autoradiographic intensities. Although we only used this method to approximate differences in levels, it is a method that has been successfully applied in previous semiquantitative studies in yeast (Zhang et al., 1993). Furthermore, we have directly confirmed the use of exposure time as a relative gauge of immunoreactivity in this study by performing controls with different volumes of identical lysates loaded on the same gels (data not shown).

Ubiquitination Assays

The mutant HMG-R plasmids were transformed into RHY278 (a his3Δ200 lys2–801 ade2–101 ura3–52 met2 hmg1::LYS2 hmg2::HIS3 and selected for Ura prototrophy. The resulting strains were transformed with Yep112 (Ellison and Hochstrasser, 1991), which expresses an HA-tagged ubiquitin, and selected for Trp prototrophy. Ubiquitination assays were performed with these strains similarly to what was previously described (Hampton and Bhakta, 1997). Briefly, cells were grown in 20-ml cultures to an OD600 of 0.2. CuSO4 was added to a final concentration of 100 μM, and the cultures were incubated for 60 min at 30°C. To those cultures indicated, ZA was added to a final concentration of 10 μg/ml after 50 min of incubation with CuSO4, and the cultures were incubated the final 10 min at 30°C. Cells were pelleted by centrifugation and resuspended in 100 μl SUME + protease inhibitors (10 mM PMSE, 100 μg/ml N-tosyl-l-phenylalanine chloromethyl ketone, 100 μg/ml leupeptin, and 100 μg/ml pepstatin) + N-ethylmaleimide (5 mM). The cells were lysed by vortexing three times for 1 min. Cell lysates were clarified by centrifugation, and the lysates were added to 500 μl IP buffer + protease inhibitors + N-ethylmaleimide. Anti-HMG-R antibodies (40 μl) were added to the lysates and incubated at 4°C overnight. Protein A Sepharose beads (60 μl, 10% wt/vol) were added to the lysate, and the beads were incubated at room temperature for 2 h. The beads were pelleted by centrifugation and then washed once with IP buffer and twice with wash buffer, after which 40 μl 2× urea sample buffer (8 M urea, 4% SDS, 10% β-mercaptoethanol, 0.125 M Tris, pH 6.8) were added and the beads were incubated at 55°C for 10 min.

Immunoprecipitates were immunoblotted as previously described (Hampton and Rine, 1994). Briefly, either 5-μl or 35-μl samples were resolved on 8% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with either the 9E10 anti-myc antibody or the 12CA5 anti-HA antibody, respectively.

FACS Analysis and Fluorescence Microscopy

Strains for FACS analysis were grown in supplemented minimal medium and analyzed while in log phase (OD600 between 0.2 and 0.5). Living cells were analyzed by flow microfluorimetry using a FACScan (Becton Dickinson, Palo Alto, CA) analytical flow microfluorimeter with settings for fluorescein-labeled antibody analysis. Data were analyzed with CellQuest software. Histograms were

<table>
<thead>
<tr>
<th>Table 1. Descriptions of plasmids and strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid or strain</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Plasmid</td>
</tr>
<tr>
<td>pRH423</td>
</tr>
<tr>
<td>pRH469</td>
</tr>
<tr>
<td>pRH477</td>
</tr>
<tr>
<td>pRH498</td>
</tr>
<tr>
<td>pRH499</td>
</tr>
<tr>
<td>pRH502</td>
</tr>
<tr>
<td>pRH503</td>
</tr>
<tr>
<td>pRH506</td>
</tr>
<tr>
<td>pRH555</td>
</tr>
<tr>
<td>pRH561</td>
</tr>
</tbody>
</table>

Parent strains   Genotype
RHY468          a his3Δ200 lys2–801 ade2–101 ura3–52 met2 hmg1::LY52 hmg2::HIS3
RHY519          a his3Δ200 lys2–801 ade2–101 HMG2cd met2 hmg1::LY52 hmg2::HIS3

New strains   Protein expression b
RHY536          RHY468 expressing 2-1,26
RHY537          RHY468 expressing 2-1,16
RHY539          RHY468 expressing 2-1,27,54
RHY623          RHY468 expressing Hmg2p
RHY626          RHY468 expressing 2-1,1,24
RHY522          RHY519 expressing Hmg2p-GFP
RHY544          RHY519 expressing 2-1,1,54-GFP
RHY545          RHY519 expressing 2-1,27,54-GFP
RHY548          RHY519 expressing 2-1,12,12,54-GFP

Degradation Assays

Cycloheximide-chase assays were performed as previously described (Hampton and Rine, 1994), except that the lysis buffer was 1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, and 10 mM EDTA (abbreviated SUME). Briefly, yeast strains were grown in 25 ml supplemented minimal media to an approximate optical density (OD600) of 0.3. Cells were pelleted by centrifugation and resuspended in 6 ml of fresh supplemented minimal media. Cycloheximi-

* All proteins contain the catalytic domain and myc epitope, except those labeled GFP, which have the catalytic domain replaced with GFP and they do not contain a myc epitope.

b Catalytic domain constructs integrated at the HMG2 genomic locus using BamHI cut plasmid; GFP constructs integrated at the ura3-52 genomic locus using Stul cut plasmid.

