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Amino Acid Substitutions in Membrane-Spanning Domains of Hol1, a Member of the Major Facilitator Superfamily of Transporters, Confer Nonselective Cation Uptake in *Saccharomyces cerevisiae*

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Selection for the ability of *Saccharomyces cerevisiae* cells to take up histidinol, the biosynthetic precursor to histidine, results in dominant mutations at *HOL1*. The DNA sequence of *HOL1* was determined, and it predicts a 65-kDa protein related to the major facilitator family (drug resistance subfamily) of putative transport proteins. Two classes of mutations were obtained: (i) those that altered the coding region of *HOL1*, conferring the ability to take up histidinol; and (ii) *cis*-acting mutations (selected in a mutant *HOL1-1* background) that increased expression of the Hol1 protein. The ability to transport histidinol and other cations was conferred by single amino acid substitutions at any of three sites located within putative membrane-spanning domains of the transporter. These mutations resulted in the conversion of a small hydrophobic amino acid codon to a phenylalanine codon. Selection for spontaneous mutations that increase histidinol uptake by such *HOL1* mutants resulted in mutations that abolish the putative start codon of a six-codon open reading frame located approximately 171 nucleotides downstream of the transcription initiation site and 213 nucleotides upstream of the coding region of *HOL1*. This single small upstream open reading frame (uORF) confers translational repression upon *HOL1*; genetic disruption of the putative start codon of the uORF results in a 5- to 10-fold increase in steady-state amounts of Hol1 protein without significantly affecting the level of *HOL1* mRNA expression.

Histidine biosynthesis in *Saccharomyces cerevisiae* is a multistep pathway in which the final step is the conversion of histidinol to histidine by histidinol dehydrogenase. Although histidine auxotrophs defective in upstream steps of this pathway contain histidinol dehydrogenase, they are incapable of utilizing supplemented histidinol to satisfy their auxotrophic requirement because of their inability to take up this compound. We previously showed that selection for the ability of *S. cerevisiae* cells to take up histidinol led to the isolation of dominant mutations at the *HOL1* locus (12). Although similar mutations in *Salmonella* species mapped to the histidine permease (26), the *HOL1* locus is genetically distinct from *HIP1*, *GAP1*, and *CAN1*, loci which encode the permeases that mediate histidine transport in *S. cerevisiae* (1, 18, 34).

To further characterize the role of *HOL1* in transport we have undertaken a combined molecular and genetic analysis. *HOL1* was found to encode a polytopic membrane protein related to the Car1/Cyhr family of putative transporters. In *S. cerevisiae* the most closely related proteins are those that belong to Multidrug Resistance Protein Family 1 (25). We show that dominant gain-of-function mutations at *HOL1* consist of single amino acid substitutions in putative membrane-spanning domains which confer indiscriminate uptake of both monovalent and divalent cations. This is the first evidence of transport by this class of transporter in *S. cerevisiae*, since none of the physiological substrates of Multidrug Resistance Protein Family 1 is yet known. Second-site mutations that increase *HOL1-1* transport-related phenotypes abolish a single small upstream open reading frame (uORF), resulting in increased steady-state levels of Hol1 protein.

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MATERIALS AND METHODS

Strains and media used. Yeast and bacteria used in this study are listed in Table 1. Growth media and routine genetic techniques were as described by Sherman et al. (31). YNB AA-His medium lacking histidine was supplemented with different concentrations of histidinol as required. YNB AA-Ura is a medium lacking uracil. LS (low salt) medium contains essentially no potassium and was prepared as previously described (13). KCl was added as needed to obtain the required concentration of potassium. NaOH and HCl were used to adjust the final pH of yeast extract-peptone-dextrose (YPD) and YNB media. NH_4OH was used to adjust the final pH of LS media. Solid LiCl was added to media prior to sterilization. Yeast transformation was performed by treatment of cells with lithium acetate (17) or by electroporation (4).

Plasmid constructions. Plasmid pMW18, carrying the wild-type *HOL1* allele, was constructed by subcloning the 2.8-kb *Hin*dIII-*Cla*I fragment from pRG167 (12) into the corresponding sites of pRS316 (32). Plasmid pMW13, containing the *HOL1-1* allele, was generated by subcloning the 2.8-kb *Hin*dIII-*Cla*I fragment from pRG174 (12) into plasmid pRG415-2, a *URA3*-containing centromeric plasmid containing a pUC19-derived polycloning site (11a). The 2.8-kb *Hin*dIII-*Cla*I fragment from pRG148 (12) carrying the *HOL1-1*,*-101* double mutant allele was subcloned into pGem7Z (Promega) to produce plasmid pMW1. Plasmid pMW43, capable of generating the *hol1*Δ*2* null allele by gamma deletion (32), was constructed by subcloning the *Hin*dIII-*Eco*RI fragment from pMW1 and the *Cla*I-*Xho*I fragment from pRG131 (12) into the polycloning region of pRS304 (12). Plasmid pMW62 containing the single *HOL1-101* mutation was constructed by replacing the 1,322-bp *Pvu*II-*Cla*I fragment of pRG148 (12) with the *Pvu*II-*Cla*I fragment of pMW18. Plasmid pRG165-1 is an integrative plasmid (YIp5) that contains a 1.1-kb *Hin*dIII-*Eco*RI fragment encompassing the 5′ portion of *HOL1* including the *HOL1-101* mutation (12).

Cloning of *HOL1* and *HOL1-1* alleles. Plasmid pRG158, containing a 400-bp

TABLE 1. *S. cerevisiae* and *E. coli* Strains[a]

Strain	Genotype
E. coli	
DH5α	F$^-$ ϕ80d*lac*ZΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*) U169 λ$^-$
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_k^- m_k^-) *relA1 supE44* Δ(*lac-proAB*) [F′ *tra*Δ*36 proAB laq*I^qZΔM15]
S. cerevisiae	
MW15	*MAT*α *ura3-52 his 3*Δ*200 his4-15 trk1*Δ *trk2*Δ*::HIS3 hol1*Δ*1*
R757	*MAT*α *ura3-52 lys9 his4-15*
R657	*MAT***a** *ura3-52 his4-15 HOL1-1*
R704	*MAT***a** *ura3-52 his4-15 HOL1-1,-101*
R1714	*MAT***a** *ura3-52 trp1*Δ*1 his4-15*
R1843	*MAT*α *ura3-52 trp1*Δ*1 his4-15*
MW71	*MAT***a** *ura3-52 trp1*Δ*1 his4-15 hol1*Δ*2::TRP1*
MW72	*MAT*α *ura3-52 trp1*Δ*1 his4-15 hol1*Δ*2::TRP1*
MW118	MW15/pHOL1 (pMW18)
MW121	MW15/pHOL1-1,-101 (pMW11)
MW122	MW15/pHOL1-1 (pMW13)
BJ2168	*MAT***a** *ura3-52 trp1*Δ*1 leu2 prc1-401 prb1-1122 pep4-3 gal2*
MW137	BJ2168/pCEN-HOL1-1,-101,-6HA (pMW119)
MW150	BJ2168/p2μ-HOL1-1,-101,-6HA (pMW117)
MW147	BJ2168/pRS316
LH75	BJ2168/pCEN-HOL1-1,-6HA (pLH18)

[a] Strain BJ2168 was a gift from A. Atkins and M. Culbertson. All other *S. cerevisiae* strains were generated in our laboratory.

