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ARTICLES

Genetic and Biochemical Analysis of the Transmembrane Domain of *Arabidopsis* 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

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Abstract We have examined the amino terminal membrane anchoring domain of *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A reductase (Hmg1p), a key enzyme of the isoprenoid biosynthetic pathway. Using both in vitro and in vivo approaches, we have analyzed a series of recombinant derivatives to identify key structural elements which play a role in defining Hmg1p transmembrane topology. Based on our results, we have proposed a topological model for Hmg1p in which the enzyme spans the lipid bilayer twice. We have shown the two transmembrane segments, designated TMS1 and TMS2, to be structurally and functionally inequivalent in their ability to direct the targeting and orientation of reporter proteins. Furthermore, we provide evidence indicating both the extreme amino terminal end and carboxyl terminal domain of the protein reside in the cytosol. This model therefore provides a key basis for the future examination of the role of the transmembrane domain in the targeting and regulation of Hmg1p in plant cells. J. Cell. Biochem. 65:443–459.

Key words: *Arabidosis thaliana;* HMG CoA reductase; Hmg1p; transmembrane domain; protein

In plants, the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) catalyzes one of the first committed steps in the isoprenoid biosynthetic pathway, the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonic acid (MVA). This key intermediate is activated by a series of phosphorylation events and subsequently undergoes decarboxylation to generate isopentenyl pyrophosphate, the biosynthetic precursor for a diverse array of both essential and secondary metabolites, including sterols, chlorophyll, dolichol, carotenoids, phytoalexins, and growth regulators [Bach, 1987; McGarvey and Croteau, 1995]. Given the fundamental roles these isoprenoid compounds play in plant growth and development, understanding their biosynthetic regulation will provide important insights into plant metabolism and its control. Since mevalonate synthesis is a reaction that is common to the synthesis of all isoprenoids, the study of its regulation represents a logical first step in beginning to understand the overall coordinate control of the pathway. Although studies in plants have shown HMG CoA reductase activity to respond to a variety of external stimuli [Brooker and Russell, 1975, 1979; Wong et al., 1982], little progress has been made in identifying the biochemical basis for this control.

In mammals, the overall regulation of the isoprenoid biosynthetic pathway, and in particular cholesterol biosynthesis, has been shown to be closely linked to the control of a single HMG CoA reductase gene [Reynolds et al., 1984]. Multivalent control via negative feedback from both sterol and nonsterol derived signals has been shown to regulate HMG CoA reductase at the levels of transcription [Osborne et al., 1985; Sudhof et al., 1987a,b), translation [Reynolds et al., 1984], and protein stability [Chun et al., 1990; Meigs et al., 1996]. The basis of some of these regulatory processes is in part mani-

Abbreviations used: endo H, endo-β-N-acetylglucosamine; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; MVA, mevalonic acid; PMSF, phenylmethylsulfonylfluoride; TFMS, trifluoromethane sulfonic acid.

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fested through structural features of the enzyme itself [Chun et al., 1990; Roteilman et al., 1992; Chun and Simoni, 1992; Kumagai et al., 1995]. Comprised of two distinct structural domains, mammalian HMG CoA reductase consists of a cytoplasmic carboxyl terminal domain containing the catalytic site of the enzyme and an amino terminal domain consisting of eight membrane spanning regions which anchors the protein to the endoplasmic reticulum (ER). While the amino terminal domain is not necessary for catalysis, it has been shown to contain the information necessary for targeting and retention in the ER [Skalnik et al., 1988] and establishing topological orientation [Liscum et al., 1985; Olender and Simoni, 1992; Roteilman et al., 1992] as well as for playing a role in protein stability [Jingami et al., 1987; Chun et al., 1990; Chun and Simoni, 1992]. How the amino terminal domain mediates these functions still remains unknown.

In the yeast *Saccharomyces cerevisiae*, two isoforms [Basson et al., 1986] of HMG CoA reductase, designated HMG1 and HMG2, have been identified. Both isoforms have been shown to be capable of meeting the need for HMG CoA reductase activity [Basson et al., 1986]. Elegant biochemical and genetic analyses have provided evidence that the proteins encoded by both genes have a membrane-anchoring amino terminal domain responsible for their subcellular localization [Basson et al., 1988] and for protein stability [Hampton and Rine, 1994]. Although the amino terminal domains from both HMG CoA reductases have been shown to be similar to each other [Basson et al., 1988], they show little sequence homology to any mammalian enzyme. Strikingly, the predicted structural architecture of the transmembrane domain is remarkably similar between the yeast and mammalian enzymes, despite the lack of conserved sequences. Moreover, expression of mammalian HMG CoA reductases complements mutations in HMG1 and HMG2 and provides the MVA essential for yeast viability [Basson et al., 1988].

Similar to *Saccharomyces cerevisiae*, the plant *Arabidopsis thaliana* recently has been shown to produce two isoforms of HMG CoA reductase [Caelles et al., 1989], Hmg1p and Hmg2p, which are encoded by the HMG1 and HMG2 genes, respectively. Studies of these genes have shown them to be differentially expressed [Enjuto et al., 1994, 1995]. At present, the subcellular localization of these enzymes has not been determined, but both enzymes can be cotranslationally inserted into microsomal membranes in vitro [Caelles et al., 1989; Enjuto et al., 1995; Campos and Boronat, 1995], suggesting they may both reside as integral membrane proteins in the ER. Computer based analysis of the primary amino acid sequence of these enzymes predicts a structurally simple transmembrane domain, consisting of two stretches of hydrophobic amino acids that may span the membrane once or twice [Caelles et al., 1989; Learned and Fink, 1989]. Recently, support for a two-membrane spanning segment model for plant HMG CoA reductases has been proposed [Campos and Boronat, 1995; Denbow et al., 1996] using data generated from in vitro systems. Given the structural conservation between the amino terminal domain of the yeast and mammalian HMG CoA reductase enzymes, the relatively simple architecture of the transmembrane domain of plant HMG CoA reductase is a surprising departure from that structural paradigm.

Despite the differences in the predicted structure of the transmembrane domains of the plant and animal enzymes, one of the striking features of all eukaryotic HMG CoA reductases is their association with the endoplasmic reticulum as integral membrane proteins. In mammalian cells and yeast, this localization plays a key role in the regulated expression of HMG CoA reductase.

As a first step towards delineating the structural and functional roles of the amino terminal transmembrane domain of HMG CoA reductase in plants, we have analyzed the topological features of Hmg1p. By exploiting a combination of biochemical and genetic approaches, we directly tested the transmembrane model predicted by computer based algorithms for Hmg1p using both in vitro and in vivo systems.

MATERIALS AND METHODS

Materials and Reagents

All restriction enzymes and DNA modifying proteins were purchased from New England Biolabs (Beverly, MA). An in vitro transcription kit and m7G(5′)ppp(5′)G RNA cap analog were purchased from Ambion. An in vitro wheat germ extract translation system, RNAsin, and canine pancreatic microsomes were purchased from Promega (Madison, WI). Translation grade [^{35}S] methionine was purchased from New England Nuclear/Du Pont (Boston, MA). Amplify

fluorography reagent and rainbow color protein markers were purchased from Amersham (Arlington Heights, IL). XAR-5 X-ray film was purchased from Kodak (Rochester, NY).

Phenylmethylsulfonylfluoride (PMSF), pepstatin A, chymostatin, antipain, Tween 20, and trifluoromethane sulfonic acid (TFMS) were purchased from Sigma Chemical (St. Louis, MO). Endo-β-N-acetylglucosaminidase H (endo H) was purchased from Boehringer-Mannheim (Indianapolis, IN). ECL chemiluminescence immunodetection reagents were purchased from Amersham. Horseradish peroxidase–conjugated goat antirabbit or mouse IgG were purchased from Pierce (Rockford, IL). Rabbit antiserum to β-glucuronidase (SUC2) was purchased from ClonTech (Palo Alto, CA).

Strains and Media

The yeast strain JRY0510 (*MATa his4-401 trpl-1 leu2-3 leu2-112 ura 3-52 HOL1-1*) was the parent strain for all the in vivo topology assays using the *HMG1:SUC2:HIS4* plasmids. During the course of our studies, we found that JRY0510 cells would not grow when using galactose as a carbon source. Consequently, GAL+ revertants of JRY0510 were isolated by plating cells onto yeast minimal media with 2% galactose as a carbon source. Strain SEY6210 (*MATa leu2-3,112 ura3-52 his3-Δ200 trpl-Δ901 lys2-801 suc2-Δ9 GAL*) was the parent strain used for the subcellular fractionation experiments involving the *HMG1:SUC2:HIS4* plasmids. Yeast strain FY23 (*a, ura3-52, trplΔ63, leu2Δ1, GAL2*) was the parent strain used in the expression analysis of HMG1:*uidA* chimeric fusions and 12CA5 epitope-tagged HMG1 proteins.