In-cis Determinants of Hmg2p Degradation
produced from 10,000 individual cells. To examine the effects of lovastatin on the steady-state levels of GFP reporters by FACS, the drug was added to early log-phase cultures (OD<sub>600</sub> < 0.2) to the desired final concentration and the cells grown another 3 h, so that final cultures were still in log phase.

Fluorescence microscopy on cells expressing GFP reporter fusions was performed with a Nikon Optiphot-2 microscope with epifluorescence illumination, using a 100× oil-immersion objective. GFP fluorescence was observed in living cells with a Nikon B2-A filter for fluorescein fluorescence with excitation at 450–490 nm and long-band pass emission. Images were captured and processed as described (Hampton et al., 1996a,b).

**RESULTS**

**Use of the Two HMG-R Isozymes to Reveal Determinants of Degradation and Regulation**

The degradation of Hmg2p is programmed by the transmembrane domain, with the catalytic domain having apparently little or no function in regulated degradation (Hampton and Rine, 1994; Hampton et al., 1996a). Although the Hmg1p isozyme has ~ 50% sequence identity to Hmg2p in the transmembrane region, the Hmg1p protein is quite stable. This difference in the behavior of the two isozymes implies that either specific determinants are present in the Hmg2p transmembrane region that program its regulated degradation, or Hmg1p contains determinants that program its stability. Hmg2p degradation is regulated by a signal generated from the mevalonate pathway between mevalonate and squalene (Hampton and Rine, 1994; Hampton and Bhakta, 1997). Hmg2p degradation can be uncoupled from regulation in a mutant version of Hmg2p called 6 myc-Hmg2p (Hampton et al., 1996b). This protein is rapidly degraded in an HRD-dependent manner, but its degradation is no longer regulated. Together, these observations favor models in which there are distinct determinants in the Hmg2p transmembrane region that are responsible for either degradation per se or its regulation. To test this hypothesis, we began a search for determinants of regulated degradation of Hmg2p by making chimeric recombinants between Hmg2p and the stable Hmg1p isozyme.

As in previous studies (Hampton and Rine, 1994; Hampton et al., 1996a,b), the engineered coding regions were expressed from the strong, constitutive GAPDH promoter to separate degradation from other possible modes of regulation. In addition, all HMG-R–derived coding regions had a single copy of the myc epitope tag coding region inserted between codons 618 and 619 in the poorly conserved HMG-R linker region, which allowed efficient detection using the 9E10 antibody (Figure 1) (Hampton and Bhakta, 1997). The addition of the single myc tag at this position had no effect on the ability of the Hmg2p protein to provide the essential HMG-R activity or to undergo regulated, HRD-dependent degradation.

*A Necessary Determinant for Hmg2p Degradation*

Studies of HMG2/HMG1 recombinants revealed a critical determinant of degradation in the first 26 amino acids of the Hmg2p protein. A chimeric Hmg2p with the first 26 amino acids replaced by those from Hmg1p, named 2–11–26 (Figure 1), was extremely stable when expressed in yeast. When protein synthesis was inhibited by the addition of cycloheximide to cells expressing 2–11–26, the amount of 2–11–26 was unchanged after a 4-h incubation (Figure 2A, right panel). In contrast, the amount of normal Hmg2p declined 10-fold in the same experiment (left panel). To test the stability of 2–11–26 further, we took advantage of a recently discovered way to stimulate the degradation of Hmg2p (Hampton and Bhakta, 1997). The degradation rate of normal Hmg2p can be stimulated by the addition of the squalene synthase inhibitor ZA. Inhibition of squalene synthase causes a...
buildup of a mevalonate-derived signal for degradation, most likely derived from FPP. This effect of ZA can be directly observed as the establishment of a new log-phase, steady-state level of Hmg2p that is three- to fourfold lower in treated cells than in untreated cells. Use of ZA allowed a more detailed analysis of the behavior of 2–11–26. In particular, if 2–11–26 were still subject to regulated degradation that was simply slower, then ZA would be expected to hasten its degradation. When a log-phase culture of a strain expressing normal Hmg2p was treated with ZA, the steady-state level of Hmg2p dropped fourfold after 3 h (Figure 2B, left panel). In contrast, the same treatment of the strain expressing 2–11–26 had no effect on the steady-state level of 2–11–26 (Figure 2B, right panel). Thus, it appeared that replacement of the first 26 amino acids of Hmg2p with those from Hmg1p resulted in a protein that was unable to enter the HRD degradation pathway, rather than being a slowly degraded substrate.

