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# Genetic and Biochemical Evaluation of Eucaryotic Membrane Protein Topology: Multiple Transmembrane Domains of *Saccharomyces cerevisiae* 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

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**Both 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase isozymes of the yeast *Saccharomyces cerevisiae* are predicted to contain seven membrane-spanning domains. Previous work had established the utility of the histidinol dehydrogenase protein domain, encoded by *HIS4C*, as a topologically sensitive monitor that can be used to distinguish between the lumen of the endoplasmic reticulum and the cytoplasm. This study directly tested the structural predictions for HMG-CoA reductase by fusing the HIS4C domain to specific sites in the HMG-CoA reductase isozymes. Yeast cells containing the HMG-CoA reductase-histidinol dehydrogenase fusion proteins grew on histidinol-containing medium if the HIS4C domain was present on the cytoplasmic side of the endoplasmic reticulum membrane but not if the HIS4C domain was targeted to the endoplasmic reticulum lumen. Systematic exchanges of transmembrane domains between the isozymes confirmed that both isozymes had equivalent membrane topologies. In general, deletion of an even number of putative transmembrane domains did not interfere with the topology of the protein, but deletion or duplication of an odd number of transmembrane domains inverted the orientation of the protein. The data confirmed the earlier proposed topology for yeast HMG-CoA reductase, demonstrated that the yeast enzymes are core glycosylated, and provided in vivo evidence that the properties of transmembrane domains were, in part, dependent upon their context within the protein.**

The rate-limiting step of cholesterol biosynthesis, the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid, is catalyzed by HMG-CoA reductase EC1.1.1.34) (34; reviewed in reference 7). In mammalian cells, this pathway is feedback controlled by modulating synthesis and degradation of the enzyme in response to exogeneous sterols (8, 9, 14, 21). HMG-CoA reductase is an integral membrane glycoprotein of the endoplasmic reticulum (ER) (26) lacking a cleavable signal sequence (6). Membrane association is mediated by sequences in the amino half of the protein (27).

Mammalian cells have a single structural gene for HMG-CoA reductase, whereas the yeast *Saccharomyces cerevisiae* has two structural genes, *HMG1* and *HMG2*, each of which encodes fully functional isozymes (5). Either gene is sufficient for all aspects of the yeast life cycle. Sequence comparisons of the two isozymes reveals that they are very similar in their C-terminal domains, which harbor the catalytic activity of the enzyme (4). Less conservation is observed in the N-terminal halves of the two yeast isozymes, with segments of homology observed between some potential membrane-spanning domains and between some loops separating potential membrane-spanning domains. Neither yeast isozyme exhibits any primary sequence homology to the corresponding amino-terminal domain of the mammalian protein. Nevertheless, the structure proposed for both yeast proteins and for the mammalian protein predicted the existence of seven membrane-spanning α helices (4, 27). In contrast, the homologous protein from *Arabidopsis thaliana* harbors only one potential membrane-spanning domain (25) and other resident ER membrane proteins require only a single transmembrane domain to be anchored in the membrane. It is thus likely that the elaborate structure of the membrane-associated region of the mammalian and yeast HMG-CoA reductase proteins serves other functions in addition to being a membrane anchor. In fact, this region in the mammalian protein controls the stability of the protein in response to sterol (17).

The structural model for yeast HMG-CoA reductase was constructed entirely on predictions made with the aid of computer-based algorithms. This paper describes direct tests of several aspects of this structural model. By fusing a truncated version of the HIS4 protein (HIS4C) containing the histidinol dehydrogenase domain to specific transmembrane domains of HMG-CoA reductase, it was possible to evaluate the topology of specific positions within the protein in vivo. The results strongly suggest that both yeast HMG-CoA reductase isozymes are integral membrane proteins that span their cognate membranes seven times. Furthermore, homologous sequences in the two isozymes reside in topologically equivalent compartments within the cell. These data also provide information on the glycosylation of yeast HMG-CoA reductase and on the orientation independence of its transmembrane domains.

## MATERIALS AND METHODS

**Stains and media.** *Escherichia coli* strain DH5αF′ (*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 φ80dlacZΔM15 Δ[lacZYAargF]*U169) from Bethesda Research Laboratories, Inc. was used for plasmid transformations, and strain CJ236 (*dut-1 ung-1 thi-1 relA1* pCJ105) (23, 24) was used for the preparation of uridine-labeled template DNA. Yeast

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strain FC2a (*MATa his4-401 trp1-1 leu2-3 leu2-112 ura3-52 HOL1-1*) was derived from FC2-12b (11) by HO-mediated mating type interconversion and was the standard host to test *HIS4C* expression of different fusion proteins. FC2a cells were transformed with plasmid DNA according to the lithium-acetate method (20), and the *URA3*-marked plasmids were selected for on supplemented minimal plates lacking uracil and containing 2% glucose and required amino acids and bases at 30 mg/liter. $Ura^+$ transformants were streaked on supplemented minimal medium plates lacking histidine but containing 6 mM histidinol. Yeast strain JRY1597 (*MATa hmg1::LYS2 hmg2::HIS3 lys2-801 his3Δ200 ura3-52 ade2-101 met*) was grown in the presence of 5 mg of mevalonolactone per ml and was used to test *HMG2* expression of the plasmid series pB. Growth on histidinol or mevalonolactone medium was scored as positive if individual cells were capable of forming normal single colonies within 4 days. Growth indicated as negative indicated that no discernable single colonies were formed. Microbiological culture media were from Difco Laboratories; histidinol and all other chemicals were from Sigma Chemical Co.