*Cla*I-*Xho*I fragment located downstream of the cloned *HOL1-1,-101* allele (12), was integrated into the genomes of wild-type *HOL1* (strain R757) and mutant *HOL1-1* (strain R657) cells by transformation. Genomic DNA from these transformants was prepared, digested with *Hin*dIII, ligated to recircularize plasmids, and transformed into *Escherichia coli*, yielding plasmids pRG167 (*HOL1*) and pRG168 (*HOL1-1*).

Isolation and characterization of new *HOL1* mutants. Two strategies were used to isolate spontaneous mutants that conferred uptake of histidinol. In the first, single colonies of a wild-type *HOL1* strain (R757; Table 1) were allowed to develop on permissive medium (YPD) and then replica plated to YNB AA-His–2.5 mM histidinol. To ensure independence of the mutations, only a single Hol$^+$ mutant was picked from a replica-plated colony. Since all of the Hol$^+$ mutants were dominant, plasmid integration was used to determine which of them harbored alleles of the *HOL1* locus. Plasmid pRG165-1 is an integrative plasmid that contains the 5′ portion of *HOL1* including the *HOL1-101* mutation. For those Hol$^+$ mutants that harbored a mutation at the *HOL1* locus, integration of pRG165-1 generated a *HOL1-X,-101* double mutant that was easily identified by its ability to grow on a very low concentration (0.25 mM) of histidinol. Seven independent Hol$^+$ mutants appeared to harbor mutations at *HOL1* on the basis of this preliminary assessment. Plasmids harboring the entire *HOL1* region were recovered from genomic DNA prepared from pRG165-1 integrants by digestion with *Xho*I followed by circularization with T4 DNA ligase and transformation into *E. coli*. The DNA sequence of the entire *HOL1* region for each of the mutant alleles was determined.

In a second selection, plasmid-borne mutant *HOL1* alleles were isolated from a *hol1*Δ*2* strain (MW71) that harbored the *HOL1-101*-expressing centromeric plasmid pMW62. Independence of mutant alleles was again ensured by picking only a single mutant that arose from a wild-type colony that was replica plated to YNB-His–2.5 mM histidinol medium. Mutant *HOL1* alleles isolated by this selection were recovered directly by transformation of genomic DNA preparations into *E. coli*. The presence of a mutant *HOL1* allele on the plasmids recovered by either of these selection strategies was confirmed by their ability to confer a Hol$^+$ phenotype to a *hol1*Δ*2* recipient (strain MW71) upon transformation.

DNA sequence analysis. The DNA sequence of the 2.8-kb fragment containing the *HOL1-1,-101* double mutation was determined from a set of overlapping deletions generated by exonuclease III (Pharmacia) digestion of plasmid pMW1. A set of 15 oligonucleotide primers based upon the sequence of the *HOL1-1,-101*-containing fragment was used to determine the DNA sequences of wild-type, *HOL1-1*, and other mutant *HOL1* alleles. DNA sequencing reactions were performed by the dideoxy-chain termination method (28).

Construction of epitope-tagged Hol1p. To generate a *HOL1-1,-101* allele containing multiple copies of the hemagglutinin (HA) epitope (21) a *Not*I site was inserted by oligonucleotide-directed mutagenesis immediately following the last codon in the *HOL1* open reading frame. A 118-bp fragment encoding a triple HA epitope (gift from M. Rose) was subcloned into this site. One clone contained a fortuitous insertion of two correctly oriented copies of this fragment resulting in the addition of six copies of the epitope. This hextuply-tagged *HOL1-1,-101* allele was recovered and subcloned into centromeric and high-copy plasmids, creating pRS316-*HOL1-1,-101,-6HA* (pMW119) and pRS426-*HOL1-1,-101,-6HA* (pMW117). Plasmid pLH18, expressing an epitope-tagged *HOL1-1* allele, was constructed by replacing the 1.3-kb *Eco*RI fragment in pMW119 (*HOL1-1,-101,-6HA*) with that from pMW18 (*HOL1*).

Immunoblot analysis. *S. cerevisiae* protein was prepared as described previously (3) and HA-tagged proteins were detected by immunoblotting with monoclonal antibody 12CA5 (Berkeley Antibody Company). To control for protein loading, Sec61 (33) was detected with a polyclonal antibody (gift from R. Schekman).

Nucleic acid hybridizations. Nucleic acid hybridization analysis was performed as described by Maniatis et al. (23). Hybridization probes were prepared by labeling with [α-^{32}P]dCTP using the random oligonucleotide priming method (10). *S. cerevisiae* RNA was prepared by disruption of cells in the presence of glass beads (3) and polyadenylated [poly(A)$^+$] RNA was enriched by retention on oligo(dT) cellulose columns.

[$^{45}Ca^{2+}$] uptake assays. [$^{45}Ca^{2+}$] was measured by incubating cells in the presence of $CaCl_2$ at concentrations from 25 μM to 15 mM with a specific radioactivity of 0.5 μCi/μM of Ca^{2+}. Logarithmically growing cells were harvested by centrifugation and washed twice with 50 mM Tris-succinate (pH 5.9) followed by aeration at 30°C for 4 to 6 h in the same buffer, then washed twice and kept on ice. One milliliter of assay buffer (50 mM Tris-succinate [pH 5.9]) with the appropriate $CaCl_2$ concentration was prewarmed to 30°C. An aliquot of 10^8 cells was added to begin the assay. Samples of 10^7 cells were removed at various intervals, collected on 0.45-μM-pore-size filters, and washed three times with 5 ml of ice-cold buffer. The filters were dried and the amount of radioactivity contained on each filter was determined by liquid scintillation in a Beckman LS 7000 counter. The values of triplicate assays at each $CaCl_2$ concentration were averaged. Data are expressed as the initial rate of [$^{45}Ca^{2+}$] uptake over the initial linear portion of uptake (picomoles per 10^7 cells per minute) versus the concentration of $CaCl_2$ in the assay buffer.

For competition assays, the rate of [$^{45}Ca^{2+}$] uptake in the presence of a fixed concentration of $CaCl_2$ (0.5 mM) with 0, 0.5, 2.0, or 5.0 mM $CaCl_2$, $MgCl_2$, or $MnCl_2$ as competitor was determined. The amount of radioactivity contained in the cells after 0, 0.5, 2, and 5 min was measured. Results are expressed as percent transport by division of the initial transport rate in the presence of competitor by the rate in absence of competitor. The calculated values are plotted with respect to the concentration of nonradioactive competitor.