All yeast cells were grown on yeast minimal medium (YNB): 2–4% carbon source, 0.67% Bacto-yeast nitrogen base minus amino acids (Difco Laboratories, Detroit, MI), and the indicated amino acid supplements. Final concentrations of supplements were 0.20 g/l His, 0.013 g/l Ade, 0.13 g/l Leu, 0.08 g/l Trp, and 0.022 g/l Ura. Yeast as transformed with DNA using the LiOAc method as described by Ito et al. [1983].

Construction of Plasmids

The yeast–*E. coli* shuttle vector pYES-2 was purchased from Invitrogen (La Jolla, CA) and the pGUSN358 → S plasmid from ClonTech. To create ERP 87, we subcloned a cytosolic form of *Arabidopsis* cyclophilin (~0.7 Kb insert of pCG22) into pBluescript KS− as a Bgl II and Xba 1 insert. To obtain a full-length mRNA transcript of HMG1, we subcloned the corresponding 2.2Kb cDNA into the pGEM3Z plasmid as a EcoR1 fragment to create ERP 5.

The *uidA* insert of GUS (N358 → S) was subcloned in frame to HMG1 DNA segments already present in pBluescript KS− to construct the HMG1:*uidA* (N358 → S) gene fusions. The resultant HMG1:*uidA* (N358 → S) gene fusions were subsequently subcloned into the yeast expression vector pYES-2 to generate the D0, D1, D2, and D12 chimeric fusions.

HMG CoA reductase transmembrane spans D0, D1, and D12 were amplified from an *Arabidopsis thaliana* HMG CoA reductase cDNA using the polymerase chain reaction (PCR). Oligonucleotides were designed to introduce flanking restriction enzyme sites into the resulting PCR products: HindIII at the 5′ end and BamHI at the 3′ end. To generate the D0, D1, and D12 HMG1 DNA segments, we synthesized the following primers: (P0) 5′-CCGGATCCTCCGTTGGAGTTGTTGTT-3′; (P1) 5′-CCGGATCCGATAGTGACGACGTGAAGAG-3′; (P12) 5′-CCGGATCCGGCACGTGAGATAAATGACT-3′; (P3) 5′-CCAAGCTTCTCAGATATTTCAGAGGT-3′. The P3 primer, complementary to a 5′ untranslated region, was used in conjunction with one other primer (P0, P1, P12) to amplify the desired DNA segments. Following amplification, each DNA sample was extracted with phenol:chloroform, blunt-ended with Klenow polymerase, and ligated using T4 DNA ligase. The PCR products were digested with BamHI and HindIII and subcloned into pBluescript KS−. Positive recombinants containing D0, D1, and D12 in pBluescript KS− were digested with BamHI and HindIII and the resultant inserts subcloned into the analagous sites of the yeast–*E. coli* shuttle vector pYES-2. An in-frame *SUC2:HIS4* translational fusion (Sma1/Sph1 insert of ERP 10) was subcloned into the EcoRI (blunt-ended) and Sph1 sites of the D_n:pYES-2 constructs. The resultant tripartite *HMG1:SUC2:HIS4* gene fusions were designated pHMG1D0, pHMG1D1, and pHMG1D12. Each construct consisted of an 5′ region of HMG1 followed by approximately 320 codons of the mature yeast invertase gene (*SUC2*) and the C-terminal 715 codons of histidinol dehydrogenase (HIS4).

To generate the pHMG1D2 construct, we employed single-stranded DNA mutagenesis [Kunkel et al., 1987]. The PΔ1 primer, 5′-GCG-

CTTCCTCTTCCGTTATATCCTCTTCAC-GTCGTC-3′, was designed to delete most of the putative D1 transmembrane spanning segment. The resulting HMG1D2 DNA segment was subcloned into pYES-2 to generate pHMG1D2, as described previously.

Yeast Growth for In Vivo Topology Assays

Yeast cultures were grown at 30°C on synthetic complete medium containing 1X YNB, minus amino acids supplemented with the appropriate additions. Raffinose, galactose, or glucose was used as a carbon source as indicated.

In order to perform in vivo topology assays, we grew HMG1:SUC2:HIS4 JRY0510 transformants as follows: 10 ml cultures containing 3% raffinose, 1X YNB minus amino acids, plus the appropriate additions were inoculated from a single yeast colony and incubated at 30°C, 250 rpm, until reaching logarithmic phase (OD_{600nm} 0.5–1.0). Cells were harvested by centrifugation (5 min, 1,100 *g* at room temperature [RT]) and supernatants decanted. Pellets were resuspended in 5 ml deionized H_2O, washed, and centrifuged. The supernatants were decanted and the pellets resuspended in a final volume of 1X YNB, minus amino acid mix to give 1 OD_{600nm} unit per milliliter. Concentrated yeast solutions were serially diluted 1:9 in 1X YNB, minus amino acid mix, and approximately 7.5 ul of each dilution was pipeted onto yeast plates containing 2% galactose, 1X YNB, amino acid additions plus or minus 7.5 μM histidinol. In addition, a parallel series of dilutions was aliquotted onto plates containing glucose as a carbon source. Yeast cultures were incubated at 30°C for 6 days prior to scoring. Growth of yeast cells at a particular dilution was denoted with a + sign and no growth with a − sign.

In Vitro Transcription and Translation of pHMG1D$_n$ DNA Templates

The pHMG1D$_n$ (n = 0, 1, 2, 12) series constructs and control DNAs were transcribed as follows. Purified CsCl DNAs were digested with the restriction enzyme of choice and precipitated with ethanol. The linearized DNA templates were used to make capped RNAs according to the vendor's instructions (mMessage in vitro translation kit; Ambion, Austin, TX). Synthesized RNAs were precipitated by adding sodium acetate and ethanol and incubating at −80°C for 1 h. Following centrifugation in a Brinkmann (Westbury, NY) model 5415C centrifuge (15 min, 14,000 rpm, 4°C), the nucleic acid pellets were resuspended in 50 μl of RNAse-free H_2O and the OD_{260nm} of the solution determined. Typically the yield was between 5 and 15 μg of RNA. Samples were stored in ethanol at −80°C until use.

pHMG1D0, D1, D2, D12 DNAs were digested with Xba 1, resulting in DNA templates containing approximately 1.3–1.6 Kb of coding sequence. ERP 87 and ERP 5 were digested with Xho1 to generate 0.7 and 2.2 Kb templates, respectively. Approximately 1 nmol of in vitro synthesized RNA was translated by adding it to the following mixture: 4 ul of a 1 mM mix of each amino acid except methionine (Promega); 25 uCi of >1,175 Ci/mmol translational grade [^{35}S] methionine (NEN); 25 ul of amino acid–depleted, nuclease-treated wheat germ extract (Promega); 3.7 ul 1M KOAc; 20 units RNAsin; H_2O to 50 ul. The mixture was incubated at 25°C for 1–2 h.

The in vitro translation system was supplemented with microsomes by including 2–5 ul of canine pancreatic microsomes (Promega) in the translation mixture.

Fractionation of In Vitro Synthesized Protein Products

In vitro synthesized protein products were fractionated into membrane-bound and soluble fractions as follows: 50 μl of the translation mixture was loaded on top of a 75 μl cushion of 15% (w/v) sucrose in TNE (50 mM Tris, pH 7.5, 500 mM NaCl, and 25 mM EDTA). The microsomes were pelleted by centrifugation in a Beckman (Fullerton, CA) TL-100 ultracentrifuge for 15 min at 190,000*g* in a TL-100.1 rotor. Approximately 125 μl of supernatant was transferred to a new tube containing an equal volume of 2X sample prep buffer (2X SPB). To release the lumenal contents of the microsomes, we resuspended the pellet in 50 μl 50 mM Na_2CO_3, pH 11.0. The mixture was layered on top of a 100 μl 15% (w/v) sucrose cushion in TNE. The microsomes were centrifuged as above except the duration of centrifugation was increased to 45 min. The supernatant (150 μl) was mixed with an equal volume of 2X SPB, while the pellet was resuspended in a volume equal to that of the original translation mixture.

SDS-PAGE and Fluorography

Whole cell extracts or protein products synthesized in the in vitro translation system were

separated by SDS–polyacrylamide gel electrphoresis (SDS-PAGE) as follows. The proteins were denatured by heating at 100°C for 3 min and the samples loaded into a lane of a SDS-PAGE gel (8% or 12% acrylamide, as indicated). The SDS-PAGE gel and the electrophoresis buffer were prepared according to the method of Laemmli et al. [1970]. The gels were run at 20 mA, fixed in 25 parts isopropanol, 65 parts H_2O, and 10 parts glacial acetic acid, incubated in Amplify (Amersham), dried, exposed to X-ray film, and developed.