**The Stabilizing 1–26 Amino Acid Replacement Did Not Affect Localization**

At present it is thought that the ER is the site of degradation of HMG-R in both mammals and yeast (Chun et al., 1990; Hampton and Rine, 1994). It is possible that mislocalization of HMG-R protein from the ER to some other cellular compartment could alter its degradation. Thus, one explanation for the stabilizing effect of the first 26 amino acids in 2–11–26 was that the replacement caused mislocalization of the protein to a compartment in which the protein is protected from degradation.

To examine the effect of the 1–26 replacement on localization of Hmg2p, we made the identical replacement in the coding region for Hmg2p-GFP. The Hmg2p-GFP protein has the natural Hmg2p catalytic domain replaced with the autofluorescent GFP reporter. Hmg2p-GFP is subject to the same regulated, HRD-dependent degradation as Hmg2p (Hampton et al., 1996a,b). The cellular distribution of Hmg2p-GFP is very similar to the distribution of wild-type Hmg2p (Koning et al., 1996; Hampton et al., 1996a) and thus provides a direct, noninvasive, in vivo assessment of cellular localization.

Hmg2p-GFP with the first 26 amino acids replaced with those from Hmg1p, called 2–11–26-GFP (Figure 1), was stable in comparison to the degraded Hmg2p-GFP, when examined by cycloheximide-chase (Figure 3A) or after addition of ZA to growing cells (data not shown). Thus, the 1–26 replacement had the predicted effects on the stability of the Hmg2p-GFP reporter protein. The subcellular distribution of 2–11–26-GFP was examined by fluorescence microscopy and was indistinguishable from that of unmodified Hmg2p-GFP. In strains expressing either protein, fluorescence was observed in both nuclear and peripheral ER staining, as well as bright patches of membrane proliferations (Figure 3B). Thus, it appeared that replacement of the first 26 amino acids of Hmg2p with those from the stable Hmg1p had no effect on the localization of the resulting protein, but limited its recognition by the HRD degradation machinery.

**A Determinant of Regulation**

The above experiments revealed a small sequence determinant required for normal degradation. We also tested chimeras for any effect on the regulation of Hmg2p degradation. Manipulations of the mevalonate pathway by various enzyme inhibitors can alter the rate of Hmg2p degradation. Hmg2p is stabilized in cells treated with the HMG-R inhibitor lovastatin and is more rapidly degraded in cells treated with the squalene synthase inhibitor ZA (Hampton and Rine, 1994; Hampton and Bhakta, 1997; and see above). Therefore, our search for determinants of regulation involved testing Hmg2p/Hmg1p chimeras for altered degradation in response to these drugs.

Replacement of the first transmembrane span of Hmg2p with that from Hmg1p resulted in a mutant of Hmg2p, called 2–127–54 (Figure 1), that was less sensitive to lovastatin-induced stabilization. The degradation of normal Hmg2p was blocked by the addition of lovastatin in a cycloheximide-chase experiment (Figure 4A, left panel). In contrast, the degradation of 2–127–54 was only slightly attenuated by the same treatment (Figure 4A, right panel). Furthermore, the steady-state level of 2–127–54 was significantly lower than the level of normal Hmg2p. This lower steady-state level presumably reflected an increased tendency of 2–127–54 to enter the HRD degradation pathway (see below), implying reduced regulation of degradation of 2–127–54 relative to normal Hmg2p.