Primer extension analysis. Primer extension analysis was performed to determine the 5′ end of *HOL1* mRNA from congenic *HOL1-1* (R657) and *HOL1-1,-101* (R704) cells (Table 1) essentially as described by Ausubel et al. (3). The reaction products were separated on a DNA sequencing gel along with the products of a sequencing reaction using the same oligonucleotide primer. The 5′-most nucleotide of oligonucleotide HOL1 PE (5′-GGAAGAATAGATGTT AAATGTGCCAGGAATATAGTCCG-3′) corresponds to position +66 with respect to the *HOL1* coding sequence and was used to approximate the transcriptional start site. The 5′-most nucleotide of oligonucleotide HOL1 PE3 (5′-TTTCGAGAGGAGGTTCAGTAAATATTACTT CAGAGACTG-3′) corresponds to position −274 with respect to the *HOL1* coding sequence and was used to more accurately map the *HOL1* transcriptional start site.

Nucleotide accession sequence number. The accession number for the *HOL1-1,-101* sequence is L42348.

RESULTS

***HOL1* mutants suppress the K$^+$ requirement of *trk1*Δ *trk2*Δ cells.** We previously showed that dominant mutations at *HOL1* conferred increased uptake of histidinol and sodium (12). To determine if mutations in *HOL1* might also confer increased K$^+$ transport, the effects of *hol1*Δ*2*, *HOL1-1*, and *HOL1-1,-101* alleles in cells that contained disruptions of the K$^+$ transporter genes *TRK1* and *TRK2* were tested. Deletion of *TRK1* and *TRK2* severely impairs K$^+$ uptake, resulting in an approximately 1,000-fold increase in the amount of potassium in the medium required for growth: wild-type cells can grow on media containing 0.1 mM potassium, while *trk1*Δ *trk2*Δ cells require approximately 30 to 50 mM potassium to support growth (20).

To determine first if the wild-type Hol1 protein is involved in potassium transport, the potassium requirements of *trk1*Δ *trk2*Δ *hol1*Δ*2* (MW15) cells expressing the wild-type *HOL1* allele from plasmid pMW18 were compared with cells of the same strain that harbored vector sequences. No significant differences in the growth rates of these on K$^+$-limiting media

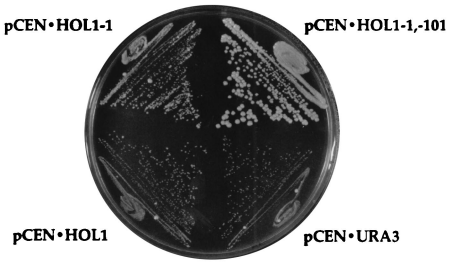

FIG. 1. Effect of mutations in *HOL1* on the phenotype of *trk1*Δ *trk2*Δ cells. *trk1*Δ *trk2*Δ cells bearing vector sequences (pRS316) or the wild-type (*HOL1*; pMW18), single mutant (*HOL1-1*; pMW13), or double mutant (*HOL1-1,-101*; pMW11) *HOL1* alleles carried on centromeric plasmids were streaked out to allow development of single colonies on YNB AA-Ura medium containing 7 mM potassium. Colonies were photographed after growth at 30°C for 4 days. See Table 1 for complete genotypes.

were observed (data not shown). In contrast, when plasmids expressing the mutant *HOL1-1* or *HOL1-1,-101* alleles were introduced into the *trk1*Δ *trk2*Δ *hol1*Δ*2* recipient, they partially suppressed the *trk1*Δ *trk2*Δ phenotype (Fig. 1). Thus, the mutant Hol1 transporter appears able to transport K^+.

Divalent ion uptake by mutant Hol1p. High concentrations of Ca^{2+} added to the growth medium inhibited the ability of *HOL1-1* and *HOL1-1,-101* cells to utilize histidinol and also suppressed their hypersensitivity to Li^+ (unpublished results). These results suggested that Ca^{2+} might also be transported by the wild-type or mutant Hol1 proteins. Cells harboring the *hol1*Δ*2* mutation or overexpressing the wild-type *HOL1* allele from a multicopy plasmid exhibited essentially indistinguishable rates of Ca^{2+} uptake, suggesting that the wild-type Hol1 protein does not normally transport Ca^{2+} (Fig. 2A). However, the rate of [$^{45}Ca^{2+}$] uptake by *hol1*Δ*2* cells expressing the *HOL1-1,-101* allele from a single-copy plasmid was significantly increased compared to *hol1*Δ*2* cells harboring the vector alone (Fig. 2A). In separate experiments Mg^{2+} and Mn^{2+} were shown to be capable of inhibiting [$^{45}Ca^{2+}$] uptake by *HOL1-1,-101* cells in a manner as efficient as isotopic Ca^{2+} (Fig. 2B). These results suggested that divalent cations in addition to Ca^{2+} are either transported by *HOL1* mutants or can block the uptake of Ca^{2+}.

***HOL1* encodes a predicted membrane protein.** The DNA sequence of the 2.8-kb fragment that contains the *HOL1-1,-101* allele was determined (accession no. L42348) and found to contain a single large open reading frame of 1,758 nucleotides which predicts a 586-amino-acid protein with a molecular mass of 65.2 kDa. Hydrophobicity analysis (9, 16, 22) of the inferred amino acid sequence of the *HOL1* open reading frame identified 12 putative membrane-spanning segments (Fig. 3), suggesting that Hol1 is an integral membrane protein. The putative architecture of Hol1p within the membrane resembles the commonly observed 12 transmembrane classes of transporters which include a relatively large hydrophilic region between putative membrane-spanning domains 6 and 7.

Comparison of the predicted Hol1 protein sequence with sequences contained in the major databases by using the NCBI BLASTP network search program revealed that Hol1 shares sequence similarities with a group of proteins in the Car1/Cyhr family of putative transporters that include *bcr* from *E. coli* (5), Car1 from *Schizosaccharomyces pombe* (19), Bmrp from *Candida albicans* (11), and Cyhr from *Candida maltosa* (29). A comparison of the inferred Hol1 sequence with that of Car1 reveals few gaps but only 18% identity (Fig. 4). The inferred protein sequences of several other open reading frames in the genome of *S. cerevisiae* (ybp3, yb30, yhk8, and ybl8) exhibit similar degrees of sequence conservation with Hol1. The members of the Cyhr/Car1 family are predicted to contain 12 membrane-spanning domains, and several have been implicated in the transport of a variety of drugs: *E. coli bcr* mutants are hypersensitive to bicyclomycin (5), *S. pombe car1* mutants exhibit increased sensitivity to amiloride (19), and *S. cerevisiae* cells expressing the *C. albicans cyhr* gene gain increased resistance to cycloheximide (11). However, to date none of the normal substrates for any of the members of the Car1/Cyhr family have yet been identified.