**Construction of pA and PAΔ7.** A truncated copy of the *HIS4* gene, lacking the N-terminal 33 codons, was inserted as a 3.1-kilobase (kb) *Bam*HI-*Sph*I fragment from YCp502 (11) into the unique *Bam*HI-*Sph*I sites of pUC118 to generate pCS1. An in-frame *SUC2-HIS4C* fusion was then created by inserting the 1.4-kb *Sma*I-*Bcl*I fragment from pRB722 (C. Kaiser, personal communication) into the unique *Sma*I-*Bam*HI sites of pCS1 to produce pCS2. The resultant gene fusion contained the first 307 codons of the mature form of invertase, followed by the C-terminal 767 codons of *HIS4*. The *SUC2-HIS4C* gene fusion from pCS2 was then cloned as a *Sst*I-*Sph*I fragment into the polylinker of the yeast shuttle vector YEp352 (19), creating pCS3. Two tripartite *HMG1-SUC2-HIS4C* fusion genes were then constructed by fusing the N terminus of *HMG1* to *SUC2-HIS4C* at each of two different points within the membrane domain of *HMG1*. In the first instance, the N-terminal 525 codons of *HMG1* were fused in frame to *SUC2-HIS4C* by inserting the 2.5-kb *Eco*RI fragment from pJR301 (after filling in the ends with Klenow fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates) into the unique *Sma*I site of pCS3, thus generating pA. The second fusion was constructed by using an *Exo*III deletion derivative of pJR301, namely, pJR301Δ5.11. In this case, the N-terminal 463 codons of *HMG1* were fused to *SUC2-HIS4C* by inserting the 2.3-kb *Eco*RI-*Hin*dIII fragment from pJR301Δ5.11 (again after filling in the ends as described above) into the *Sma*I site of pCS3 to generate pAΔ7.

**Site-directed mutagenesis.** Oligonucleotide-directed mutagenesis was performed as previously described (23, 24). To introduce the sites at HMG1 domains, the *Eco*RI-*Sph*I fragment containing the *HMG1-HIS4C* fusion of plasmid pA was inserted into pEMBL18 (10; as described in reference 4). For the *HMG2* mutagenesis, pJR417, consisting of pEMBL18 with a 4.9-kb *Eco*RI fragment with *HMG2*, was used (4). The plasmids were propagated in strain CJ236 and were isolated as uridine-labeled single-strand DNA upon superinfection with phage R408 (from Stratagene). Synthetic mutagenic oligonucleotides flanking the mismatch by 12 perfectly matching residues were annealed, and these primers were extended in vitro in the presence of deoxynucleoside triphosphates, Klenow fragment of DNA polymerase I, and T4 ligase. The DNA was used for transformation of DH5αF′, and successful mutagenesis was monitored by restriction analysis of plasmid DNA from *Escherichia coli* transformants.

**Reconstruction of fusion genes.** To test the in vivo effects of the introduced restriction sites on *HIS4C* expression, the *Sph*I-*Sal*I fragments carrying the *Xho*I site mutations in the *HMG1-HIS4C* fusion gene were isolated and used to replace the *Sph*I-*Sal*I fragment in plasmid pA, in which the *HIS4C* distal *Sph*I site was previously destroyed by filling in as described above. These plasmids (pA1 to pA7) were transformed into strain FC2a, and $Ura^+$ transformants were streaked on histidinol-containing medium. To test the effects of the *Sal*I restriction sites on *HMG2* expression, the mutated *Eco*RI fragments were inserted all in the same orientation into YEp352. These plasmids were transformed into the mevalonate auxotrophic strain JRY1597, and $Ura^+$ transformants were checked for growth in the absence of mevalonate. Growth characterized as positive indicated that individual cells could form normal single colonies within 4 days of incubation; growth characterized as negative indicated that individual cells were completely incapable of forming a visible colony in this period of time.

*HMG2-HMG1-HIS4C* fusion genes (plasmid series pX) were constructed by triple ligation: *Eco*RI-*Sal*I fragments containing *HMG2* domains, *Xho*I-*Sph*I segments containing the *HMG1-HIS4C* fragments, and the large *Sph*I-*Eco*RI fragment of YEp352 were ligated in equimolar amounts. The pAD plasmid series was constructed from the pA series by ligating appropriate *Xho*I-*Sal*I fragments with each other.

**Immunoprecipitation of fusion proteins.** FC2a transformants were grown at 30°C to mid-log phase (optical density at 600 nm, 0.5 to 1.0) in minimal medium supplemented with 5% glucose and required amino acids. The cells (4 optical density units at 600 nm) were washed in fresh medium, resuspended in 1 ml of the medium, and labeled for 30 min with Tran$^{35}$S *E. coli* lysate (Amersham Corp.) (50 μCi/optical density unit of the cells) in the presence or absence of 10 μg of tunicamycin per ml. Excess methionine and cysteine were present during the chase. The cells were killed in 10 mM $NaN_3$, washed, and suspended in 100 μl of 8 M urea–1% sodium dodecyl sulfate (SDS), and a mixture of proteinase inhibitors was added. Cells were broken with glass beads by vortexing at room temperature for 90 s. After a 10-min incubation at 55°C, the supernatant fraction was recovered upon centrifugation. The beads were washed with 50 μl of the same solution, and the pooled supernatant fractions were diluted into 1 ml of IP buffer (15 mM $NaPO_4$ [pH 7.5], 150 mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% deoxycholate). After preadsorption with *Staphylococcus aureus* cells (IgGsorb; The Enzyme Center), a rabbit antiinvertase antiserum (33) was added together with protein A-Sepharose and incubated overnight at 4°C. The beads were washed three times in IP buffer and three times in 2 M urea–0.2 M NaCl–1% Triton X-100–100 mM Tris (pH 7.5), followed by one wash in 0.5 M NaCl–20 mM Tris (pH 7.5)–1% Triton X-100 and two washes in 10 mM Tris (pH 7.5)–50 mM NaCl. The antibody-antigen complex was dissociated by heating for 10 min to 55°C in 2× Läemmli sample buffer. Endo-β-*N*-acetylglucosaminidase H treatment was done overnight at 37°C in 150 mM sodium citrate (pH 5.5)–5 mM $NaN_3$–1 mg of bovine serum albumin per ml–50 mM β-mercaptoethanol, followed by immunoprecipitation. Labeled protein was electrophoretically separated on a 6% SDS-polyacrylamide gel; the gel was fixed in 25% isopropanol–10% acetic acid and incubated in Amplify (Amersham) prior to drying and fluorography at −70° by using Kodak XAR film.