Immunoblotting

Whole cell lysates for immunoblot analysis were prepared as follows: 50 ml cultures of logarithmic phase yeast cells (OD_{600nm} 0.5–1.0) growing on 3% raffinose were inoculated with a stock galactose solution to give a final concentration of 4% galactose. Cells were allowed to continue to grow (30°C, 250 rpm, ~12–16 h) overnight and were harvested by centrifugation (SS34 rotor, 10 min at 1,400*g* at 6°C). The supernatants were decanted and the pellets resuspended in ice-cold deionized H_2O. The samples were centrifuged as stated above and resuspended in 10 ml sorbitol buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM TrisHCl, pH 7.4), centrifuged, and resuspended in 4 ml sorbitol buffer. Approximately 2 ml of sterile glass beads (0.4–0.6 mm; Sigma) was added to each sample and the tubes vortexed vigorously for 30 s. The vortexing procedure was repeated a total of eight times prior to centrifuging samples for 5 min at 2,000*g*. Supernatants were mixed with an equal volume of 2X SPB and heated for 3 min at 100°C. Samples were briefly centrifuged to clear any particulate matter and the proteins separated by SDS-PAGE. Proteins in the gel were electrophoretically (~12 h, 20V at 15°C) transferred to a nitrocellulose membrane. Following blotting, the nitrocellulose membrane was incubated in phosphate buffered saline (PBS) (100 mM sodium phosphate pH 7.5, 100 mM NaCl) with 0.1% Tween 20, 5% Carnation nonfat milk for 1–2 h at room temperature. Immobilized proteins were probed by incubating with a 1:2,000 dilution of primary antiserum in PBST (1% milk and 0.1% Tween 20) at room temperature for 1 h. The nitrocellulose membrane was extensively washed in PBST, incubated with a 1:2,000 dilution of horseradish peroxidase–Goat anti-rabbit serum, washed, and developed with an ECL chemiluminescence immunodetection kit (Amersham).

Chemical and Enzymatic Deglycosylation

Yeast protein extracts containing recombinant HMG1p, tagged with the hemaglutinin epitope, T154 and T579, were chemically deglycosylated using the method of Karp et al. [1982]. Yeast protein extracts (0.5–1.0 mg) were concentrated in vacuo in screw-capped tubes. A solution of 90% trifluoromethanesulfonic acid (TFMS) and 10% anisole was prepared, capped under nitrogen, and placed on ice; 500 µl of the TFMS: anisole mix was added to each sample on ice, gently mixed, capped under nitrogen gas, and incubated on ice for 6 h. The TFMS: anisole-treated samples were transferred to corex centrifuge tubes containing 10 ml of pyridine:ether (1:9 v/v) in a dry-ice acetone bath, centrifuged in a clinical centrifuge (approximately 1,100*g*) for 5 min at room temperature. Supernatants were decanted and the pellets resuspended in 5 ml of 0.1 M NH_4HCO_3. Samples were dialyzed overnight (approximately 16 h) at 4°C against 4 L of 0.1 M NH_4HCO_3. Dialysis was then continued against 4 L of 5 mM NH_4HCO_3 for 24 h with several buffer changes. At the completion of dialysis, samples were frozen and dried down to completeness. The deglycosylated protein samples were resuspended in 1X SPB for analysis.

For enzymatic deglycosylation, yeast protein extracts were precipitated with 10% trichloroacetic acid (w/v) and the protein pellets resuspended in 50 µl of 50 mM sodium citrate, pH 5.6, 0.2% SDS, 0.05% sodium azide, 1 mM PMSF, and 20 ug/ml pepstatin A. Prior to starting enzyme digestions, the samples were heated for 10 min at 100°C and allowed to cool to room temperature. For each enzymatic deglycosylation 10–20 mU of endo H was added and the samples incubated for 36 h at 37°C.

RESULTS

HMG1:SUC2:HIS4 Gene Fusions as a Reporter of Membrane Protein Topology

Sequence analysis of genes encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from a wide variety of higher plants revealed a surprising feature of this highly conserved enzyme. Unlike the mammalian or fungal HMG

CoA reductase proteins that exhibit multiple transmembrane spanning segments within the complex architecture of the amino terminal domain (Fig. 1), the secondary structure of the transmembrane domain of the plant enzyme is predicted by computer alogorithm to be comparatively simple [Learned and Fink, 1989]. As seen in Figure 1, the hydropathy profile of *Arabidopsis* Hmg1p reveals two highly hydrophobic stretches of amino acids embedded within the amino terminal region of the protein, consistent with a model in which the protein is anchored to the endoplasmic reticulum by either one [Learned and Fink, 1989] or two [Caelles et al., 1989] transmembrane spanning segments. In contrast, the hydropathy profile of yeast HMG CoA reductase shown in Figure 1 is characteristic of the seven or eight membrane-spanning segments that comprise the transmembrane domain of the animal and fungal enzymes. Moreover, genetic [Sengstag et al., 1990] and biochemical [Wright et al., 1988; Olender and Simoni, 1992] studies have shown that the amino terminal domain of the yeast and animal enzymes serves not only to target and anchor HMG CoA reductase to the ER membrane but also to coordinate MVA-regulated enzyme turnover [Gil et al., 1985; Chun and Simoni, 1992; Hampton and Rine, 1994]. As a first step towards understanding the structural and functional roles that the HMG CoA reductase transmembrane domain plays in plants, we took advantage of a combination of biochemical and molecular genetic approaches to test the structural models for *Arabidopsis* HMG CoA reductase and to characterize the contributions of specific structural motifs to Hmg1p topology.

As shown in Figure 1, the amino terminal domain of *Arabidopsis* HMG1p contains two highly hydrophobic stretches of amino acids, either one or both of which could potentially span a membrane. These two regions have been designated TMS1 and TMS2, and include amino acid residues 53–69 and 98–114, respectively (Fig. 2). As a means of systematically evaluating the ability of these segments to determine the topological features of a membrane protein, we constructed a series of chimeric genes in which specific portions of the transmembrane domain from Hmg1p were fused to yeast histidinol dehydrogenase, an enzymatic reporter used to monitor orientation of the protein in the endoplasmic reticulum [Deshaies and Schekman, 1987]. A schematic representation highlighting the *HMG1* sequences amplified by PCR and used in the construction of these gene fusions is shown in Figure 2. The exact protein regions of Hmg1p encoded by each construct and the nomenclature used to identify them are as follows: D0 (+1-+20aa); D1 (+1-+92aa); D2 (+1-+56aa, +86-+122aa), and D12 (+1-+124aa). These amplified segments of *HMG1* were fused in frame to portions of the yeast invertase (*SUC2*) and histidinol dehydrogenase (*HIS4*) genes to create the pHMG1D$_n$ series of constructs. The fragment of the yeast invertase gene present in these chimeric genes introduces a immunogenic determinant into the fusion protein, while the presence of HIS4 at the C-terminus of the protein can be used as a reporter of topological space in Saccharomyces cerevisiae.

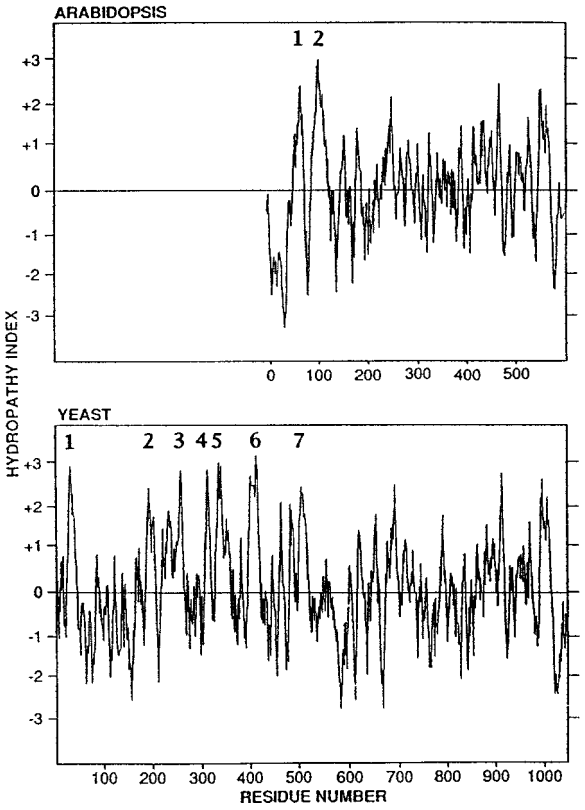

Fig. 1. Hydropathy plots of the amino acid sequences of *Arabidopsis* and yeast HMG-CoA reductase proteins. The average hydrophobicity of each amino acid residue was calculated [Kyte and Doolittle, 1982] over a window of nine amino acids and was plotted as a function of amino acid position. The graphs were aligned to maximize structural similarities. The labeled peaks indicate the membrane-spanning regions in the aminoterminal domain of the proteins. The hydropathy plot of *Arabidopsis Hmg1p* was replotted from Learned and Fink [1989] and the yeast Hmg1p plot from Basson et al. [1986].