Regulation of degradation can also be examined by observing the change in the steady-state level of Hmg2p in the presence of various drugs that inhibit mevalonate pathway enzymes (Hampton and Bhakta, 1997, and above). That is, the addition of lovastatin to growing cells increases the half-life of Hmg2p degradation and results in an increase in the steady-state level of Hmg2p. Conversely, the addition of ZA to growing cells decreases the half-life of Hmg2p degradation and results in a decrease in the steady-state level of Hmg2p. Treatment of the strain expressing 2–127–54 with drugs that altered the mevalonate pathway caused the expected qualitative changes in the steady-state levels of the protein. In strains expressing either normal Hmg2p or 2–127–54, addition of ZA caused an expected drop in the steady-state level of each protein (Figure 5A, ZA lanes). Furthermore, incubation of the strains in increasing doses of lovastatin caused increases in the steady-state levels of both nor-
mal Hmg2p and 2–127–54. However, while the level of the normal Hmg2p was maximally increased by the addition of 0.2 μg/ml lovastatin, the amount of lovastatin required to maximally increase the level of 2–127–54 was 1 μg/ml (Figure 5A).

One difficulty in comparing the effects of lovastatin on the degradation of normal Hmg2p with the poorly regulated 2–127–54 is that the two proteins are present at different levels in the two strains. Thus, the associated HMG-CoA reductase activity, and therefore the sensitivity to lovastatin, was different between the strains being examined. To compare the effects of lovastatin on the stability of Hmg2p and 2–127–54 at identical levels of HMG-R activity, we constructed a strain in which the enzymatically active, stable, soluble Hmg2p catalytic domain was expressed from the constitutive GAPDH promoter as the sole source of essential HMG-R catalytic activity. The resulting strain (RHY519) was then transformed with plasmids that express either the normal Hmg2p-GFP or the analogous GFP reporter with the 2–127–54 transmembrane region (Figure 1). In this way, Hmg2p-GFP or 2–127–54-GFP was expressed in strains with constant and identical HMG-CoA reductase activity. The expected similarity of the HMG-R activity between the two strains was confirmed both by immunoblotting for catalytic domain expression and by testing the lovastatin sensitivity of the two strains (our unpublished data). The effect of lovastatin on the steady-state levels of 2–127–54-GFP or Hmg2p-GFP was then compared using these strains.

FACS analysis of the two strains revealed that the steady-state level of 2–127–54-GFP was three- to four-fold lower than that of Hmg2p-GFP, as was the case between 2–127–54 and Hmg2p examined above. Addition of lovastatin to growing cultures of either strain caused a time-dependent increase in the steady-state levels of fluorescence in either strain, indicated by a

Figure 3. Stabilizing replacement of the first 26 amino acids of Hmg2p-GFP did not affect cellular localization of the protein. (A) Cycloheximide-chase assay of strains expressing Hmg2p-GFP or the stabilized chimera 2–11–26-GFP. After addition of cycloheximide at 0 h, lysates were prepared at the indicated times and immunoblotted with anti-GFP antibody. (B) Fluorescence microscopy of living, log-phase cultures of strains expressing Hmg2p-GFP or the stabilized chimera, 2–11–26-GFP.
rightward shift to higher fluorescence in the fluorescence histograms. However, this stabilizing effect on 2–127–54-GFP required significantly more lovastatin (Figure 5B). For example, 0.8 μg/ml lovastatin was needed to shift the levels of 2–127–54-GFP by twofold, whereas the same shift for Hmg2p-GFP required only 0.1 μg/ml. Similarly, saturation of the effect of lovastatin occurred at 3.2 μg/ml for 2–127–54-GFP, whereas saturation for Hmg2p-GFP occurred at only 0.4 μg/ml. In addition, these amounts of lovastatin caused the steady-state levels of Hmg2p-GFP to increase fivefold, whereas the 2–127–54-GFP levels increased only threefold. Because these strains had identical levels of HMG-R catalytic activity, this analysis confirmed that 2–127–54 was still regulated by alterations in the mevalonate pathway, but required significantly stronger inhibition of the pathway to be stabilized.

The Poorly Regulated 2–127–54 Was a HRD Pathway Substrate
We have proposed that the regulation of Hmg2p, and related proteins such as Hmg2p-GFP, occurs at the level of Hmg2p itself rather than on the HRD gene-encoded degradation machinery (Hampton et al., 1996b). Thus, degradation of less regulated or unregulated Hmg2p mutants should still be HRD pathway dependent. Alternatively, it is possible that 2–127–54 had a lower steady-state level and was less regulated because it was degraded through a different pathway. Therefore, it was important to test whether the poorly regulated 2–127–54 was still a substrate of the HRD pathway. Various mutants of the HRD pathway have been discovered, and each of these mutants results in constitutive stabilization of wild-type Hmg2p (Hampton et al., 1996b). One of these mutants, hrd1–1, results in complete stabilization of wild-type Hmg2p and is useful to assess HRD pathway dependency. Therefore, to examine whether 2–127–54 was a substrate of the HRD pathway, we constructed isogenic hrd1–1 strains that expressed either normal Hmg2p or 2–127–54 as the only source of HMG-R. We also constructed hrd1–1 or HRD1 versions of the GFP reporter strains that expressed the free Hmg2p catalytic domain along with the appropriate GFP reporter. These strains were then used to assess the role of the HRD pathway in the degradation of 2–127–54.