## RESULTS

**Nomenclature.** A number of plasmids encoding recombinant protein molecules were used in this study. The pA series contained the *HMG1* gene and mutant alleles of it. The pB series contained the *HMG2* gene and mutant alleles of it. The pX series contained recombinants between *HMG1* and *HMG2* and a capital D, such as pAD, referred to a duplication or deletion within the coding sequence.

**HIS4C as a reporter gene for membrane protein topology.** *HIS4* encodes a trifunctional cytoplasmic protein consisting of proteolytically separable catalytic domains (22). Fusion proteins consisting of sequences of other proteins at the N terminus and the HIS4C domain at the C terminus retain histidinol-dehydrogenase activity (11, 32). Yeast cells that have a deletion of the genomic *HIS4* locus but that express a plasmid-encoded HIS4C protein domain in the cytoplasm are able to grow on minimal medium supplemented with histidinol, provided the strain also carries the *HOL1-1* mutation necessary for efficient histidinol uptake. Fusion of a signal sequence to HIS4C results in translocation of the fusion protein through the ER membrane (11). As a consequence, *his4* mutant cells containing such a fusion are unable to grow on histidinol-containing medium, due perhaps to the inability of the substrate(s) or product(s) of the enzyme to cross the ER membrane or to the extensive glycosylation and possible inactivation of the enzyme (11). The successful use of HIS4C as a reporter of the topological space in which that protein resides and the precedent of similar approaches to study the topology of membrane proteins in *E. coli* (26) prompted us to use HIS4C as a reporter of the topology of discrete portions of HMG-CoA reductase.

The structural model predicted that the HMG1 and HMG2 proteins both contained seven membrane-spanning domains, designated M1 through M7. The catalytic domains of both yeast proteins were likely to be on the cytoplasmic side of the ER membrane, by analogy to mammalian HMG-CoA reductase. To test this assumption regarding the orientation of the HMG1 protein, a fusion gene was constructed in which the sequences encoding the catalytic domain of HMG1 were replaced by the HIS4C domain, as described in Materials and Methods. The resulting fusion gene present on plasmid pA (Fig. 1) contained the seven putative *HMG1* transmembrane domains M1 through M7, the *HIS4C* domain, and a part of the invertase gene (*SUC2*), which introduced an immunogenic determinant into the resulting fusion protein. A second fusion protein (pAΔ7) was constructed by fusing the *HIS4C* fragment to the putative lumenal loop between transmembrane domains M6 and M7. Plasmids coding for either fusion protein were introduced into strain FC2a, and transformants were tested for their ability to grow on histidinol-containing medium (henceforth referred to as the $His^+$ or $His^-$ phenotype). As indicated in Fig. 1, transformants containing pA were $His^+$, whereas transformants containing pAΔ7 were $His^-$. Both types of transformants expressed a fusion protein that was immunoprecipitated with an antiserum directed against invertase (Fig. 2).

An immunoreactive species of 170 kilodaltons was evident in transformants containing pA, in good agreement with the calculated molecular weight of the fusion protein. The mobility of that species was slightly affected when glycosylation was blocked by tunicamycin during protein synthesis, indicating that the protein was core glycosylated. In this orientation, only two potential N-glycosylation sites between M6 and M7 (4) were exposed to the ER lumen, giving rise to a barely detectable mobility shift upon tunicamycin treatment (compare lanes 1 and 2 in Fig. 2). A similar tunicamycin-dependent shift in mobility was observed with the wild-type HMG1 protein (data not shown). Therefore, in pA transformants, the HIS4C domain of the fusion protein was apparently located on the cytoplasmic side of the membrane, allowing conversion of histidinol to histidine, thus producing a $His^+$ phenotype (Fig. 3). In contrast, a species of much higher molecular weight was observed in transformants containing pAΔ7 (Fig. 2, lane 3). The slower mobility was due to glycosylation, since the band was shifted to a faster-migrating form (lane 4) when tunicamycin was present during protein synthesis. Thus, the catalytic domain of the fusion protein was located in the ER lumen, producing a $His^-$ phenotype. As a consequence, many of the Asn-X-Ser/Thr

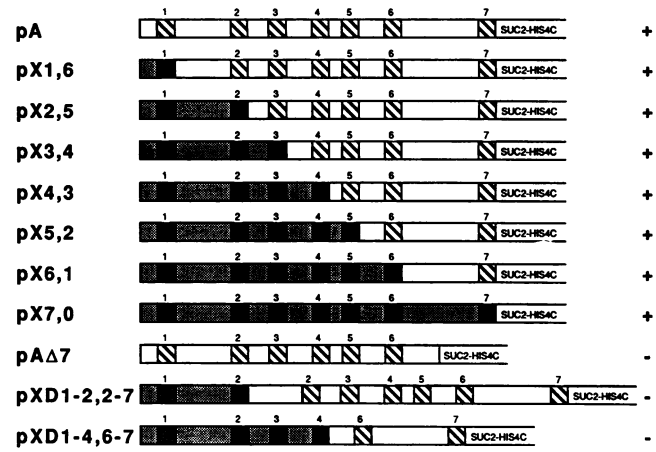


FIG. 1. First series of fusion proteins constructed for this study. All contain a segment of yeast invertase (*SUC2*) and histidinol dehydrogenase (*HIS4C*). Blank and striped areas denote *HMG1* sequences; shaded and stippled areas denote *HMG2* sequences. The striped and stippled boxes denote the transmembrane domains M1 through M7, identified by the numbers 1 through 7. The signs + and − indicate whether or not a particular fusion gene expressed in FC2a supported growth on histidinol-containing medium. The distances between putative transmembrane domains are roughly to scale.