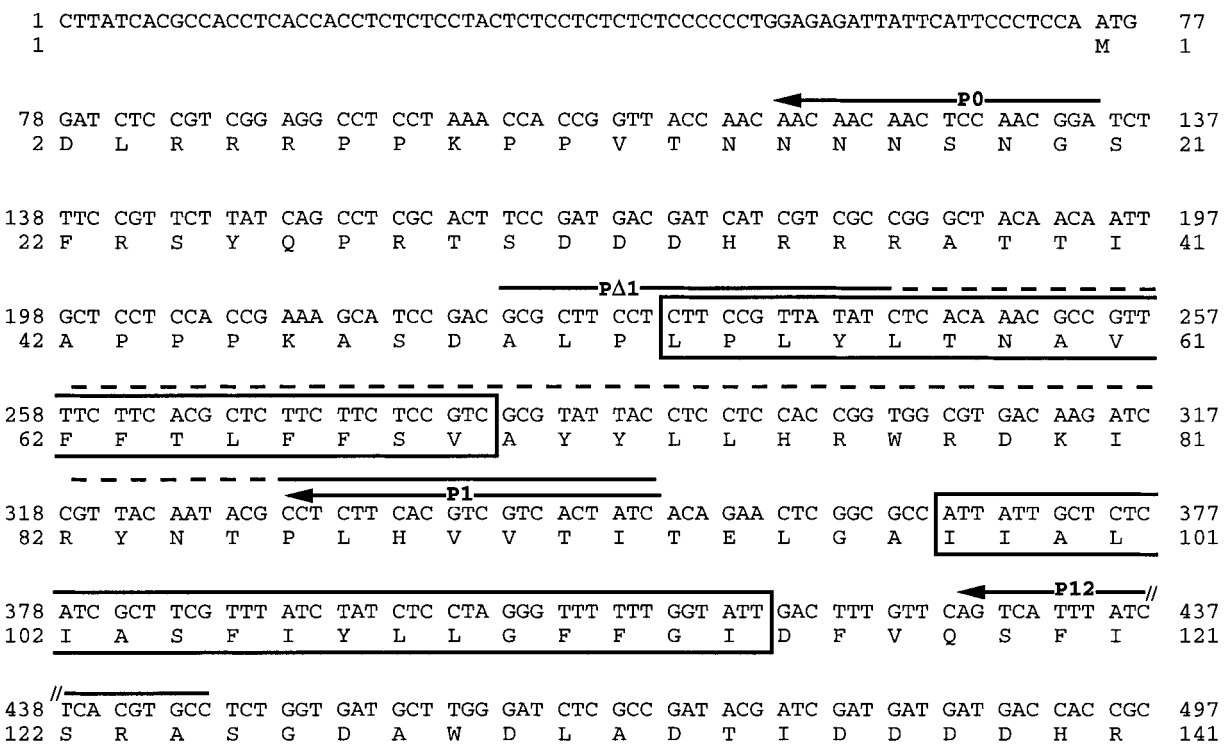

Fig. 2. The nucleotide and amino acid sequence of *Arabidopsis* HMG-CoA reductase. The 5′ nucleotide sequence of *Arabidopsis* HMG1 and its corresponding amino acid sequence is shown. The two putative membrane spanning domains, TMS1 and TMS2, encompass amino acids 53–69 and 98–114, respectively. To create three of the four 5′ end segments from HMG1, we employed the polymerase chain reaction. A common 5′ end oligonucleotide primer (P3) was used in conjunction with either P0, P1, or P12 to obtain the D0, D1, and D12 segments, respectively. Arrows labeled P0, P1, and P12 correspond to the regions of complementarity with the oligonucleotides P0, P1, and P12, respectively. To construct the D2 segment, we designed an oligonucleotide primer to delete the D1 transmembrane span using single-stranded DNA mutagenesis. The solid line above the PΔ1 oligonucleotide primer corresponds to the primer's nucleotide sequence, while the dashed line represents the portion of DNA deleted upon mutagenesis. For a more detailed description of the construction and subcloning of the D0, D1, D2 and D12 segments, see Materials and Methods.

Analysis of HMG1:SUC2:HIS4 Topology in a Coupled Translation-Translocation System

RNA transcribed in vitro from truncated pHMG1D$_n$ templates was used to program a wheat germ lysate and direct the synthesis of radiolabeled protein. As a first test of the fidelity of this heterologous system, translation in the cell-free extract was carried out in the presence of canine microsomes and the resulting protein products fractionated by centrifugation. By using the sequential procedure outlined in Figure 3A, followed by SDS PAGE, we can distinguish between soluble, lumenal, and intrinsic membrane proteins synthesized in the wheat germ lysate. To establish that the coupled in vitro translation/translocation system could correctly target proteins synthesized in the extract, full-length transcripts derived from the *HMG1* and cyclophilin (ERP87) cDNAs were translated in the presence of canine pancreatic microsomes. As shown in Figure 3B, Hmg1p was found exclusively in the membrane fraction (Fig. 3B, lane 4) as a polypeptide migrating with an apparent MW of 66 kilodaltons (calculated MW 66 kDa) but was absent in the supernatant fraction (Fig. 3B, lane 3). In contrast, the ERP87 translation product, a cytosolic form of *Arabidopsis* cyclophilin (calculated MW 22 kDa), was detected in the supernatant fraction (Fig. 3B, lane 1) as a protein migrating with an apparent MW of 21.5 kDa but was excluded from the membrane fraction (Fig. 3B, lane 2). These results are consistent with the predicted residence for these proteins [Learned and Fink, 1989; Enjuto et al., 1994; Lippuner et al., 1994] and demonstrate that the heterologous in vitro system can faithfully target and translocate *Arabidopsis* proteins to the proper subcellular location.

In order to determine if the transmembrane domain of *Arabidopsis* Hmg1p was necessary and sufficient for in vitro targeting and integra-

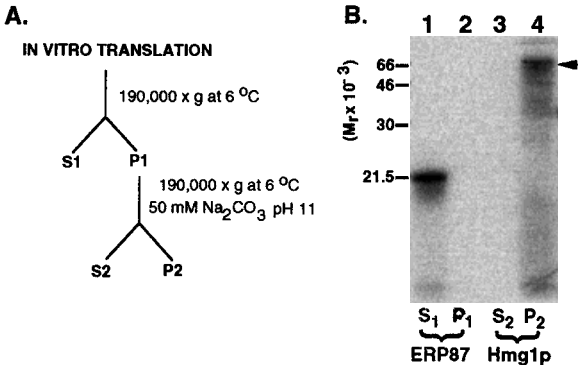

Fig. 3. Fractionation of in vitro translated proteins generated in the presence of canine microsomes using a wheat germ cell-free system. **A:** Flow diagram illustrating fractionation of in vitro translated proteins by two sequential centrifugation steps. Supernatant fractions are denoted by S and pellet fractions by P. **B:** In vitro synthesized mRNAs coding for *Arabidopsis* HMG1 (Hmg1p; calculated MW 66 kDa) and cyclophilin (ERP87: calculated MW 22 kDa) were translated in the presence of canine pancreatic microsomes. Aliquots containing equilvalent amounts of supernatant (*lanes 1,3*) and pellet (*lanes 2,4*) fractions were isolated as described in A. This autoradiogram is of a 12% acrylamide SDS-PAGE gel that was exposed to Kodak XAR-5 film for 15 h. Translation, fractionation, and SDS-PAGE were performed as described in Materials and Methods. Molecular mass standards (rainbow markers; Amersham) include bovine serum albumin (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (21.5 kDa).

tion in the microsomal membranes, we used RNA derived from $pHMG1D_n$ templates to direct protein synthesis in the translation/translocation reaction (Fig. 4A). Following fractionation, the in vitro translation products were analyzed by SDS-PAGE and autoradiography. As shown in Figure 4B, the proteins synthesized in these reactions exhibit the predicted mobilities and range in size from approximately 47.3 to 58.7 kDa depending on the portion of the Hmg1p transmembrane domain encoded in the fusion gene. Moreover, although the fusion proteins synthesized in these in vitro reactions share a common carboxyl terminus encoded by *SUC2:HIS4* sequences, they contain different portions of the Hmg1p amino terminal domain and display markedly different properties, as revealed by fractionation of the in vitro reactions carried out in the presence of microsomes. Although all the fusion proteins were found to separate into both soluble and membrane fractions following centrifugation (Fig. 4B), this distribution probably reflects, at least in part, the translocation capacity in the in vitro system. As shown in Figure 4C, both the HMG1D0 and HMG1D1 (calculated MW of 47.3 and 55.2 kDa, respectively) ^{35}S-labeled proteins appear predominantly in the S_2 supernatant (Fig. 4C, lanes 1, 3) following release of the lumenal contents of the microsomes by treatment with 50 mM Na_2CO_3, pH 11, while only a quantitatively minor amount of these proteins was associated with microsomal membranes (Fig. 4C, lanes 2, 4). However, based on this experiment, we cannot distinguish between nonspecific association of the HMG1D0 and HMG1D1 polypeptides with the microsomes and the entry of these proteins into the secretory pathway. In either case, the Hmg1p sequences present in the HMG1D0 and HMG1D1 proteins appear to lack key structural information required for proper targeting and localization in the endoplasmic reticulum membrane. Conversely, the HMG1D2 and HMG1D12 (calculated MW of 55.2 and 58.7 kDa, respectively) ^{35}S-labeled proteins were found to be enriched in the membrane fraction associated with the P_2 pellet (Fig. 4C, lanes 6, 8), with lesser amounts of the polypeptides capable of release into the S_2 supernatant fraction by alkali treatment (Fig. 4C, lanes 5, 7). Based on these results, the Hmg1p subdomains contained within the D2 and D12 proteins appear to be sufficient in vitro to both guide and to anchor these proteins in the microsomal membranes.