The HRD1 gene was required for degradation of 2–127–54 (Figure 6A). In the HRD1 strain (middle panel), 2–127–54 was degraded in a 4-h cycloheximide-chase experiment; whereas the level of 2–127–54 was essentially unchanged after 4 h in the hrd1–1 strain (right panel). The hrd1–1 mutation also caused an elevation of the initial steady-state level of 2–127–54 that would be expected from constitutive stabilization of the protein.

To observe the effects of the hrd1–1 mutation more quantitatively, HRD1 and hrd1–1 strains that expressed the appropriate GFP reporter were compared by FACS. In strains with the normal HRD1 gene, the steady-state level of 2–127–54-GFP was three- to fourfold lower than that of Hmg2p-GFP (Figure 6B). In contrast, the presence of the hrd1–1 allele caused a significant increase in the fluorescence of each strain. Importantly, the steady-state levels of 2–127–54-GFP and Hmg2p-GFP were identical to each other in the hrd1–1 background, such that the fluorescent histograms were virtually superimposable (Figure 6B). These data implied that the different steady-state levels of the two proteins in HRD1 strains were due to differences in HRD-dependent degradation. Furthermore, the identical steady-state levels of the two proteins in a hrd1–1 strain indicated that HRD-dependent degradation was the only, or at least primary, mechanism by which the levels of the two proteins differed.

Effects of the 1–26 or 27–54 Replacements on the Level of Hmg2p Ubiquitination
Proteins are often targeted for degradation by covalent attachment of ubiquitin (Hochstrasser, 1995). We have previously demonstrated that Hmg2p undergoes regulated ubiquitination that appears to be correlated with the physiological control of Hmg2p degradation (Hampton and Bhakta, 1997). When signals that promote Hmg2p degradation are high, as when cells are incubated with ZA, the levels of Hmg2p ubiquitination are increased. Because the Hmg2p mutants described above had altered degradation phenotypes,
we directly tested whether the 1–26 or the 27–54 amino acid replacements had any effect on Hmg2p ubiquitination. The appropriate proteins were expressed in yeast strains that coexpressed ubiquitin containing a HA epitope tag (Ellison and Hochstrasser, 1991) to facilitate detection of ubiquitin conjugates. The assay was conducted by immunoprecipitating the HMG-R under study with anti-HMG-R antibodies and then subsequently assaying for covalently linked ubiquitin by immunoblotting the precipitated protein with the anti-HA 12CA5 antibody, as described (Hampton and Bhakta, 1997). The same immunoprecipitates were also separately immuno-blotted with the anti-myc 9E10 antibody to evaluate the total amount of myc-tagged Hmg2p that was immunoprecipitated.

Using this approach, we evaluated the ubiquitination of normal Hmg2p, stable 2–11–26, and poorly regulated 2–127–54 in the absence or presence of ZA. As previously described, Hmg2p is ubiquitinated, and the proportion of the ubiquitinated Hmg2p was significantly increased by brief incubation with ZA (Figure 7, top panel, Hmg2p). In contrast, the stable 2–1–26 showed no ubiquitination in either condition (top panel, 2–1–26). This is consistent with the high stability of the protein even in the presence of added ZA. Conversely, the poorly regulated 2–127–54 had a higher proportion of ubiquitination than the normally regulated Hmg2p (top panel, 2–127–54). Addition of ZA caused a modest increase in the amount of 2–127–54 ubiquitination. Again, these observations are well correlated with the degradative behavior of 2–127–54, which was still regulated but more prone to undergo degradation at a given level of mevalonate pathway flux. Thus, modifications in the Hmg2p amino acid sequence that altered the regulated degradation of Hmg2p caused appropriate and significant changes in the regulated ubiquitination of the modified proteins.

Interaction of the Two Determinants
The above experiments indicated that there were distinct portions of Hmg2p responsible for degradation and regulation. Specifically, the first 26 amino acids of Hmg2p were necessary for degradation, whereas the next 28 amino acids were necessary for optimal regu-
translation of that degradation. If amino acids 27–54 of Hmg2p were truly involved in the regulation of Hmg2p degradation, then any mutation that stabilized Hmg2p should similarly stabilize 2–11–26. Thus, the replacement in 2–11–26 should stabilize the poorly regulated 2–127–54. Accordingly, a recombinant coding region was prepared with the first 54 codons of HMG2 replaced by those from HMG1. This chimeric protein, referred to as 2–11–54 (Figure 1), had both the stabilizing 1–26 replacement and the deregulating 27–54 replacement (Figure 1).