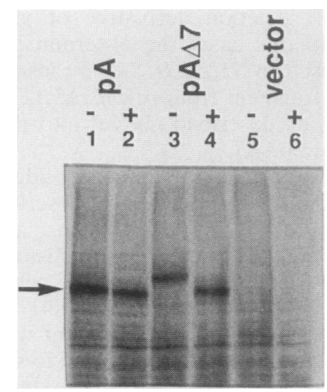


FIG. 2. Immunoprecipitation of fusion proteins pA and pAΔ7. Yeast cells expressing pA (lanes 1 and 2), pAΔ7 (lanes 3 and 4), or the vector YEp352 alone (lanes 5 and 6) were radiolabeled in the absence (indicated by a minus sign in lanes 1, 3, and 5) or presence (indicated by a plus sign in lanes 2, 4, and 6) of tunicamycin. Cell extracts were treated with antiinvertase serum, and the immunoprecipitates were electrophoretically separated on a 6% SDS-polyacrylamide gel. The arrow points to the 170-kilodalton pA fusion protein.

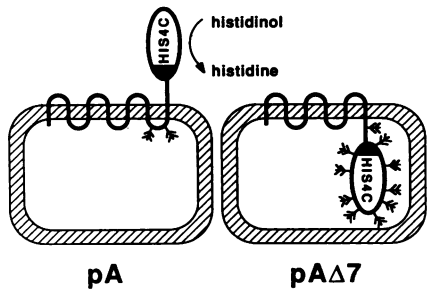


FIG. 3. An in vivo assay for membrane protein topology. The rounded vesicles represent ER, with the ER membrane shown as a diagonally striped area surrounding the ER lumen. The fusion proteins pA and pAΔ7 are anchored in the membrane by multiple transmembrane domains. The catalytic domain (HIS4C) in pA was present on the cytoplasmic side of the membrane, where it converts histidinol to histidine. In pAΔ7, the HIS4C domain was present in the ER lumen, was glycosylated by several carbohydrate chains, and was probably physically separated from its substrate histidinol. Therefore, pA transformants exhibited a $His^+$ phenotype, whereas pAΔ7 transformants exhibited a $His^-$ phenotype. The black area represents the SUC2 part present in both fusion proteins. This figure graphically demonstrates the scheme used in the assessment of membrane protein topology. In general, fusion proteins with an odd number of membrane-spanning domains conferred a $His^+$ phenotype, whereas an even number of transmembrane domains conferred a $His^-$ phenotype.

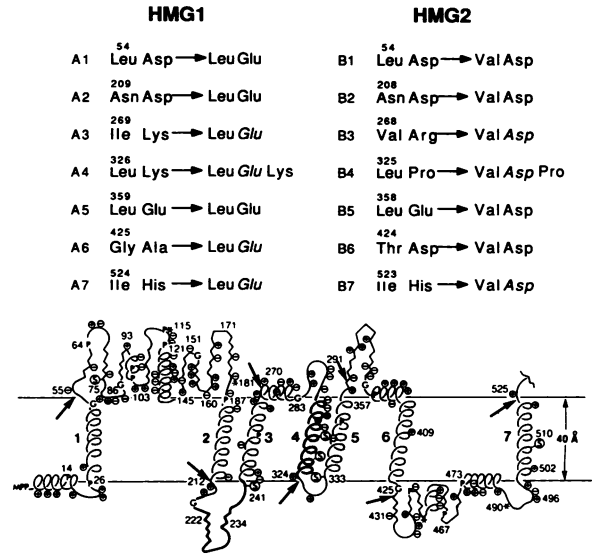


FIG. 4. Site-directed mutagenesis of *HMG1* and *HMG2* sequences. *Xho*I restriction sites were introduced into the fusion gene pA at all seven sites (indicated by arrows in the lower panel) where transmembrane domains exit the lipid bilayer. Amino acid changes leading to the fusion proteins pA1 through pA7 are indicated in the upper part of the figure. *Sal*I restriction sites were introduced at homologous locations in the *HMG2* sequence, giving rise to pB1 through pB7. Nonconservative changes in the two proteins are indicated in italics. The numbers denote positions of particular residues in both proteins. The structural model for HMG1 is redrawn from reference 4, with an alternative numbering of the transmembrane domains. The catalytic domain is not represented, yet would extend cytoplasmically from residues 525 through 1054.

sites in the *SUC2* and *HIS4C* part of the protein became accessible for glycosylation, resulting in a substantial increase in the apparent molecular weight of the fusion protein (Fig. 3).

The different phenotypes of pA and pAΔ7 transformants and the glycosylation pattern of the proteins encoded by these plasmids provided strong evidence that the putative seventh transmembrane domain (M7) of HMG1 protein indeed spanned the ER membrane, since deletion of that domain resulted in a change in the topology of the fusion protein. Furthermore, that result supported the inference made earlier that the catalytic domain of the yeast HMG1 isozyme was located on the cytoplasmic side of the ER membrane.

**The HMG1 and HMG2 isozymes are very similar in structure.** In order to test the existence of the remaining six putative transmembrane domains in HMG1 as well as the putative transmembrane domains in HMG2, restriction sites were introduced into both genes by in vitro mutagenesis. Every restriction site was introduced into the gene at positions where each transmembrane domain in the corresponding protein was predicted to exit from the lipid bilayer. The positions of the restriction sites are indicated in the structural model shown in Fig. 4. The mutations in the HMG1 membrane region were constructed in the *HMG1-HIS4C* fusion gene. The mutations of the HMG2 membrane-associated region were constructed in *HMG2*. All restriction sites were introduced in the same reading frame, allowing homologous exchanges to be made between the two isozymes and facilitating deletion of selected domains. Figure 4 summarizes the changes that were introduced in the primary structure of the proteins in order to obtain the desired restriction sites.

The mutated *HMG1-HIS4C* fusion genes carrying *Xho*I restriction sites at individual domains were expressed in strain FC2a, and growth of transformants was scored on histidinol-containing medium (Fig. 5). Most of the site mutations did not affect the $His^+$ phenotype. The mutation at transmembrane domain six (pA6) caused a slightly slower growth of the transformant on histidinol-containing medium and had no effect on medium containing histidine. The effect of the *HMG2* site mutations was evaluated in an *hmg1 hmg2* double mutant strain (JRY1597), which was consequently a mevalonate auxotroph. None of the *HMG2* site mutations affected the expression or activity of HMG2 protein, since all seven site mutations present on pB1-pB7 provided equivalent mevalonic acid-independent growth (Fig. 5).