In Vivo Analysis of HMG1:SUC2:HIS4 Membrane Protein Topology in Yeast

As a complementary strategy for addressing the question of membrane topology of *Arabidopsis* Hmg1p, we took advantage of our ability to express functionally active *Arabidopsis* Hmg1p in *Saccharomyces cerevisiae* [Learned and Fink, 1989] and to engineer chimeric proteins in which sequences from the Hmg1p transmembrane domain are fused to a reporter enzyme whose activity is sensitive to topological space. When the histidinol dehydrogenase domain of the yeast *HIS4* gene is expressed as a fusion protein in the cytoplasm of *his4* mutant yeast, these cells are capable of growing on minimal media supplemented with histidinol (Fig. 5A). However, if histidinol dehydrogenase is targeted to the lumen of the endoplasmic reticulum by virtue of its fusion to a functional signal sequence, the conversion of histidinol into histidine will not take place, and the cells will be unable to grow on histidinol-supplemented media (Fig. 5A). The successful use of HIS4C fusion proteins to discriminate between the cyto-

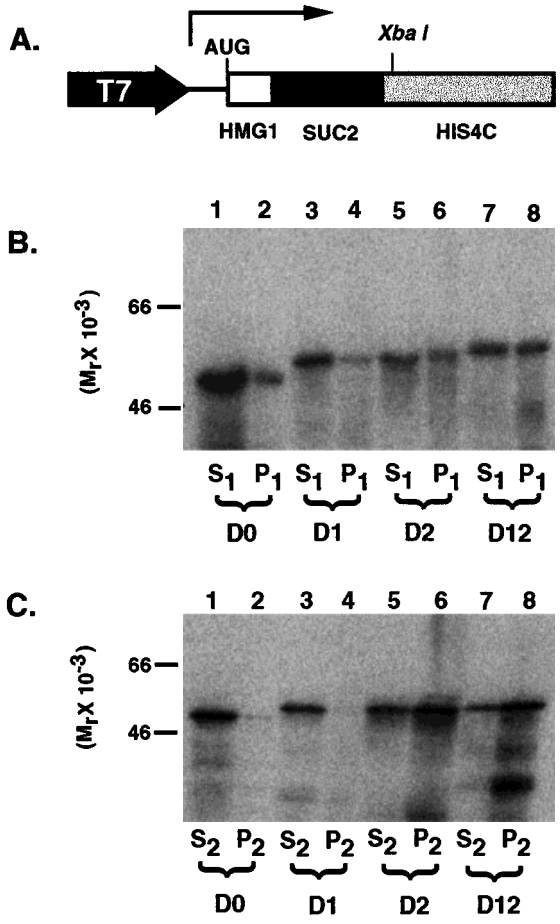

Fig. 4. Fractionation of in vitro translated HMG1:SUC2:HIS4 chimeric proteins. **A:** Schematic representation of the *HMG1:SUC2:HIS4* templates used for the in vitro transcription of mRNAs. Segments of the 5′ end of HMG1 (white) were fused to the invertase (SUC2, black) and histidinol dehydrogenase (HIS4, grey) reporter genes to create translational fusions. The resultant tripartite gene fusions were under the control of the T7 RNA polymerase promoter. Truncated mRNAs of D0, D1, D2, and D12 were made from DNA templates restriction digested with Xba 1, yielding transcripts which code for proteins of approximately 47.3, 55.2, 55.2, and 58.7 kDa, respectively. **B:** One-step fractionation of in vitro translated proteins coding for D0, D1, D2, and D12. Equivalent amounts of the S_1 (*lanes 1,3,5,7*) and P_1 fractions (*lanes 2,4,6,8*) were separated on a 12% acrylamide SDS-PAGE gel and exposed to X-ray film for 1 day. Molecular masses were estimated using rainbow color markers (Amersham). **C:** Two-step fractionation of in vitro translated proteins coding for D0, D1, D2, and D12. The S_2 (*lanes 1,3,5,7*) and P_2 fractions (*lanes 2,4,6,8*) were isolated as described previously. This is an autoradiogram of a 12% acrylamide SDS-PAGE gel exposed to X-ray film for 1 day. Following the initial centrifugation step, the P_1 pellet of each sample was treated with alkali as described in Materials and Methods.

plasmic and lumenal space in yeast [Deshaies and Schekman, 1987; Sengstag et al., 1990] prompted us to try a similar approach in analyzing the amino terminal domain of *Arabidopsis* Hmg1p.

By using expression of the chimeric HMG1:SUC2:HIS4 reporter proteins in *Saccharomyces cerevisiae*, we began to examine the characteristics of the membrane topology of these fusion proteins conferred by TMS1 and TMS2, both individually and in concert with one another. The pHMG1D_n constructs, represented schematically in Figure 5B, were transformed into the yeast strain JRY0510 (*his4C*) and tested for their ability to grow in the presence and absence of histidinol. As indicated in Figure 5C, transformed yeast cells expressing fusion proteins containing the D0, D2, and D12 subdomains from *Arabidopsis* Hmg1p were capable of growth on selective media supplemented with histidinol. In marked contrast, strains carrying the *HMG1D1:SUC2:HIS4* fusion gene grew poorly in the presence of histidinol (Fig. 5C). The growth of these strains on histidinol was completely dependent on the galactose-induced expression of the *HMG1:SUC2:HIS4* fusion genes; when glucose replaced galactose as the carbon source, none of the pHMG1D_n transformants could grow in the absence of histidine. However, when the media was supplemented with histidine, all of these yeast strains were capable of vigorous growth independent of carbon source (data not shown). As a positive control for growth on histidinol-containing media, we used a previously characterized gene fusion [Sengstag et al., 1990], designated pA, which contains a 5′ end region of yeast HMG1 fused to SUC2:HIS4C. The pA transformant has been shown to have its carboxyl-terminal reporter protein residing in the cytosol [Sengstag et al., 1990] and to complement the histidine auxotrophy in *his4* yeast. A second gene fusion, pAΔ7, contains a deletion of one of the yeast Hmg1p transmembrane spans, resulting in a reversal of its topological orientation so that the HIS4C protein now resides in the ER lumen. As shown in Figure 5C, yeast strains carrying this construct did not survive the histidinol selection. These data confirm the competence of this in vivo assay and our ability to use the histidine growth phenotype to distinguish between cytoplasmic and lumenal orientations of membrane proteins carrying the HIS4C catalytic domain.

Consequently, the ability of transformed yeast to grow in the presence of histidinol is consistent with a cytoplasmic residence for histidinol dehydrogenase when HIS4C was fused to the D0, D2, or D12 subdomains from *Arabidopsis* Hmg1p. In order to accommodate both this data and the results of the in vitro translation experi-