The 2–11–54 protein was stable and showed essentially no decrease in a 4-h cycloheximide-chase experiment (Figure 8A, right panel). As in the experiments with the hrd1–1 allele, the replacement of the first 26 amino acids of 2–127–54 with those from Hmg1p also caused a significant increase in the steady-state level of the protein. Furthermore, in spite of the presence of the 2–1,54 region that imparted a greater sensitivity to mevalonate-derived degradation signals, 2–1,54 was refractory to ZA-stimulated degradation (Figure 8B, right panel). Therefore, it appeared that amino acids 1–26 were required for the degradation of 2–127–54, and the lower steady-state level of 2–127–54 was attributed to its greater propensity to undergo regulated degradation by the HRD pathway.

A Phenotype of Poor Regulation

The availability of the poorly regulated 2–127–54 allowed us to assess directly the phenotypic consequences of altered Hmg2p degradation. This information is useful in understanding the role that regulated degradation plays in mevalonate pathway control and will facilitate the search for mutants deficient in normal regulation of Hmg2p stability.

Lovastatin is a competitive inhibitor of HMG-R, and the drug blocks cell growth and division in a dose-dependent manner. In cells expressing normal Hmg2p, the growth-slowing effects of lovastatin are blunted by an increase in the steady-state levels of Hmg2p caused by regulatory slowing of Hmg2p degradation (Hampton et al., 1996b). When cells express the unregulated 6 myc-Hmg2p as their only source of HMG-R, they are significantly more sensitive to lovastatin than comparable strains expressing only the normally regulated Hmg2p (Hampton et al., 1996b). This heightened lovastatin sensitivity of a 6 myc-Hmg2p–expressing strain was proposed to be a phenotypic consequence of unregulated degradation, since the induction of HMG-R normally caused by lovastatin cannot occur. This model predicts that heightened sensitivity to lovastatin should be a generally observed phenotype in any strain expressing an HMG-R that is less prone to regulatory stabilization. Accordingly, the lovastatin sensitivity of strains expressing the poorly regulated 2–127–54 was evaluated to further test this idea.

Otherwise isogenic strains that expressed different chimeric variants of Hmg2p were compared for growth in the presence or absence of lovastatin (Figure 9). Aliquots of twofold serially diluted cultures were placed onto media with (200 μg/ml) or without lovastatin and allowed to grow. As predicted from above, the strain expressing the poorly regulated 2–1,54 was more sensitive to lovastatin than an identical strain expressing normally regulated Hmg2p (Hampton et al., 1996b). This heightened lovastatin sensitivity of a 6 myc-Hmg2p–expressing strain was proposed to be a phenotypic consequence of unregulated degradation, since the induction of HMG-R normally caused by lovastatin cannot occur. This model predicts that heightened sensitivity to lovastatin should be a generally observed phenotype in any strain expressing an HMG-R that is less prone to regulatory stabilization. Accordingly, the lovastatin sensitivity of strains expressing the poorly regulated 2–1,54 was evaluated to further test this idea.

Otherwise isogenic strains that expressed different chimeric variants of Hmg2p were compared for growth in the presence or absence of lovastatin (Figure 9). Aliquots of twofold serially diluted cultures were placed onto media with (200 μg/ml) or without lovastatin and allowed to grow. As predicted from above, the strain expressing the poorly regulated 2–1,54 was more sensitive to lovastatin than an identical strain expressing normally regulated Hmg2p, as indicated by the rapid drop off of growth in the dilution series on lovastatin (bottom panel; 2–1,54). In the absence of drug, the growth of each strain was identical (top panel) and indicated that each strain had a similar cell density, dilution, and plating efficiency in the absence of the drug. Comparisons of the twofold dilutions revealed that the strain expressing 2–1,54 had ap-
proximately eightfold lower plating efficiency on this dose of lovastatin.