The site mutants were further used to construct homologous exchanges between *HMG1* and *HMG2* transmembrane domains. These exchanges are referred to as the pX series, and the number(s) before the comma refers to the presumptive transmembrane domains contributed by the *HMG2* sequence; the number(s) after the comma refers to presumptive transmembrane domains contributed by the *HMG1* sequence. In the fusion proteins pX1,6 to pX7,0 (Fig. 1), increasing numbers of *HMG1* domains were replaced with corresponding *HMG2* domains. Upon transformation of the recombinant plasmids into FC2a, a $His^+$ phenotype was observed for all recombinants. This result indicated that every fusion protein was oriented with the HIS4C catalytic domain on the cytoplasmic side of the ER membrane. Therefore, the two isozymes possessed very similar structures and corresponding parts of the membrane-associated region were exchangeable. The simplest interpretation of these data was that if a given segment in the HMG1 protein constituted a transmembrane domain, then its counterpart in the HMG2 protein must be capable of spanning a membrane as well. Surprisingly, some of the fusion proteins (pX2,5 to

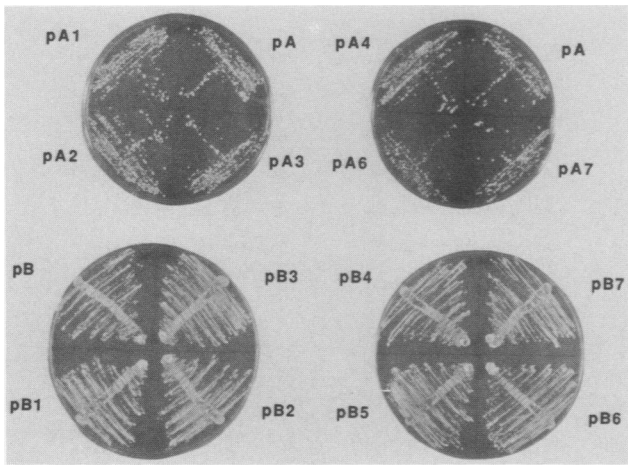


FIG. 5. Phenotypes of the *HMG1-HIS4C* and the *HMG2* site mutants. In the upper panel, strain FC2a was transformed with the parental plasmid pA and the *HMG1* site mutants pA1 through pA7. $Ura^+$ transformants were streaked for single colonies on plates containing histidinol and were incubated at 30°C for 7 days. Plasmid pA5 was not included in the analysis, since the mutation leading to the *Xho*I site was silent (see Fig. 4). In the lower panel, strain JRY1597 was transformed with the parental plasmid pB and the *HMG2* site mutants pB1 through pB7 and $Ura^+$ transformants were selected on plates containing mevalonolactone. $Ura^+$ clones were then streaked on plates lacking mevalonolactone, and growth was determined after 2 days at 30°C. Note that the *HMG1-HIS4C* fusion genes and the *HMG2* genes were all present in the same orientation on plasmid YEp352. In parallel experiments, FC2a and JRY1597 lacking plasmids were unable to grow in the absence of histidinol or mevalonolactone, respectively.

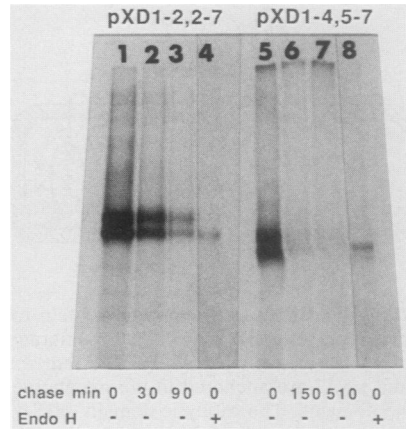


FIG. 6. Immunoprecipitation of pXD1-2,2-7 and pXD1-4,6-7 proteins. FC2a cells expressing pXD1-2,2-7 (lanes 1 through 4) or pXD1-4,6-7 (lanes 5 through 8) were radiolabeled. Crude extracts were prepared immediately (lanes 1 and 5) or after a chase in nonradioactive medium (lanes 2, 3, 6, and 7). The length of the chase was 30 min (lane 2), 1.5 h (lane 3), 2.5 h (lane 6), and 8.5 h (lane 7). Proteins were immunoprecipitated and electrophoretically separated on a 6% SDS-polyacrylamide gel. A small sample of the immunoprecipitated protein from lanes 1 and 5 was digested with endo-β-*N*-acetylglucosaminidase H, followed by an additional immunoprecipitation prior to electrophoresis (lanes 4 and 8).

pX7,0) conferred a thermosensitive phenotype for growth on histidinol-containing medium. At 34°C these transformants did not grow, whereas pA and pX1,6 showed normal growth. The temperature-sensitive growth was relieved by supplementing the medium with histidine. At 25°C, however, growth was normal for all transformants. The reason for the thermosensitive expression of histidinol-dehydrogenase activity in these cases was unclear.

**Duplication of domain M2 or deletion of domain M5 changed the topology of the fusion protein.** In order to test the existence of other putative transmembrane domains, we constructed the fusion proteins pXD1-2,2-7 and pXD1-4,6-7 (Fig. 1). The former protein contained the M2 domains of both HMG1 and HMG2 proteins, thus harboring eight potential transmembrane domains. The latter protein lacked the putative domain M5 and thus contained only six potential transmembrane domains. If the structural predictions for domains M2 and M5 were correct and if each transmembrane domain were capable of inserting into the membrane in either orientation, the HIS4C domains of these proteins should be located within the ER lumen. Expression of both recombinants in FC2a indeed resulted in a $His^-$ phenotype, arguing that both M2 and M5 functioned as transmembrane domains. Lack of HIS4C activity was not due to a failure of the cell to express the fusion proteins, since both were detected by immunoprecipitation (Fig. 6, lanes 1 and 5) and by immunoblotting (data not shown). Both fusion proteins exhibited two major species of different molecular weight. The slower-migrating species represented a glycosylated form, since this band disappeared upon digestion with endo-β-*N*-acetylglucosaminidase H (Fig. 6, lanes 4 and 8). Furthermore, the slower-migrating species was absent when glycosylation was inhibited by tunicamycin treatment of the cells, whereas the faster-migrating species was unaffected (data not shown). Since the addition of M2 or the removal of M5 resulted in a topological reorientation of a significant fraction of the fusion protein molecules, M2 and M5 were capable of functioning as transmembrane domains.