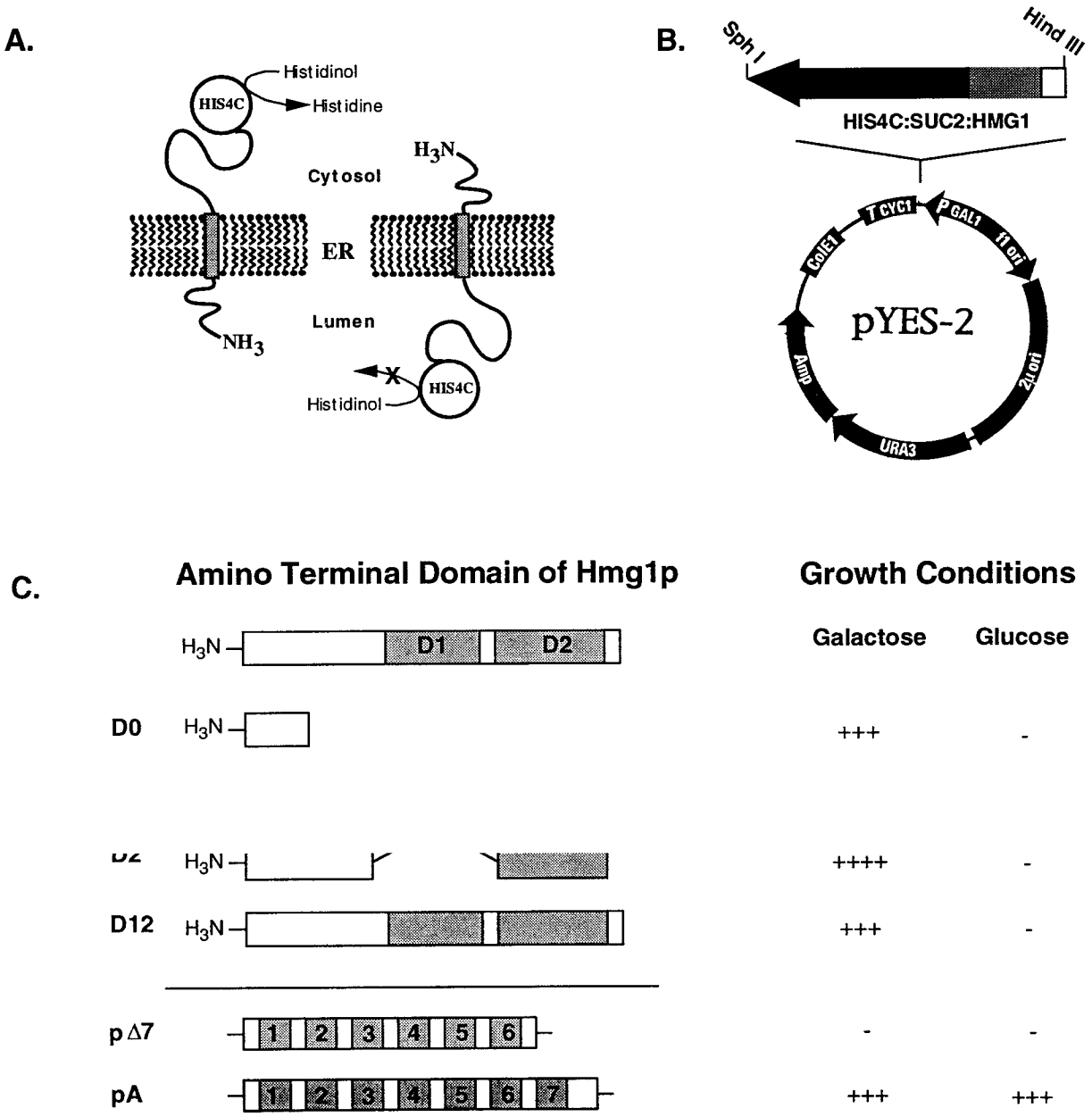

Fig. 5. In vivo assay for membrane topological space using HMG1:SUC2:HIS4 transformants in the yeast strain JRY0510. **A:** Schematic representation of the histidinol dehydrogenase (*HIS4*) in vivo assay for membrane protein topology. The ER membrane is shown as a linear phospholipid bilayer with the ER lumen and cytosol designated. Illustrating how HIS4 acts as a reporter of topological space, an integral membrane protein with one membrane-spanning domain (thatched rectangle) is shown fused to the HIS4 reporter gene. To differentiate between whether the HIS4 protein resides in the cytosol or ER lumen, the gene fusions of interest are transformed into a yeast strain with a *his* minus phenotype (i.e., JRY0510). If the HIS4 protein is present on the cytoplasmic side of the membrane, it can convert histidinol into histidine, thus allowing yeast growth. However, if the HIS4 protein is present in the ER lumen, transformants will exhibit a *his* minus phenotype due to glycosylation or possibly the inability of the histidinol substrate to cross the ER membrane. **B:** Construction of *HMG1* chimeric gene fusions used to analyze membrane protein topology. Segments consisting of the 5′ end of *HMG1* (white) were fused to *SUC2* (grey) and *HIS4* (black). The tripartite gene fusions were subcloned as Hind III/Sph I inserts into the yeast–*E. coli* shuttle vector pYES-2. Expression of the resulting gene fusions was under the control of the GAL1 promoter. **C:** Growth of HMG1:SUC2:HIS4 JRY0510 transformants in the presence or absence of histidinol. The left-hand portion of the panel schematically illustrates the putative Hmg1p membrane-spanning segments encoding each of the D0, D1, D2, and D12 constructs. The gene fusions pA and pAΔ7 are yeast *HMG1:SUC2:HIS4* gene fusions used as positive and negative controls, respectively. The + and − signs to the right of each construct denote whether or not a transformant grew. A detailed description of how yeast cells were grown and scored is presented in Materials and Methods.

ments presented in Figures 3 and 4, we speculate that the HMG1D0 fusion protein resides in the cytoplasm, whereas the fusion proteins containing the D2 and D12 subdomains are integral membrane proteins. In contrast, yeast expressing the HMG1D1 protein appeared incapable of converting histidinol to histidine, suggesting that the HIS4 reporter enzyme is localized in the lumen of the endoplasmic reticulum.

In Vitro and In Vivo Analysis of HMG1 Glycosylation

The results described in the previous sections provide direct evidence for the active role of TMS2 in anchoring Hmg1p to the endoplasmic reticulum and in determining membrane protein orientation and, furthermore, indicate that the isolated TMS1 cannot function as a transmembrane span within the HMG1D1 fusion protein. However, the data do not allow us to evaluate the structural role of TMS1 when it appears in concert with TMS2 in the context of the native transmembrane domain (TMD). As a means of confirming the orientation and topology determined by sequences in the TMD, we devised a series of experiments to distinguish between alternative topological models for *Arabidopsis* Hmg1p. Because we have established that the carboxyl terminal domain of the HMG1D12:SUC2:HIS4 fusion protein resides in the cytoplasm, we can discriminate between a transmembrane domain consisting of one or two membrane-spanning segments by determining the topological space occupied by the extreme amino terminal region of *Arabidopsis* Hmg1p.

To this end, we took advantage of the existence of two overlapping consensus glycosylation sites at amino acid positions +16 and +19 in the extreme amino terminus of the native Hmg1p and the recognition that such sequences could serve as sites for the covalent attachment of carbohydrates in the event they were translocated across the endomembrane and made accessible to the enzymatic machinery responsible for N-linked glycosylation in the lumen of the endoplasmic reticulum. In order to exploit this diagnostic biochemical event, we needed a small, simple reporter protein that could be monitored by immunochemical methods but which could be glycosylated only at positions +16/+19 in Hmg1p. Consequently, we constructed a second series of chimeric genes that consisted of an in-frame fusion between a mutant form of *uidA* from *E. coli* (in which the asparagine residue in the consensus N-glycosylation site at 358 was converted to serine [Farrell and Beachy, 1990] and the 5′ region of HMG1. Following transformation of the *pHMG1:uidA358* constructs into yeast, crude cellular extracts were prepared and the proteins subjected to immunoblot analysis. As shown in Figure 6B, β-glucuronidase fusion proteins containing the D0, D2, and D12 subdomains (Fig. 6B, lanes 1,3,4) all migrated as a single species that corresponded to their calculated molecular mass (~70.2, 78.1, and 81.6 kDa, respectively). In contrast, proteins displaying two slightly different relative mobilities could be detected with anti-β-glucuronidase antibodies in samples isolated from yeast expressing the HMG1D1:uidA358 protein (Fig. 6B, lane 2, black and white arrowheads; calculated MW 78.1 kDa). One possible explanation for the appearance of this second protein species is the core glycosylation of the amino terminus upon translocation of D1 across the ER membrane. As shown in Figure 6C, enzymatic deglycosylation of HMG1D1:uidA358 by treatment with endo-β-N-acetylglucosaminidase H (endo H) converts the doublet detected in the crude extract (Fig. 6C, lane 1) into a single discrete band (Fig. 6C, lane 2). This result is consistent with the idea that the HMG1D1:uidA358 protein is inappropriately translocated into the lumen of the ER, where it is subject to carbohydrate modification. In contrast, no changes in the electrophoretic mobility of the D12 protein were observed following treatment with endo H (Fig. 6C, lanes 3,4). These results provide evidence that in vivo the extreme amino terminus of the HMG1D1:uidA358 protein is exposed to the ER lumen, while the amino terminus of the complete Hmg1p TMD present in the HMG1D12 segment is not available for glycosylation and resides in the cytoplasm.

Finally, to confirm the localization of the amino terminus in the native *Arabidopsis* Hmg1p, we examined the glycosylation state of the polypeptide both in vitro and in transformed yeast cells. First, RNA transcribed in vitro was used to program a wheat germ lysate and direct the synthesis of Hmg1p. As shown in Figure 7A, the electrophoretic mobility of Hmg1p (black arrowhead) was identical whether the RNA was translated in vitro in the absence (Fig. 7A, lane 3) or presence (Fig. 7A,