The heightened lovastatin sensitivity caused by expression of 2–127–54 could be suppressed by stabilization of Hmg2p, either through in-cis or in-trans means. A strain expressing the in-cis stabilized 2–11–54, which has the additional stabilizing 1–26 replacement, along with the poorly regulating 27–54 replacement, was as resistant to lovastatin as a strain expressing normal Hmg2p (bottom panel, 2–11–54). Similarly, the presence of the stabilizing hrd1–1 mutation in a strain expressing 2–127–54 (bottom panel, 2–127–54, hrd1–1) restored the lovastatin resistance to that of a strain expressing normal Hmg2p. Thus, enhanced lovastatin sensitivity caused by expression of the poorly regulated 2–127–54 could be attributed to the increased tendency of this protein to enter the HRD degradation pathway.

Other Necessary Determinants of Degradation and Regulation

To determine whether amino acids 1–54 from Hmg1p were sufficient for regulated degradation, reciprocal replacements of the N-terminal regions of stable Hmg1p with regions from Hmg2p were expressed and studied. To provide the best comparison to native Hmg2p, a chimeric Hmg2p with the entire N-terminal transmembrane region (amino acids 1–523) replaced with that from Hmg1p (amino acids 1–524) was first constructed and used as the “parent” protein for the reciprocal replacements of the Hmg1p transmembrane domain (Figure 1). In this way, any contributions to regulated degradation caused by the Hmg1p linker regions or catalytic domains would be eliminated. The linker region and the C-terminal catalytic region do not appear to be required for the isozyme-specific degradative behavior of Hmg2p (Hampton and Rine, 1994; Hampton et al., 1996a). Nevertheless, these regions have not been rigorously studied, and controlling for possible effects of these two domains allowed us to assess precisely the sufficiency of the determinants under study.

As expected from previous studies, when the entire Hmg2p transmembrane region (amino acids 1–523, Figure 1) was replaced with that from Hmg1p (amino acids 1–524), the resulting protein, 2–1, 524, was stable in all assays (Figure 10A, right panel, and our unpublished data). Because the stabilizing 1–26 amino acids from Hmg1p were included in this protein, it was not surprising that the larger chimera was stable. However, replacement of amino acids 1–26 of the Hmg1p N terminus with those from Hmg2p did not result in degradation (our unpublished data). In a series of chimeras with increasing lengths of Hmg1p sequence from the N terminus, degradation was first observed when amino acids 1–211 of Hmg2p were used to
DISCUSSION

Our data indicated that the regulated degradation of Hmg2p had specific amino acid determinants for degradation and its regulation. Small sequence alterations affected, sometimes drastically, the degradative behavior of Hmg2p. It appeared that precise sequence information was required for the acquisition of stability when mevalonate-derived signals for degradation were lowered, and distinct sequence information was required to program the destruction of Hmg2p by permitting access to the HRD-encoded degradation machinery.

A necessary determinant of HRD-dependent degradation was found in the first 26 amino acids of Hmg2p. Examination of Hmg2p-GFP with the identical replacement indicated that the replacement did not affect cellular distribution of the resulting protein. Thus, the stabilizing effect of the replacement was probably not through gross mislocalization of the protein to a protected compartment, but rather was due to an inability of the chimera to enter efficiently the HRD degradation pathway. This stabilization was observed even in the presence of ZA, which stimulates the degradation of normal Hmg2p. The stabilized 2–1–26 had 16 amino acids that differed from the original sequence. At present, we are investigating whether the replacement added a stabilizing factor or removed a determinant of degradation.

Since altering the first 26 amino acids of Hmg2p sharply diminished degradation, it is possible that this region may be a ubiquitination site. The stabilized 2–1–26 did show decreased ubiquitination, indicating that amino acids 1–26 in normal Hmg2p may indeed contain a site for ubiquitination. Lysine is the usual linkage site for the degradative addition of ubiquitin to targeted molecules, and therefore the loss of a lysine would be one way to disrupt ubiquitination. However, the stable 2–1–26 has all of the original lysines, and an extra one at position 9. Thus, although these amino acids may participate, perhaps directly, in the degradation of Hmg2p, the stabilization cannot be explained by the loss of a critical, ubiquitinated lysine. Whatever the function of the first 26 amino acids, other features of the protein must work in concert with this region because it alone was not sufficient to program the degradation of Hmg2p (our unpublished data), nor could this portion of Hmg2p impart full degradation to 2–1–26. We are further investigating the role of the determinants described above, and others, in the ubiquitination and degradation of Hmg2p.

A different portion of the Hmg2p sequence, amino acids 27–54, contained a determinant required for normal regulation of Hmg2p degradation. In particular, 2–127–54 was more prone to undergo degradation by the HRD pathway at a given level of mevalonate pathway activity than was normal Hmg2p. This increased tendency for degradation was reflected in a decreased steady-state level of 2–1–27–54. The lower steady-state level required a functional HRD1 gene.