Effect of *hol1*Δ null alleles. The DNA sequence of *HOL1* revealed that the *hol1*Δ mutation generated previously (12) resulted in an in-frame deletion and thus could express an altered and perhaps partially functional protein. To better test the conclusion that *HOL1* is a nonessential gene, a deletion-disruption allele, *hol1*Δ*2*, was constructed; this replaced 850 nucleotides of the *HOL1* open reading frame with the *URA3* gene (see Materials and Methods). The *hol1*Δ*2* mutation was

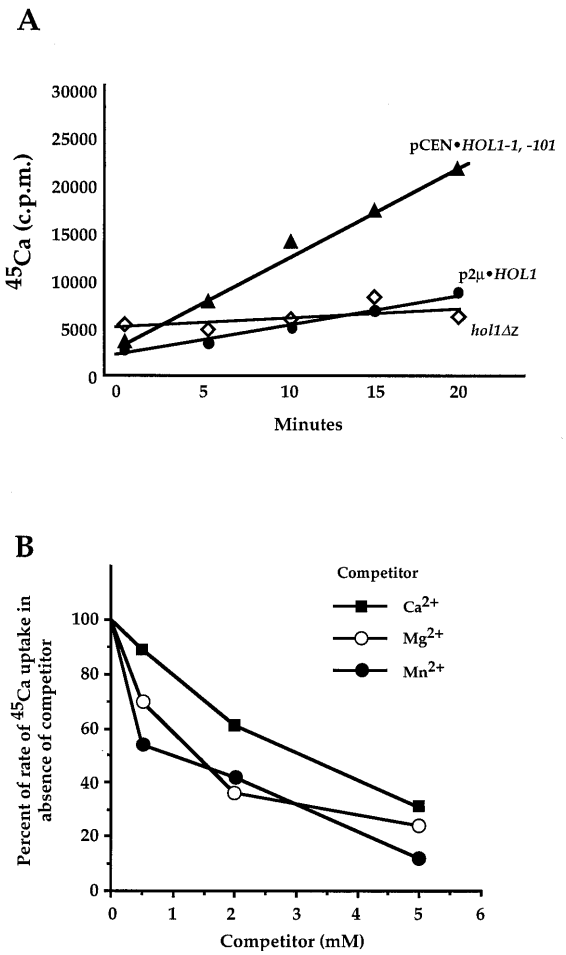

FIG. 2. ^{45}Ca uptake by *HOL1-1,-101*, *HOL1*-overexpressing, and *hol1*Δ*2* cells. (A) Uptake of ^{45}Ca was measured in *hol1*Δ*2* cells and in *hol1*Δ*2* cells that harbored plasmids expressing the mutant (*HOL1-1,-101*) or wild-type (*HOL1*) alleles. The wild-type allele was carried on a multicopy plasmid to maximize the ability to detect transport activity. Total calcium concentration in this experiment was 0.5 mM. (B) Competition for ^{45}Ca uptake by Ca^{2+}, Mg^{2+}, and Mn^{2+}. Initial rates of uptake by *HOL1-1,-101* cells (strain R704; see Materials and Methods for details) were measured in the presence and absence of the indicated competitor ions as indicated.

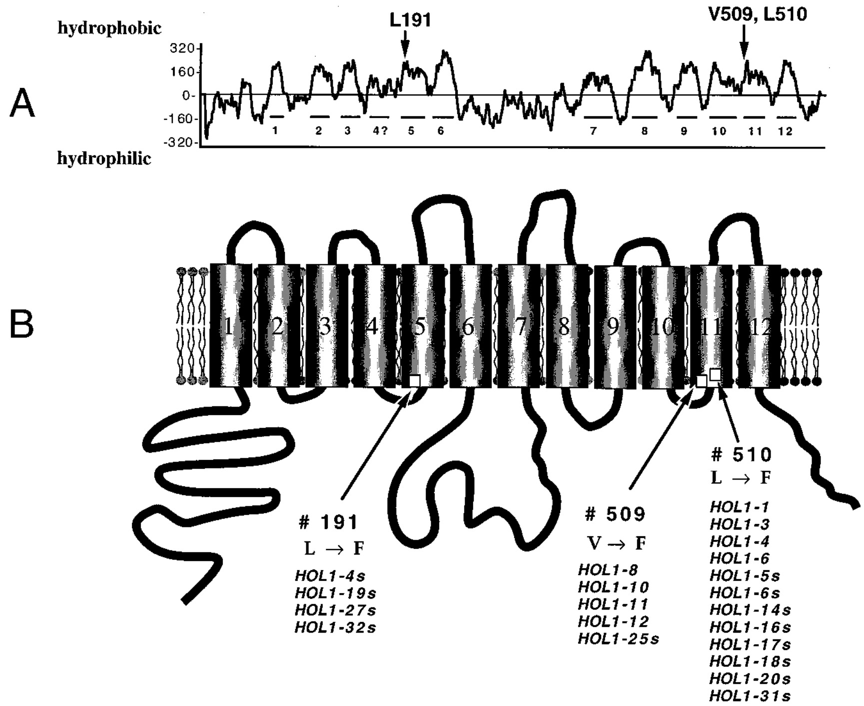

FIG. 3. Topological analysis and model of inferred protein sequence of *HOL1*. (A) Hydropathy plot (16) of inferred protein sequence of *HOL1* using a window of six amino acids. Solid bars below hydrophobic peaks indicate putative membrane-spanning domains. Sites of dominant *HOL1* mutations are indicated by arrows. (B) Topological model based on hydrophobicity plot above. Locations and identities of independent *HOL1* mutations are indicated at amino acids 191, 509, and 510. L, leucine; F, phenylalanine; V, valine. See Materials and Methods for details of isolation and identification of mutations.

generated in cells that harbored the *HOL1-1,-101* allele and resulted in the loss of the Hol$^+$ phenotype in these cells. Haploid cells containing the *hol1Δ2* allele are viable and show no obvious growth-related phenotypes (see below).