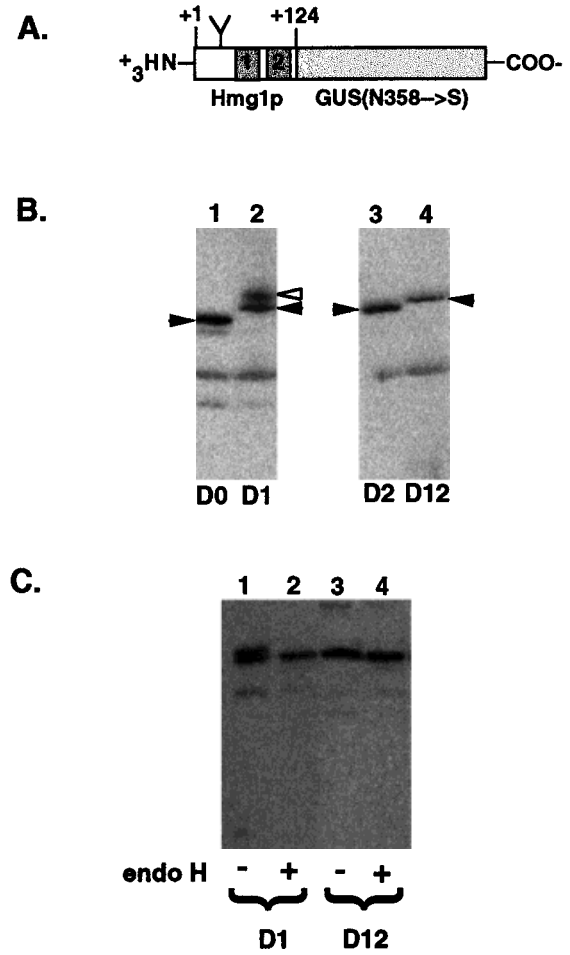

Fig. 6. Western blot analysis of yeast protein extracts containing HMG1:*uidA* (N358 → S) chimeric proteins. **A:** Schematic representation of the *HMG1:uidA* (N358 → S) gene fusions constructed for in vivo analysis in the yeast strain FY23. 5′ end segments of *HMG1,* identical to those incorporated in the *pHMG1D$_n$* series constructs, were fused to the *uidA* (N358 → S) reporter gene. The resultant fusions were placed under the control of the *GAL1* promoter in the yeast expression vector pYES-2. The translational start site is indicated by +1, and the end of the largest *HMG1* segment, D12, is denoted by +124 (most C-terminal amino acid encoded by HMG1). Consensus N-glycosylation sites at amino acid positions 16 and 19 are represented with a Y. **B:** Equivalent amounts of each yeast protein extract from *HMG1:uidA* (N358 → S) transformants were separated on an 8% acrylamide SDS-PAGE gel, blotted onto a nitrocellulose membrane, and probed with rabbit antiglucuronidase serum (ClonTech). Proteins were visualized using the ECL system and molecular masses estimated using rainbow color markers. *Lanes 1–4* contain the D0, D1, D2, and D12 fusion proteins, respectively. The calculated MWs for these fusion proteins are 70.2, 78.1, 78.1, and 81.6 kDa, respectively. Black arrowheads denote unglycosylated proteins and the white arrow a glycosylated protein species. **C:** Western blot of yeast protein extracts containing the D1 and D12 fusion proteins enzymatically deglycosylated with endo H. *Lanes 1,3.* Control samples which did not undergo endo H treatment. *Lanes 2, 4:* Samples treated with endo H as outlined in Materials and Methods. Electrophoresis and protein detection were as described in B.

lane 4) of canine pancreatic microsomes. As a positive control for glycosylation, yeast a-factor was also translated in vitro in the absence (Fig. 7A, lane 1) or presence (Fig. 7A, lane 2) of canine pancreatic microsomes. As expected, the unmodified form of a-factor (Fig. 7A, lane 1, grey arrowhead; calculated MW of 18.6 kDa) becomes glycosylated (Fig. 7A, lane 2, white arrowhead; apparent MW of 30.0 kDa) upon addition of microsomes, resulting in a dramatic shift in its electrophoretic mobility. These data suggest that Hmg1p does not serve as an efficient substrate for glycosylation in vitro despite its association with the membrane fraction and the modifying capability of the microsomal preparation.

Second, to examine the glycosylation state of Hmg1p in vivo, we constructed two epitope-tagged forms of *Arabidopsis* HMG1 (T154 and T579) and introduced them into yeast. Both of these HMG1 alleles complement *hmg1hmg2* mutations in HMG CoA reductase–deficient yeast and encode enzymatically competent proteins [Re and Learned, unpublished results]. Protein extracts were prepared from yeast expressing either HMG1T154 or HMG1T579, treated with endo H, and analyzed by immunoblotting. In Figure 7B, we show that treatment of the crude yeast extract with endo H did not alter the electrophoretic mobility of either the HMG1T154 (Fig. 7B, lane 4; calculated MW of 67.1 kDa) or HMG1T579 (Fig. 7B, lane 6; calculated MW of 67.1 kDa) polypeptides when compared directly to undigested samples (Fig. 7B, lanes 3,5). These results indicate that neither recombinant Hmg1p was glycosylated in an N-linked high-mannose form. As an independent test of carbohydrate modification, we also subjected these yeast extracts to chemical deglycosylation by treatment with TFMS (Fig. 7C). Despite the effective removal of carbohydrate modifications by this treatment, as monitored by periodic acid–Schiff base staining following electrophoresis (data not shown), no change in mobility was observed for either epitope-tagged Hmg1p (Fig. 7C, lanes 3–6) after deglycosylation. Thus, the lack of glycosylation of T154 and T579 Hmg1p indicates the amino termini of these proteins are inaccessible for carbohydrate modification and reside in the cytoplasm.

DISCUSSION

In this article, we examine the topological features of the membrane-anchoring domain of

Arabidopsis Hmg1p, one of the key enzymes in the isoprenoid biosynthetic pathway. By examining a series of recombinant derivatives of *Arabidopsis* Hmg1p both in vitro and in vivo, we identified key structural elements within the TMD that play functional roles in endomembrane targeting and in determining the topological orientation of HMG CoA reductase in the endoplasmic reticulum. Based on results of both genetic and biochemical analyses, we propose a model for Hmg1p membrane protein topology that provides a framework for the functional dissection of the transmembrane domain and its contributions to aspects of *Hmg1p* targeting and regulation in *Arabidopsis*.

Amino terminal derivatives of the putative transmembrane domain of *Arabidopsis* Hmg1p were fused to reporter proteins as a powerful and systematic approach to analyze membrane protein topology and targeting. By monitoring the ability of this collection of *HMG1:SUC2:HIS4C* gene fusions to direct the synthesis of chimeric proteins whose orientation and targeting is determined by the Hmg1p TMD and whose enzymatic activity is sensitive to compartmental localization, we have identified regions of the amino terminal domain that define the topological orientation of this membrane protein.

Sequence analysis of the *Arabidopsis HMG1* gene revealed a surprisingly simple architecture for the transmembrane domain, consisting of the amino terminal one-fourth of the protein which contains two short hydrophobic stretches of amino acids, designated TMS1 and TMS2 (Fig. 2). Interestingly, only one of these two segments, TMS2, is predicted to adopt the amphipathic α-helical structure that appears as a common spanning motif in transmembrane proteins [Klein et al., 1985; Popot and Engelman, 1990]. In this report, we have examined the relative contributions of TMS1 and TMS2 to Hmg1p targeting and topology and show experi-

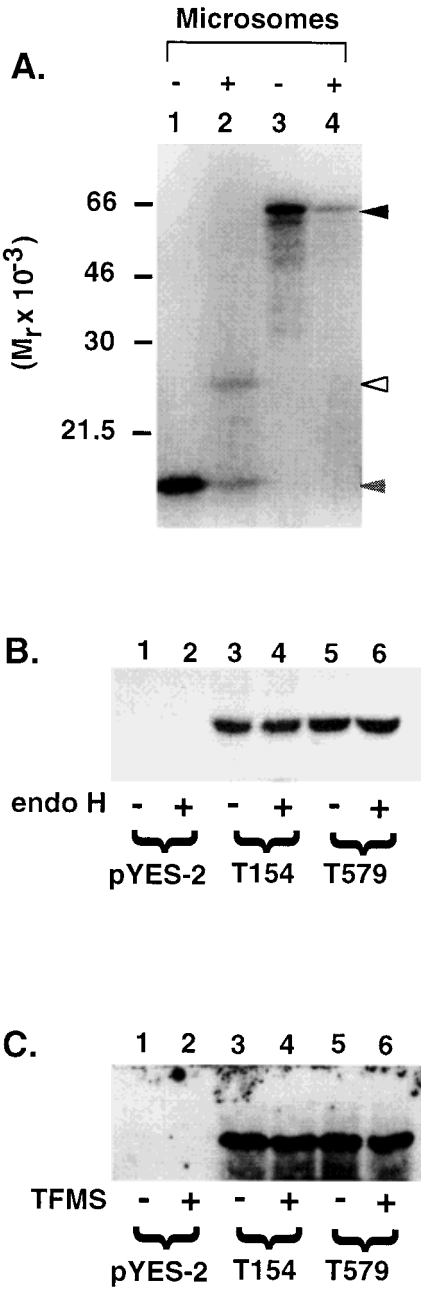

Fig. 7. In vitro and in vivo analysis of Arabidopsis Hmg1p glycosylation. **A:** In vitro translation reactions of HMG1 and yeast a-factor mRNA transcripts in the absence (*lanes 1,3*) and presence (*lanes 2,4*) of canine pancreatic microsomes. The grey arrowhead denotes unglycosylated a-factor (calculated MW 18.6 kDa), the white arrowhead glycosylated a-factor (apparent MW 30.0 kDa), and the black arrowhead Hmg1p (calculated MW 66 kDa). This is an autoradiogram of a 12% acrylamide SDS-PAGE gel exposed to X-ray film for ~7 h. Lanes containing samples translated in the presence of canine pancreatic microsomes are denoted with a + and those in the absence with a −. Translation, SDS-PAGE, and fluorography were performed as described in Materials and Methods. **B:** Western blot analysis of endo H–treated yeast protein extracts containing T154 or T579 epitope-tagged Hmg1p (calculated MW 67.1 kDa). Equivalent amounts of endo H–treated (*lanes 2,4,6*) and untreated (*lanes 1,3,5*) samples were separated on an 8% acrylamide SDS-PAGE gel, blotted onto a nitrocellulose membrane, and probed with a mouse anti-12CA5 serum (Babco, Berkeley, CA). Protein bands were visualized using an ECL detection kit. **C:** Yeast samples containing T154 and T579 protein extracts were chemically deglycosylated using trifluoromethane sulfonic acid (TFMS). Equivalent aliquots of TFMS-treated (*lanes 2,4,6*) and untreated (*lanes 1,3,5*) samples were electrophoretically separated, transferred to nitrocellulose, and detected as described above.

mentally that, as predicted, these two segements are neither structurally nor functionally equivalent.