Degradation of 2–127–54 was still affected by altering the mevalonate pathway, it also appeared that amino acids 27–54 of Hmg2p provided a necessary, but not sufficient, determinant for regulation of Hmg2p degradation, since the presence of this sequence did not allow regulation of 2–1212–524.
An intriguing general feature of the regulatory response of Hmg2p degradation emerged from these studies. It was noted that in the presence of cycloheximide, stabilization of either normal Hmg2p or the poorly regulated 2-127-54 required much more lovastatin than comparable experiments when cycloheximide was not used. In the absence of cycloheximide, a steady-state dose-response curve (Figure 5A) revealed that 0.2 \( \mu g/ml \) lovastatin allowed full stabilization of normal Hmg2p. In contrast, a dose-response curve in the presence of cycloheximide (data not shown) demonstrated that 25 \( \mu g/ml \) of lovastatin was required to give full stabilization. This difference in the amount of lovastatin required to give complete stabilization was also observed with the poorly regulated 2-127-54, indicating that this may be a general feature of Hmg2p regulation. This effect of cycloheximide on Hmg2p regulation is not understood at present, but may indicate the involvement of a short-lived protein in the coupling of mevalonate pathway signals to Hmg2p degradation.

Regulation of Hmg2p degradation appears to occur by altering the ability of Hmg2p to enter the HRD pathway. Since the first 26 amino acids were required for HRD-mediated degradation, we tested whether the degradation of 2-127-54 similarly required this sequence. When both determinants were replaced simultaneously, the resulting chimera 2-11-54 was significantly stabilized, as measured in a cycloheximide chase assay and by observing the lovastatin sensitivity of strains expressing 2-11-54 as the sole source of HMG-R. Thus, the degradation of the poorly regulated 2-127-54 required the same critical 1-26 determinant for degradation as normal Hmg2p.

The portion of the sequence altered in 2-127-54 has been predicted to be a transmembrane span (Basson et al., 1988). It is possible that the key amino acids are present in the lipid portion of the ER membrane. This
Conceivably, the first transmembrane span controls degradation by direct interaction with a mevalonate pathway product that controls degradation. There are numerous examples of small molecule recognition by determinants in transmembrane spans (Kim et al., 1992; Cascieri et al., 1995), and this type of direct mechanism has been proposed to operate in the regulation of mammalian HMG-R by sterols (Hua et al., 1996). Alternatively, Hmg2p stability may be controlled by proteins that transduce the regulatory signal from the mevalonate pathway through, perhaps, direct interaction with determinants such as those in 27–54 or other features of the Hmg2p molecule. Presumably, such intermediary proteins, if they exist, will be revealed by studies to discover genes required for normal regulation of Hmg2p stability.

The $HRD$-encoded proteins themselves do not appear to be the target of regulation in the degradation of Hmg2p (Hampton et al., 1996b, and above). Rather, Hmg2p stability appears to be regulated by alteration of its access to the continuously active HRD machinery. The poorly regulated 2–127–54 was more prone to enter the HRD degradation pathway at a given level of mevalonate pathway activity, implying that amino acids 27–54 contained some information necessary to impose regulated stability on Hmg2p.

The determinants described above have a very small number of amino acid differences between the original sequence and those that altered the degradation phenotype. The precise amino acids that underlie the changes in degradative behavior may reveal the molecular mechanisms involved in regulated degradation. It appears from these studies that the degradation of Hmg2p and its regulation by the mevalonate pathway could be uncoupled in the sense that sequence determinants can be found that altered regulation but still allowed degradation. This might indicate that separate groups of genes are responsible for degradation and regulation. With this model in mind, we are currently isolating in-trans mutants deficient in regulation of Hmg2p stability but normal for HRD-dependent degradation. If successful, such screens will reveal the cellular machinery that creates and transduces the cellular signals that control Hmg2p stability.

ACKNOWLEDGMENTS

The authors thank Dr. Robert Rickert (University of California San Diego, Department of Biology) for the use of the FACScalibur flow microfluorimeter, software, and color printer. The last author further wishes to thank A. Richman for help with running protocols and for maintaining carbohydrate influx. This work was supported by American Heart Association grant 96013020 (to R.Y.H.), National Institute of Health grants DK-5199601 (to R.Y.H.) and GM-35827 (to J.D.R.), and a Searle Scholarship (to R.Y.H.). Core support was provided by a National Institute of Environmental and Health Sciences Mutagenesis Center grant (P30ES01896–12 to J.D.R.).

REFERENCES