A physiological role for the wild-type Hol1 protein was sought by comparing the growth of haploid cells harboring the wild-type *HOL1* allele with those harboring the *hol1Δ2* allele and those containing the wild-type allele expressed from a multicopy plasmid (pMW86). Experiments described in a later section confirmed that Hol1 protein is overexpressed when the *HOL1* gene is carried by a multicopy vector. Despite extensive testing, no growth difference attributable to the presence of a particular *HOL1* allele was found. These tests included those described by Purnelle et al. (27), sensitivity to extremes of salt and osmotic conditions, the presence of potentially toxic levels of various ions, different pH, the use of alternative carbon and nitrogen sources, the requirement for trace elements and vitamins, and resistance to a variety of compounds, including cy-

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          1                                                                            *                      90
Hol1      .......... .......... .......... MDKYTNRDHP DYIPGTFNIY SSQNLENGI. ....IYESKL KKTSSGVVLI PQ..
Car1      .......... .......... MASKIASLFS PSETASKDQH ENVAEDLELG TAPLNQIGIH ETNSEYDEK. KREESPEV.. ...IDISNLI
Ybp3      MQAQGSQSNV GSLRSNCSDN SLPNNHVMMH CDESSGTPHS EHNDYSYEKT NLESTASNSR EHRDNQLSRL KSEEYVVPKN QRRGLLPQLA

          91              *                             *        *                *               **          180
Hol1      PSYSPNDPLN WSSWRKLAHF GLMAFITAFT AATSNDAGAA QDSLNEIYGI SYDSMNTGAG VLFLGIGWST LFLaPFANLY GRKITYIVCT
Car1      SSDHPAHPQN WHWAKRWDIV FMFCLMQIYV IWTSNGFGSI EYSVMAQFNV SAQVATLCLS MNILGSGLGP MFLgPLSDIG GRKPVYFCSI
Ybp3      IIPEFKDARD YPPMMKKMIV FLIAFssmmg PMGTSIIFPA INSITTEFKT SVIMVNVSIG VYLLSLGVFP LWWSSLSELE GRRTTYITSF

          181        **       *       *                 *        *                  *               *         270
Hol1      TLGLFGALWF ALAKRTSDTI WSQLFVGISE SCAEAQVQLS LSDIFFQHQL GSVLTVYIMC TSIGTFLGPL IAGY.ISAFT NFRWVGWVAV
Car1      FVYTVFNISC ALPRNIVQMI ISHFIIGVAG STALTNVAGG IPDLFPEDTA GVPMSLFVWA CAGGAIGAPM ATGVDINAKY GWRWLYYINI
Ybp3      ALLFAFNIGS ALAPDINSFI ALRMLCGAAS ASVQSVGAGT VADLYISEDR GKNLSYYYLG PLLAPLLSPI FGSLLVN.RW PWRSTQWFMV

      271 *  *   *                                                                                            360
Hol1      IISGGLLITI .......... .........I FGCEETYFDR GQYMTPLTSC QSGYEDGTTL QNSDNTAVSR RKRHLDAKLS TPGAMGEKGV
Car1      IVGGFFLIVI LI........ .........  .......... .......... .......... .......... .......... IPETLPIKVI
Ybp3      ILSGCNVILL TVLLPETLRK QDSKGAIAQI LAERRIQVDN NERGEIQEDY QRG.EDETDR IENQVATLST EKHNYVGEVR DQDSLDLESH

          361                                             *       *                 *     *                   450
Hol1      DLSETAE... .......... .......... FEVNNEEEVT IPETRELIDG SK........ ......EHLK PYPKRV.... ..AILTKATN
Car1      TRYENA.... .......... .......... .......... ..KGRIVEGI PK........ ......NNLK EVLKKC.... ..KFVTT...
Ybp3      SSPNTYDGRA GETQLQRIYT EASRSLYEYQ LDDSGIDATT AQVTRIRSTD PKLARSIREN SLRKLQTNLE EQVKKVLSSN GGEIAPKQVS

          451                         *  *                   *                                           *    540
Hol1      LKGYGFKQYF KYLK...INL RMFLFPPVWL SGMFWGIQDV FLTFYLTTQE SAYYEPPWNY SDFGVAIMNV PTLIGAVIGC ICAGIVSDYF
Car1      .......... .......MGF RMMLTEPIIL SMGLYNFYAY GISYFFLTAI WPVFYDTYKM SEMGASCTYL SGFVASTLLF LYQPI.QDWI
Ybp3      AVRKVWDTFF VYFIKPLKSL HFLEYPPVAL AITFSAISFS TVYFVNMTVE YKYSRPPYNF KPLYIGLLYI PNSVTYFFAS IYGGRWVDML

          541      *     * *             *      *          *      *      *           * * *  *                 630
Hol1      VLWMARHNRG ILEAEFRLYF SIATAII.GP AGLLMFGIGT ARQWPWQAIY VGLGFVGFAW GCSGDIAMAY LMDCYPDMVL EGMVCTAIIN
Car1      FRRDKAKNNG VARPEARFTS ALFITLL.FP AGMFLPAFTC HPPFPWMSPI VGNSMVTVAN GHNWMCILNY LTDSYPLLSG SAVAAFTLPS
Ybp3      LKRYKEKY.G ILAPEARISW NVVTSVISFP IALLIFGWCL DKKCHWVTPL IGTALFGYAA MMTIGATLSY LVDSLPGKGA TGVALNNLIR

          631                          * *                *               *          700
Hol1      NTISCIFTFT CSDWLAASGT ENTYIALAVI NFGITAFALP MYYYGKRIRL ...WTKRWYL QSVNLRDGV.
Car1      FIGATVFAHV SQIMFNNMSV KWAVATMAFI SISIPFIIYT FYFFGQRIRA LSSLTGNKAL KYLPLENN..
Ybp3      QILAATAVFV TTPMLNGMGT GWAFTMLAFI VLGASSVLII LKKHGD.... ..YWRENYDL QKLYDKID..
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FIG. 4. Sequence comparison between Hol1 and two other members of the Car1/Cyhr family of putative transporters. Amino acid sequence alignment between Hol1, Car1, and Ybp3 was generated using the Pileup program from the collection of programs from the Genetics Computer Group (8).

cloheximide, amiloride, benomyl, and methotrexate (27). In addition, the mating efficiency of *hol1Δ2* × *hol1Δ2* (MW71 × MW72; Table 1) cells was compared with that of *HOL1* × *HOL1* (R1714 × R1843; Table 1) by the method of Trueheart et al. (35), and no differences were observed.

***HOL1* mutations alter putative membrane-spanning domains.** To identify the *HOL1-1* and *HOL1-101* mutations, the sequences of wild-type and *HOL1-1* alleles carried on plasmids pMW18 and pMW13, respectively, were determined and compared to the *HOL1-1,-101* sequence. These comparisons revealed that the *HOL1-1* mutation is a single C-to-T transition at position +1530 with respect to the start codon converting a leucine codon to a phenylalanine codon at position 510 (L510F). This site lies within the 11th predicted transmembrane domain (Fig. 3).

The observation that the *HOL1-1* mutation affected a putative membrane-spanning domain indicated that this region might be important for the transport of substrates across the lipid bilayer. To identify other sites within the Hol1 protein that may be similarly involved in transport and thus begin to establish a putative map of the transport pathway through Hol1, a collection of spontaneous independent *HOL1* mutants was obtained by selecting for the growth of a wild-type *HOL1* strain (R757) on medium lacking histidine and supplemented with 2.5 mM histidinol. Seven mutant *HOL1* alleles were cloned, and their sequences were determined. Two of the *HOL1* mutations were identical to *HOL1-1*, while another resulted in a G-to-T transversion at position +1530 which changed the same leucine codon at position 510 to one for phenylalanine (L510F) (Fig. 3). Four alleles resulted in G-to-T transversions at position +1525, resulting in substitution of the wild-type valine codon for phenylalanine at amino acid position 509 (Fig. 3). The V509F mutation conferred slightly more vigorous growth on 2.5 mM histidinol than did the L510F mutation (unpublished results).