The glycosylation pattern obtained with pXD1-2,2-7 and pXD1-4,6-7 differed from the one with pAΔ7, in which all of the protein was glycosylated (Fig. 2). About half of the pXD proteins were present in an unglycosylated form. To test the possibility that the unglycosylated form represented a precursor in a slow translocation event, the radiolabeled proteins were immunoprecipitated after a prolonged chase in nonradioactive medium. As shown in Fig. 6 (lanes 2, 3, 6, and 7), no conversion of the unglycosylated form to the glycosylated form was observed. Rather, both forms were equally reduced during the chase period. Thus, duplication of putative domain M2 or a deletion of putative domain M5 resulted in a topological change in at least half of the fusion proteins and led to a complete lack of growth on histidinol-containing medium at both high and low temperatures. Whatever fraction of unglycosylated molecules was present was apparently not sufficient to allow growth on histidinol-containing medium. These results strongly indicated that the structural predictions for M2 and M5 were correct.

The plasmid series pAD1-6 through pAD1 was constructed, in which increasing amounts of *HMG1* domains were deleted, starting from the most C-terminal transmembrane domain (Fig. 7). All plasmids were transformed into strain FC2a, and the His phenotype of the transformants was analyzed. The fusion gene pAD1-6 was very similar to pAΔ7 (Fig. 1), except for 39 amino acids from the last lumenal loop that were present in pAΔ7 but absent in pAD1-6. As expected, pAD1-6 transformants were $His^-$ and the fusion protein was glycosylated (data not shown).

Both the M6 and M7 domains were deleted in pAD1-5. Transformants of pAD1-5 grew on histidinol-containing medium, demonstrating that the last two putative transmem-

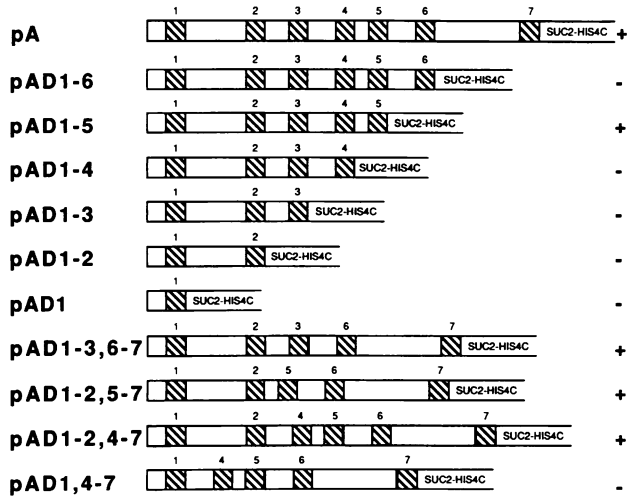


FIG. 7. Second series of fusion proteins constructed. The symbols are the same as those in the legend to Fig. 1.

brane domains were not essential for the function of the fusion protein. More importantly, this result implied that the prediction of transmembrane domain M6 was correct, since deletion of that segment in pAD1-6 rescued the HIS4C function by reorienting that domain from the ER lumen to a cytoplasmic location. Deletion of an additional domain (M5) in pAD1-5 resulted in a fusion gene (pAD1-4) that conferred a $His^-$ phenotype upon transformation into FC2a, suggesting that this deletion oriented HIS4C into the lumen of the ER. This result was in agreement with the $His^-$ phenotype of fusion protein pXD1-4,6-7, in which the M5 domain was deleted. In addition, the $His^+$ phenotype conferred by pAD1-5 together with the $His^-$ phenotype conferred by pXD1-4,6-7 indicated that domain M5 spanned a membrane as predicted.

The utility of the deletion analysis described above was limited to small deletions, since proteins with four or fewer putative transmembrane domains were unable to complement the $His^-$ phenotype of FC2a (Fig. 7). Evaluation of the remaining putative transmembrane domains relied on more subtle deletions, as described below. It is, however, worth noting that the pAD1 fusion protein was partly glycosylated, since the higher-molecular-weight form was converted to the faster-migrating species in the presence of tunicamycin (Fig. 8). This result implied that the amino terminus and the first

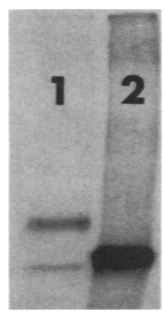


FIG. 8. Immunoprecipitation of fusion protein pAD1. Cells were radiolabeled in the absence (lane 1) or presence (lane 2) of tunicamycin. Protein was immunoprecipitated and electrophoretically separated as described above.

putative transmembrane domain were sufficient for the translocation of the HIS4C domain into the lumen of the ER. It was, therefore, plausible that the segment M1 indeed represented a membrane-spanning domain.

**Elimination of two adjacent putative transmembrane domains did not change the topology of the protein.** The evidence presented to this point indicated that domains M1, M2, M5, M6, and M7 were capable of spanning a membrane. Adding or removing single putative transmembrane domains changed the topology of the protein, placing the HIS4C domain in the ER lumen. Therefore, changing the number of transmembrane domains by two should leave the protein in its native orientation. Thus, if a putative transmembrane domain were deleted along with a known transmembrane domain and there were no change in the orientation of the catalytic domain, then the putative transmembrane domain can be inferred to, in fact, span the membrane. This strategy was applied to segments M3 and M4. The fusion protein pAD1-3,6-7 lacked both segments M4 and M5 (Fig. 7). Yeast transformants expressing this protein grew on histidinol-containing medium, indicating that the protein was inserted into the membrane with the catalytic domain oriented in the cytoplasm. Since deletion of M5 alone (pXD1-4,6-7 in Fig. 1) resulted in inverting the orientation of the catalytic domain and in a $His^-$ phenotype and deletion of segment M4 reversed the phenotype, M4 must also function as a transmembrane domain.