The chimeric reporter proteins that include TMS2, such as the HMG1D2 and HMG1D12 fusion proteins, were shown to contain the necessary structural information for proper targeting and insertion into the endoplasmic reticulum. Results from a battery of both in vivo and in vitro experiments are consistent with a membrane orientation that positions both the carboxyl-terminal domain, containing the catalytic activity, and the extreme amino terminal segment of the TMD in the cytoplasm. Surprisingly, removal of TMS1 along with most of the lumenal loop segment during construction of the HMG1D2 subdomain (amino acids 57–85) appeared to have little effect on targeting or topology of the reporter fusions containing HMG1D2. Not only is the catalytic domain of HIS4 oriented towards the cytoplasm in the HMG1D2 fusion protein (Fig. 5), but the amino terminus shows no evidence of being modified by N-linked high mannose core glycosylation (Fig. 6). Consequently, HMG1D2 appears to adopt the same membrane protein topology as the full-length TMD, including cytoplasmic localization of amino acid residues 16 and 19 at the extreme amino terminus. The biochemical and genetic analyses of these deletion constructs strongly implicate TMS2 as a key determinant for Hmg1p integration and orientation in the ER membrane, even in the absence of TMS1.

Furthermore, native Hmg1p, modified only by insertion of an epitope tag, appears to adopt a similar topological structure in the endoplasmic reticulum. These results, therefore, are consistent with the integration of Hmg1p in the ER membrane in an N-cis orientation and the amino terminal domain passing through the membrane twice and establishing cytoplasmic residence for both the amino and carboxyl-terminal regions of Hmg1p. It seems likely that TMS1 and TMS2, as defined by the highly hydrophobic peptide segments, serve as the functional elements that traverse the ER membrane, connected by a short (approximately 25 amino acids) hydrophilic loop that is sequestered in the ER lumen (Fig. 8).

In contrast, reporter proteins fused to the HMG1D0 or HMG1D1 subdomains lack key structural features resulting in mislocalization and, in the case of HMG1D1 fusion proteins,

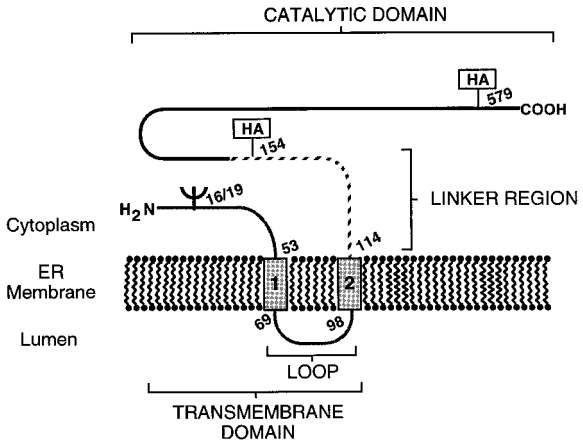

Fig. 8. Proposed model for the membrane protein topology of *Arabidopsis* Hmg1p. The primary amino acid sequence of Hmg1p is represented as follows: a solid grey line denotes the amino terminal membrane anchoring domain; the thatched line represents the linker domain and the solid black line the C-terminal catalytic domain of the enzyme. Membrane-spanning segments TMS1 and TMS2 are represented as grey rectangles with the amino acid residues which define each segment appearing next to each rectangle. The introduced epitope tags in the T154 and T579 recombinant Hmg1ps are shown as white rectangles and have intersecting lines denoting their approximate position of insertion within Hmg1p. Ψ represents the cryptic consensus N-linked glycosylation sites at amino acid positions 16 and 19. The cytoplasm and ER lumen are indicated to clearly illustrate both the extreme amino and carboxyl-termini reside in the cytoplasm. The model as shown is not drawn to scale.

translocation across the endoplasmic reticulum into the lumen. Strikingly, the amino terminal N-linked glycosylation site in the HMG1D1:uidA protein was the only polypeptide among all the constructs we tested that contained carbohydate modifications that are diagnostic of lumenal residence. Thus, the HMG1D1 subdomain, containing only TMS1, is neither retained in the ER, nor does it appear capable of spanning the membrane independently of TMS2. Thus, the HMG1D1:SUC2:HIS4 or HMG1D1:uidA proteins appear to lack the key internal insertional and stop transfer sequences that normally operate to target and orient Hmg1p in the ER membrane. This observation is consistent with reports that have implicated relative position of structural elements within a protein as an important determinant of membrane spanning capability [Peter and Spiess, 1988]. Therefore, the elimination of amino acids 93–122 from the transmembrane domain, including TMS2, results in aberrant membrane protein topology, and the deletion defines a key

functional segment within the Hmg1p transmembrane domain.

The model we have proposed for two transmembrane spans in *Arabidopsis* Hmg1p represents a significantly less complex topology than either the proposed eight transmembrane model for hamster HMG CoA reductase [Olender and Simoni, 1992; Roteilman et al., 1992] or the seven transmembrane span model for the yeast enzyme [Sengstag et al., 1990]. Nonetheless, the integral association of HMG CoA reductase with the endomembrane system is a feature of the enzyme that appears to be strikingly conserved among eukaryotes, despite the ability of the catalytic domain to function normally in the cytoplasm even in the absence of the transmembrane domain [Gil et al., 1985]. In both mammals and fungi, the amino terminal membrane domain has been shown to play an intrinsic role in determining the subcellular localization, topological orientation, and regulated turnover of the enzyme. Furthermore, in *Saccharomyces cerevisiae*, the enzymes encoded by both the HMG1 and HMG2 loci are targeted to the endoplasmic reticulum, but the evidence indicates localization within different subcompartments of the endomembrane system. In particular, the Hmg1p isoform appears to reside in the perinuclear region of the ER membrane, while Hmg2p is confined to the peripheral endoplasmic reticulum [Wright et al., 1988]. When the homologous membrane domains of the two isozymes were exchanged, the two proteins were found to have equilvalent orientations [Sengstag et al., 1990], implicating regions within the TMD as important elements for sorting the two proteins in the endomembrane. Furthermore, the noncatalytic transmembrane domain from both mammalian and fungal HMG CoA reductase enzymes has been shown to regulate enzyme turnover in response to changes in MVA availability when fused to reporter proteins [Skalnik et al., 1988; Chun and Simoni, 1992; Hampton and Rine, 1994]. Interestingly, even though the two yeast isozymes share topologically equivalent membrane domains, structural cues present in these regions of the protein may target these two isozymes for degradation by two pathways in response to distinct metabolic signals [Hampton and Rine, 1994]. The multiple roles that have been assigned to the membrane-bound domain in the yeast and mammalian enzymes raises interesting questions with regards to possible structure/function relationships. If the transmembrane domain of the plant enzyme is responsible for similar functions, the same array of structural information must reside within a much simpler amino terminal architecture. Consequently, a systematic dissection of these functional domains may be considerably more accessible in the plant enzyme than in the animal counterparts.

In fact, this study represents the first step in elucidating some of the roles played by the transmembrane domain of HMG CoA reductase in higher plants. By taking advantage of the rapid and sophisticated analyses that can be performed in the heterologous expression systems, we have identified subdomains within the TMD of Hmg1p that determine aspects of membrane protein targeting and orientation. Moreover, we have established a model for Hmg1p topology that makes specific predictions regarding the compartmentation of different portions of the protein. In particular, the amino terminal domain passes through the endoplasmic reticulum twice, establishing cytoplasmic residence for both the amino and carboxyl-terminal regions of Hmg1p. These results provide an important experimental foundation for examining the functioning of the TMD in plants and for addressing some of the key nagging questions regarding the organization and localization of isoprenoid biosynthesis in higher plants. By taking advantage of the constructs we have already characterized in vitro and in yeast and introducing them into plants for analysis, we can compare the properties of these chimeric proteins in the authentic in vivo environment and continue with the analysis of the transmembrane domain of *Arabidopsis* Hmg1p. We anticipate that these ongoing studies will continue to provide insight into both the mechanisms of membrane protein sorting as well as important new information regarding the molecular mechanisms that regulate the mevalonate pathway in higher plants.

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