We considered that the selection scheme described above may have been too stringent to identify other mutations that could also confer permeability to histidinol. To identify additional sites that may be involved in substrate transport, spontaneous independent Hol^+ mutants capable of growth on 2.5 mM histidinol were selected from *hol1Δ2* cells that harbored a centromeric plasmid expressing the *HOL1-101* allele upstream of an otherwise wild-type *HOL1* gene (strain MW71). Since the *HOL1-101* mutation increases the efficiency of *HOL1* translation (described below), this construct allowed the identification of potentially weaker *HOL1* alleles.

The DNA sequences of 13 plasmid-borne *HOL1* mutants isolated by this selection (designated *HOL1-Xs*) were determined. Nine mutations were similar to the mutations observed in the previous selection which consisted of nucleotide substitutions at codons 509 or 510 that convert them into phenylalanine codons. The remaining four, however, resulted in single nucleotide changes that converted a leucine codon to a phenylalanine codon at amino acid position 191 (L191F). L191 is predicted to lie within the fifth membrane-spanning domain (Fig. 3). Consistent with the hypothesis that this selection scheme might identify weaker *HOL1* alleles, the L191F mutants are unable to confer growth on histidinol in the absence of the *HOL1-101* mutation (unpublished results).

***HOL1* is translationally repressed by a small uORF.** Comparison of the DNA sequences of the *HOL1-1,-101* and *HOL1-1* clones revealed that the former contained a T-to-A transversion at position −214 in the 5′ noncoding region. The location of the *HOL1-101* mutation outside of the coding region was consistent with the observation that, by itself, this mutation does not confer a Hol^+ phenotype. Rather, the effect of the *HOL1-101* mutation depends on the presence of a mutation in the coding region of the gene.

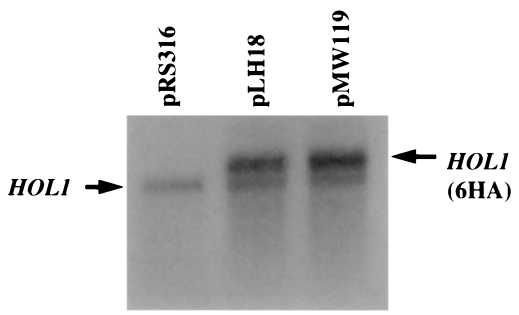

FIG. 5. Detection of *HOL1* transcripts. Poly(A)$^+$ RNA was isolated from BJ2168 *HOL1* cells containing either vector (pRS316; strain MW147), *HOL1-1,-6HA* (pLH18; strain LH75), or *HOL1-1,-101,-6HA* (pMW119; strain MW137) plasmids and probed with a ^{32}P-labeled DNA fragment contained within the coding region of the *HOL1* gene (615-bp *Kpn*I-*Kpn*I; see Fig. 1). *HOL1*, 2.5-kb chromosomal *HOL1* transcript; *HOL1* (6HA), larger transcript from plasmid-borne *HOL1-1,-6HA* or *HOL1-1,-101,-6HA* alleles; 6HA, hextuply tagged with influenza HA epitope. See Table 1 for complete genotypes.

To determine if the *HOL1-101* mutation had an effect on steady-state levels of *HOL1* mRNA, Northern (RNA) blot analysis was performed on poly (A)$^+$ RNA isolated from *HOL1* cells containing either a vector control (pRS316) or plasmids encoding epitope-tagged *HOL1-1* or *HOL1-1,-101* alleles. The transcripts expressed from centromeric plasmids encoding epitope-tagged alleles of *HOL1* (*HOL1-1,-6HA* and *HOL1-1,-101,-6HA*) were distinguishable from the mRNA transcribed from the chromosomal wild-type *HOL1* gene because of their increased length (Fig. 5). Thus, the chromosomal *HOL1* message served as an internal control in these experiments. Although the chromosomal *HOL1* transcripts were present at slightly lower levels than those expressed from the plasmids, the levels of *HOL1-1,-101,-6HA* and *HOL1-1,-6HA* RNAs were indistinguishable. This demonstrated that the stronger Hol^+ phenotype conferred upon *HOL1-1* cells by the *HOL1-101* mutation is not the result of an increase in the steady-state levels of the message.

Inspection of the *HOL1-101* mutation revealed that it abolished a putative initiation codon for a small uORF of six codons (Fig. 6). To determine if disruption of the uORF was responsible for the *HOL1-101* phenotype, additional independent mutants similar to *HOL1-101* were isolated (see Materials and Methods). DNA sequence analysis of two additional alleles revealed that one, *HOL1-102*, resulted in a single nucleotide change of A to G at position −215 and the other, *HOL1-105*, affected nucleotides −214 and −215, resulting in a nucleotide deletion and a substitution that converted the AT to a G nucleotide at this position (Fig. 6). Thus, each of three independent mutations that enhanced the Hol^+ phenotype of *HOL1-1* cells abolished the putative translational start site (5′ ATG 3′) located upstream of the presumed authentic *HOL1* initiation codon.

If mutations in the upstream ATG (uATG) affect translation of *HOL1*, this site should reside within the RNA transcript. Primer extension analysis was performed to identify the major site(s) of transcription initiation. A primer that initiated reverse transcriptase activity at position +66 with respect to the large *HOL1* open reading frame yielded a single extension product of approximately 350 to 400 nucleotides (unpublished results). To more accurately determine the site of transcript initiation, a second primer that initiated reverse transcription

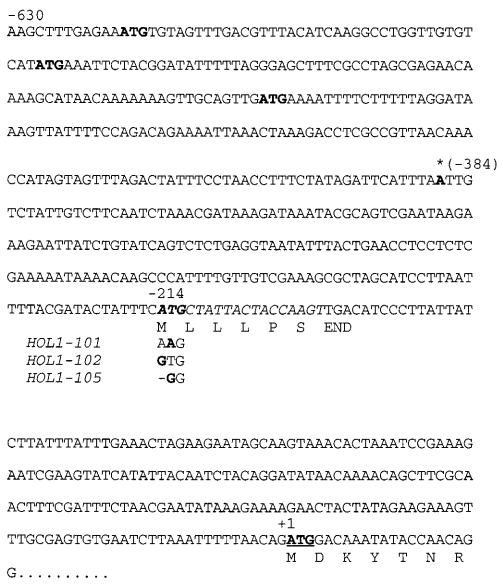

FIG. 6. DNA sequence of 5′ region of *HOL1*, showing location of *HOL1-101*, *HOL1-102*, and *HOL1-105* mutations. uORF is indicated by italics. *, major transcriptional start site; **ATG**, first ATG of *HOL1* open reading frame.