A deletion of putative domains M3 and M4 (pAD1-2,5-7 in Fig. 7) also conferred a $His^+$ phenotype in FC2a; thus the protein must be oriented with the catalytic domain in the cytoplasm. Since M4 spanned the membrane and since a deletion of both M3 and M4 did not change the orientation of the catalytic domain, M3 must have spanned the ER membrane.

The only inconsistencies among these results were obtained with the fusion proteins pAD1-2,4-7 and pAD1,4-7 (Fig. 7). In pAD1-2,4-7, a single transmembrane domain (M3) was absent, yet transformants expressing the fusion protein were $His^+$, implying that the HIS4C domain was oriented toward the cytoplasm. Alternately, a deletion of both M2 and M3 (pAD1,4-7) resulted in a fusion protein that conferred a $His^-$ phenotype. Since the earlier results indicated that M2 was a transmembrane domain (see above), these results argued against the prediction that M3 represented a transmembrane domain. However, earlier results indicated that M3 was indeed a transmembrane domain. All discrepancies could be resolved if M3 was in fact a transmembrane domain and if the ability of M4 to function as a transmembrane domain depended upon the presence of M3 (see below).

## DISCUSSION

This paper describes a genetic and biochemical analysis of the membrane-associated region of yeast HMG-CoA reductase. Yeast encodes two isozymes of HMG-CoA reductase which are essentially identical in their catalytic domains. A comparison of the membrane-associated region of the two yeast proteins reveals a mosaic of conserved and nonconserved segments (4). This study represents the beginning of a systematic effort to understand the functional implications of the structure of the membrane-associated region. Previous work established the utility of the HIS4C catalytic domain as a monitor capable of distinguishing between the cytoplasmic and lumenal compartments within a cell. When present in the cytoplasmic compartment, HIS4C catalyzes

the conversion of histidinol to histidine and thus allows growth of histidine auxotrophs. When present in the lumenal compartment, HIS4C becomes heavily glycosylated and cannot convert histidinol to histidine (11). The HIS4C domain was joined to the coding sequence of HMG1 and several HMG1-HMG2 recombinant proteins to provide a genetic measure of the topological orientation of the catalytic domain in vivo. The behavior of the fusion proteins provided evidence for the presence of seven transmembrane domains in the amino-terminal region of both HMG1 and HMG2. Subsequent analysis of the carbohydrate modifications of the fusion protein strongly supported the genetic results.

Exchanges of homologous domains in the HMG1-HIS4C fusion protein with corresponding positions in the HMG2 protein revealed that the topology of the two isozymes was very similar. In every case (pX1,6 to pX7,0; see Fig. 1), the exchange resulted in a $His^+$ phenotype. Thus homologous sequences in these two proteins were in equivalent topological orientations throughout the membrane-associated region. A combination of duplication and deletion derivatives of these proteins provided strong evidence that each of the seven domains of HMG1 predicted to span a lipid bilayer was in fact capable of functioning as a transmembrane domain. Although the behavior of domain M1 through M7 was established in the context of a fusion protein, the simplest interpretation is that M1 through M7 functioned as transmembrane domains in the intact HMG-CoA reductase proteins. In principle, it would be desirable to use the catalytic domain of HMG-CoA reductase itself as a reporter of membrane topology in order to avoid potential complications from the analysis of fusion proteins. However, HMG-CoA reductase catalytic domain remains functional in the ER lumen and both HMG-CoA and mevalonate appear to cross the ER membrane (unpublished observations). Thus, it was essential to make use of a protein domain like HIS4C, which had a distinct compartment-dependent behavior, in order to have a genetic assay for topological orientation.

Unexpectedly, proteins with more than one HMG2 domain exhibited a thermosensitive $His^+$ phenotype. This property was even found for the protein encoded by pX7,0, which contained the entire HMG2 membrane domain. Since an *hmg1* strain that expressed only HMG2 protein did not exhibit a thermosensitive phenotype, the thermosensitive phenotype must be an intrinsic property of the fusion proteins. The basis of the temperature-sensitive phenotype remains unknown.

Changing the number of transmembrane domains by one (pAΔ7; pXD1-2,2-7; and pXD1-4,6-7) resulted in a $His^-$ phenotype. In the case of pAΔ7, all of the fusion protein molecules were fully glycosylated and, hence, all must have had the catalytic domain in the ER lumen. Thus, the large carboxy-terminal part of that protein was readily translocated by the correct folding of six HMG1 domains. Further, the M7 domain or the region between M6 and M7 in the wild-type protein must have functioned as a stop-transfer domain in order to retain the catalytic domain on the cytoplasmic side of the ER membrane. In contrast, the two other fusion proteins with six and eight transmembrane domains, respectively, were present in both glycosylated and nonglycosylated species. Therefore, these deletions reversed the topological orientation of the catalytic domain in at least a fraction of the molecules. Furthermore, the unglycosylated form was not a precursor in a slow translocation process, since no interconversion was seen in a pulse-chase experiment. Whatever fraction of the catalytic domain of the protein was present on the cytosolic side, it was not sufficient to allow growth on histidinol-containing medium.