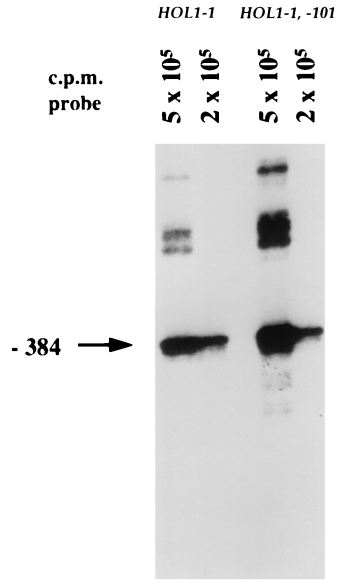

FIG. 7. Primer extension analysis of *HOL1* transcripts. Poly(A)$^+$ RNA was isolated from yeast cells and incubated with avian myeloblastosis virus reverse transcriptase and a ^{32}P-labeled oligonucleotide probe complementary to the sequence from position −313 to −274 of the 5′ untranslated region of *HOL1*. The size of the cDNA product was determined by comparison with that in a sequencing reaction using the same primer. See Materials and Methods for details. Congenic strains, R657 (*HOL1-1*) and R704 (*HOL1-1,-101*), were used.

at position −274 was used. The size of the extension product in these experiments indicated that the primary *HOL1* transcript initiates at about position −384 with respect to the large *HOL1* open reading frame (Fig. 7). Template RNA purified from cells harboring the wild-type uORF and from cells harboring the *HOL1-101* mutation which abolished the putative initiation codon of the uORF produced similar results. These experiments confirmed that the ATG which is disrupted by the *HOL1-101*, *HOL1-102*, and *HOL-105* mutations resides within *HOL1* mRNA.

In one set of experiments in which a greater amount of labeled primer was added, additional extension products greater than 500 nucleotides in length were observed (Fig. 7, compare lanes 1 and 3). Whether these represented authentic sites of transcription initiation was not determined. Potential transcription initiation from these sites would not significantly alter interpretation of the results as they, too, would include the uORF. In the same experiment an increase in the amount of extension products obtained from the *HOL1-1,-101* template RNA compared to that obtained from the *HOL1-1* template RNA was observed (Fig. 7, compare lanes 1 and 3). However, conclusions regarding levels of steady-state *HOL1* mRNA cannot be drawn from these experiments, since they were not controlled for relative amounts of template RNA.

To determine if the *HOL1-101* mutation affected the amount of steady-state Hol1 protein, plasmids expressing epitope-tagged versions of the *HOL1-1* and *HOL1-1,-101* alleles were constructed and Hol1 protein was visualized by immunoblotting with anti-HA monoclonal antibody. To allow detection of function of the epitope-tagged protein, the mutant, histidinol transport-competent alleles of *HOL1* were incorporated in both constructs. Addition of the epitope tag resulted in at least partial retention of function, since the *HOL1-1,-101,-6HA* allele conferred growth on histidinol media (unpublished results).

Heterogeneous signals of approximately 68 to 71 kDa were detected in protein samples from *HOL1-1,-6HA*- or *HOL1-1,-101,-6HA*-expressing cells (Fig. 8, lanes 2 through 6). Abolition of the putative start codon of the uORF at *HOL1* due to the *HOL1-101* mutation resulted in an approximately 5- to 10-fold increase in the steady-state levels of Hol1 (Fig. 8, lanes 3 and 5). A further increase in Hol1 protein was detected when the *HOL1-1,-101* allele was expressed from a multicopy plasmid (Fig. 8, lane 6), confirming that expression of *HOL1* from a multicopy plasmid leads to increased *HOL1* expression and indicating that the amount of Hol1 protein from extracts of the

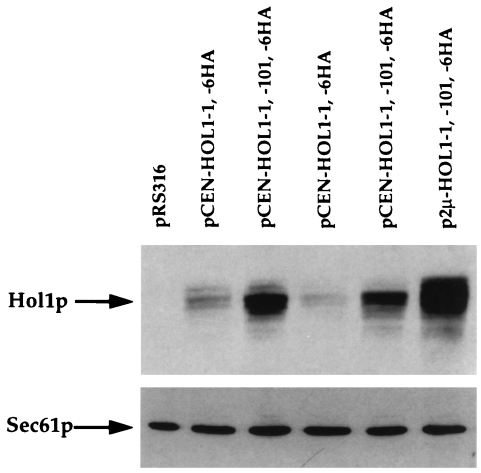

FIG. 8. Disruption of the *HOL1* uORF results in increased levels of Hol1 protein. Immunoblot of protein extracts from strains containing vector sequences only (strain BJ2168; lane 1), centromeric plasmids expressing *HOL1-1,-6HA* (strain LH75; lanes 2 and 4), *HOL1-1,-101,-6HA* (strain MW137; lanes 3 and 5), or a multicopy plasmid expressing *HOL1-1,-101,-6HA* (strain MW150). The epitope-tagged protein was detected with anti-HA monoclonal antibody. Blots were also probed with antibody against the Sec61 protein to control for the amount of protein loaded in each well.

single-copy *HOL1-1,-6HA*-expressing cells detected on the immunoblot was not saturated. In contrast, no signal was detected when protein extracts from cells that contained only the vector control were blotted (Fig. 8, lane 1).

DISCUSSION

The product of the *HOL1* gene was inferred to be a 586-amino-acid polytopic membrane protein that shares limited identity with a group of putative transporters of the major facilitator family (also known as the drug translocase family). Hol1 is most closely related to the Car1/Cyhr subfamily, members of which typically show only limited primary sequence identities. While these transporters constitute a loose-knit group, all appear to contain 12 putative membrane-spanning segments. Hol1 also contains one of the most highly conserved motifs of the facilitative transporter superfamily, the GR(R/K) motif located between membrane-spanning domains 2 and 3 (15). Although several members of the Car1/Cyhr subfamily have been implicated in the transport of hydrophobic drugs, none of their normal substrates have been identified, and their role in cell biology remains unknown.

Mutations at *HOL1* confer the uptake of histidinol and a large repertoire of cations and cationic compounds, including all of the major physiological ions. Although an indirect role for Hol1 in transport cannot be ruled out, several lines of evidence support the hypothesis that the mutant Hol1 protein is a transporter. First, the sequence of Hol1 strongly suggests that it is an integral membrane protein and the mutations that confer histidinol and cation uptake always affect putative membrane-spanning domains of Hol1. Second, the dominance of the transport phenotypes suggests a gain of function more likely for a transporter than a regulator of transport. In contrast, the *hol1Δ2* mutation does not lead to any of the Hol^+ phenotypes. Furthermore, allelic differences in the severity of the Hol^+ phenotype always correlated with severity of the other transport-related phenotypes, i.e., sensitivity to toxic ions. Finally, the newly acquired transport activities of *HOL1* mutants could be *trans*-inhibited: the ability to grow on histidinol and the Na^+ hypersensitivity phenotype of *HOL1* mutants can be inhibited by Ca^{2+} (unpublished results) and the increased Ca^{2+} uptake could be inhibited by Mg^{2+} or Mn^{2+}. Taken together, the data strongly support the notion that the gain-of-function phenotypes of *HOL1* mutants result from decreased substrate selectivity by Hol1p.