The results of these experiments leave some limitations in interpreting the properties of domains M6 and M7. As described in the context of the intact protein above, M7 can be viewed as having stop-transfer activity. Most simply, M6 would function as a signal peptide to direct the translocation of the loop between M6 and M7 and M7 would stop the transfer. Consequently, in fusion protein pAΔ7, the presence of the signal peptide in M6 and the absence of M7 would result in efficient translocation of the HIS4C domain. However, in the fusion proteins pXD1-2,2-7 and pXD1-4,6-7, transmembrane domains to the carboxy side of the duplicated or deleted segment were forced to traverse the membrane in their opposite orientation or not at all. M7, although still present, was unable to prevent the translocation of a significant fraction of the catalytic domains into the ER lumen and may even have acted as a signal peptide. Thus, the function of M7 appeared to depend, in part, upon the context in which it resided. Furthermore, it remains formally possible that an untranslocated HIS4C domain together with the noninserted domain M7 may fold in an aberrant way, interfering with HIS4C function. However, a simpler possibility was that the fraction of untranslocated molecules expressed too little cytoplasmic histidinol-dehydrogenase activity to provide a $His^+$ phenotype. These data indicated that insertion of membrane proteins with multiple transmembrane domains may be more complicated than the simple model of alternating transmembrane domains with either signal sequence or stop-transfer sequence properties, as first proposed for bovine opsin (16). Evidence that a single transmembrane domain can have different context-dependent structures has also been obtained from in vitro studies (3, 35).

There were several possible explanations for the unexpected behavior of the fusion protein encoded by pA1-2,4-7, in which deletion of putative transmembrane domain M3 resulted in maintenance of the $His^+$ phenotype, in contrast to the results obtained with duplicating M2 or deleting M5. One explanation of the $His^+$ phenotype was that segment M4 was unable to function as a transmembrane domain in the absence of M3. Indeed, the prediction that M4 functions as a transmembrane domain was less certain than for the other six domains, since several charged residues are located within the M4 membrane-spanning segment (4). Perhaps the free-energy barrier to inserting these charges in a lipid bilayer was compensated by opposite charges present in domain M3. Thus, with M3 missing, M4 would not insert (or remain) in the bilayer. Deletion of M3 would be functionally equivalent to deletion of M3 and M4, resulting in the $His^+$ phenotype of pAD1-2,4-7 transformants. The same explanation would hold for fusion protein pAD1,4-7, in which a deletion of two putative domains unexpectedly resulted in a $His^-$ phenotype. The absence of M3 might prevent M4 from spanning a membrane. In this case, the deletion of two transmembrane domain sequences would, in effect, eliminate the functioning of three transmembrane domains. An alternative explanation was based on the presence of another hydrophobic segment between M2 and M3, as indicated with a bold line in Fig. 4. This hydrophobic domain was not predicted to span a membrane due to the low probability of forming an α-helix (4). However, if it were to span the membrane, the deletion of M3, which also deleted this region, would have deleted a pair of transmembrane domains and would be expected to cause the $His^+$ phenotype. Fur-

ther experiments are necessary to distinguish among these and several other possibilities.

Mammalian HMG-CoA reductase has been predicted to have seven transmembrane domains in the amino-terminal portion of the protein, similar to the structure predicted for HMG1 (27). Although the transmembrane domains of the mammalian enzyme have not yet been analyzed in the detail presented here, the available evidence indicates a closely conserved structure between the yeast and mammalian enzymes, despite the lack of sequence homology. For example, glycosylation of both the yeast and human proteins occurs on the hydrophilic loop joining transmembrane domains six and seven. Similarly, simultaneous deletion of both predicted transmembrane domains four and five of the mammalian enzyme leaves the protein in the proper topological orientation in the ER membrane (21), as did the paired transmembrane domain deletions of the yeast enzyme reported here.

Heterologous protein fusions or sequence rearrangements have been used to analyze the topology of other eucaryotic membrane proteins (3, 13, 15, 35, 37). In some studies, membrane association of the proteins is analyzed by in vitro methods by using mRNA to program cell-free translation systems in the presence of membranes. Faithful translocation is judged by the appearance of glycosylated or protease-resistant gene products. In these experiments in which heterologous components were used, the translocated forms represented only a minor fraction of the synthesized proteins. In contrast, the approach used here involved homologous interaction of HMG-CoA reductase with its cognate membrane, in which translocation could be assayed directly in vivo. A comparable in vivo approach has been described for procaryotic organisms, involving fusions of alkaline phosphatase to multitopic membrane proteins (1, 28; reviewed in reference 29). It is worth noting the limitation of the different approaches. For example, certain alkaline phosphatase fusions had intermediate levels of alkaline phosphatase activity, perhaps due to only partial translocation analogous to pXD1-2,2-7 and pXD1-4,6-7. In vivo studies of fusion proteins provide results free of in vitro artifacts, yet because the proteins used are substantially altered forms of the native protein, their behavior is a reflection of what transmembrane domains can do rather than what they necessarily do in their native context. In this regard, the behavior of transmembrane domains of HMG1 depended in part upon their contest in the protein. In vitro studies may measure the behavior of a small fraction of the total proteins and are subject to the intrinsic limitations of the assays. Thus, it is unlikely that any one approach can fully reveal the functional aspects of each transmembrane domain in proteins that span the lipid bilayer numerous times.

Seven transmembrane domains are a common motif in other eucaryotic membrane proteins, such as rhodopsin (31), and have been suggested for the yeast pheromone receptors *STE2* and *STE3* (18, 30) and the β-adrenergic receptor (12). These proteins have in common the interaction with heterotrimeric GTP-binding proteins (G proteins) which transfer information from the outside of the cell to effectors inside the cell. The structural similarity between the membrane-associated region of HMG-CoA reductase and the G-protein-interacting receptors could be a coincidence, although the conservation of secondary structure in the absence of primary sequence conservation makes this explanation unlikely. If this structural similarity is a reflection of functional similarity, what signal could HMG-CoA reductase transmit to a cell? In both yeast and mammalian cells, increases in the amount of HMG-CoA reductase protein, even without an increase in the amount of HMG-CoA reductase activity, result in a dramatic proliferation of intracellular specialized membranes (2, 36; Wright and Sengstag, unpublished observations). In yeast cells, as little as 10-fold increased synthesis of HMG-CoA reductase from a heterologous promoter was effective in triggering increased membrane synthesis. It will be interesting to learn whether a GTP-binding protein participates in the response of cells to the level of HMG-CoA reductase protein.

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