A genetic approach was taken to gain insight into the possible transport pathway through Hol1 that might be taken by histidinol. Molecular analysis of numerous *HOL1* mutations selected by their ability to confer histidinol transport revealed a highly limited repertoire of mutable sites within the transporter. The analysis of 21 independent mutants, each selected for the ability to confer growth on histidinol, revealed structural changes in only 2 of the 12 putative membrane-spanning domains. Mutations at either V509 or L510 in membrane-spanning domain 11 confer stronger Hol^+ phenotypes than do mutations at L191 in membrane-spanning domain 5. In every case, the mutation that conferred the ability to grow on histidinol resulted in a change from a small hydrophobic amino acid (leucine or valine) to phenylalanine. Thus, while these mutations preserve the hydrophobicity of the putative membrane-spanning domain, they result in the substitution of a large side chain for a small one and are consistent with the hypothesis that the mutations increase permeability through the Hol1 transporter by decreasing substrate selectivity.

The 12 transmembrane domain superfamily of transporters that includes the facilitative transporters for glucose, tetracycline, and xylose have been postulated to have arisen by duplication of an ancestral six transmembrane element (15). Although the Hol1 protein sequence does not contain obvious repeated sequences within the amino and carboxyl terminal halves of the protein, the two positions affected by the *HOL1* mutation that give rise to histidinol transport would affect similar positions within analogous membrane-spanning segments in a transporter that consists of two functionally related halves, and these domains are the two least hydrophobic among the collection of 12. This is consistent with a model in which membrane-spanning domains 4 and 10 constitute part of a polar pathway through which the transported substrate travels. Such an arrangement for the analogous membrane-spanning domains in the superfamily of facilitative transporters, also known as the USA family because of its inclusion of uniporters, symporters, and antiporters, has recently been postulated by Goswitz and Brooker (14).

The ability to be converted into a histidinol transporter by direct selection is not a global property of polytopic membrane proteins or even of transporters specifically. Despite the analysis of a fairly large collection of independent Hol^+ mutants (12; this report), mutations were not found at genes encoding other relatives of the Car1/Cyhr transporter family or at genes encoding permeases capable of transporting histidine. Perhaps Hol^+ mutations in these transporters result in decreased protein stability or the inability to be properly localized to the plasma membrane. Alternatively, single amino acid substitutions that interfere with the associations of closely packed transmembrane helices may not generally be sufficient to confer permeability to histidinol. If so, the ability to confer a Hol^+ phenotype may be limited to transporters that are nearly capable of transporting histidinol or are even weakly capable of transporting histidinol prior to mutation. In this case, the Hol^+ mutation might simply sufficiently expand the size of cationic molecules that can be accommodated by the transporter. This could arise through an increase in the size of the pathway through which the normal solute travels or by removal of an electrostatic barrier present in the pore of the wild-type transporter. Either possibility is consistent with the observation that the mutations that lead to a Hol^+ phenotype are predicted to reside within membrane-spanning domains. However, since the Hol^+ mutations result in a change from a small hydrophobic amino acid to phenylalanine, the removal of an ionic barrier within the pore would have to be an indirect effect of these mutations on the structure of the transporter.

The energy source that drives the uptake of cations through the mutant Hol1 transporter is likely to be the large electrical potential across the plasma membrane. This is supported by the results of several recent studies. Anderson et al. (2) demonstrated that expression of Kat1, an *Arabidopsis* K^+ channel, was able to suppress the potassium uptake-defective phenotype of *trk1Δ trk2Δ* cells. Since Kat1 is only activated in *Xenopus* oocytes under conditions of extreme polarization (approximately −100 mV [30]) the membrane potential in *S. cerevisiae* must be of at least this magnitude. Indeed, Bertl et al. (6) have shown that a similar membrane hyperpolarization is sufficient to drive K^+ uptake in *S. cerevisiae* cells that express either the endogenous Trk proteins or the heterologous Kat1 potassium channel. Thus, the membrane potential in *S. cerevisiae* is sufficient to drive the nutritional uptake of cations, provided they can find a portal of entry. The mutant Hol1 protein appears to provide this opportunity in a manner that is essentially nonselective.

Expression of the protein encoded by *HOL1* is translationally inhibited. The 5′ leader of the 2.5-kb *HOL1* transcript is unusually long (approximately 384 nucleotides) compared to

the average *S. cerevisiae* leader sequence (52 nucleotides) (7). This region also contains an uORF of six codons that mediates a negative effect on the expression of *HOL1*. Three independent spontaneous mutations obtained by selection for increased Hol1-mediated transport each resulted in the disruption of the putative translational initiation codon of the uORF. One such mutation, *HOL1-101*, confers a 5- to 10-fold increase in steady-state levels of epitope-tagged Hol1 protein. Since *HOL1* mRNA levels are not increased in the *HOL1-101* mutants, this strongly suggests that the uORF confers translational repression upon *HOL1.*

Studies of the effects of uORFs on translation of RNAs indicate that they may play an important role in translational control of protein synthesis. For example, *S. cerevisiae GCN4* encodes a transcriptional activator of amino acid biosynthetic genes and has four AUG codons contained within the upstream region of its mRNA (24). In response to amino acid starvation, translation of *GCN4* is increased via *trans*-acting factors that modulate utilization of the upstream AUG codons, resulting in more efficient utilization of the *GCN4* start codon (24). *CPA1* mRNA has a single uORF of 25 codons that is important in translational expression in response to arginine (36). *CPA1* mutations which result in constitutive translational expression include amino acid codon changes within the uORF and truncations of the uORF, indicating that the peptide itself is important in this regulatory mechanism.

Translational regulation in *S. cerevisiae* has until now been observed to involve only genes that encode either a biosynthetic enzyme or a regulator of biosynthetic genes; this is the first time that a putative transporter protein has been found to be potentially regulated by translational repression. It is also the first example of spontaneous mutations that relieve translational repression by eliminating the putative start codon of an uORF. In addition, if *HOL1* is indeed regulated by the uORF, this is a substantially different scenario from that exemplified by *GCN4* (24) or *CPA1* (36) because it involves only a single uORF and one that is most likely too small to produce a peptide that could participate in the regulatory mechanism. Elucidation of the conditions under which *HOL1* is released from this potential translational repression may provide important clues regarding the identity of its normal substrate(s).

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