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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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 histidinol 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.

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 histidine to required level. YNB AA-Ura is a medium lacking uracil. LS (low salt) medium contains essentially no potassium and was prepared as previously described (13). KC1 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. NH4OH 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 HindIII-ClaI 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 HindIII-ClaI fragment from pRG174 (12) into pRS152, pRS413, and pRS415-2. Plasmid pMW62 containing the single HOL1-101 mutation was constructed by subcloning the 1.322-bp HindIII-ClaI fragment from pRG148 (12) into the corresponding sites of pRS316 (32). Plasmid pMW63 containing the single HOL1-101 mutation was constructed by replacing the 1.322-bp HindIII-ClaI fragment from pRG148 (12) with the Clal-XhoI fragment from pRS304 (12) into the polycloning region of pRS304 (12). Plasmid pMW64 containing the single HOL1-101 mutation was constructed by replacing the 1.322-bp PvuII-ClaI fragment from pRG148 (12) with the FnuI-ClaI fragment from pMC165-1. Plasmid pRG165-1 is an integrative plasmid (Yip5) containing a 1.1-kb HindIII-EcoRI 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**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F' φ80lacZ/ΔM15 endA1 recA1 hisD17 supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF) U169 λ’</td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thiD17 supE44 Δ(lac-proAB) [F’ traD36 proAB lacIqZAM15]</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td></td>
</tr>
<tr>
<td>MW15</td>
<td>MATa ura3-52 his32α00 his4-15 trk1Δ trk2Δ::HIS3 holl1Δ</td>
</tr>
<tr>
<td>R757</td>
<td>MATa ura3-52 lys9 his4-15</td>
</tr>
<tr>
<td>R657</td>
<td>MATa ura3-52 his3-15 HOL1-1</td>
</tr>
<tr>
<td>R704</td>
<td>MATa ura3-52 his3-15 HOL1-1-101</td>
</tr>
<tr>
<td>R1714</td>
<td>MATa ura3-52 trp1Δ his4-15</td>
</tr>
<tr>
<td>R1843</td>
<td>MATa ura3-52 trp1Δ his4-15</td>
</tr>
<tr>
<td>MW71</td>
<td>MATa ura3-52 trp1Δ his4-15 holl1Δ::TRP1</td>
</tr>
<tr>
<td>MW72</td>
<td>MATa ura3-52 trp1Δ his4-15 holl1Δ::TRP1</td>
</tr>
<tr>
<td>MW118</td>
<td>pMW15/pHOL1 (pMW18)</td>
</tr>
<tr>
<td>MW121</td>
<td>pMW15/pHOL1-1-101 (pMW11)</td>
</tr>
<tr>
<td>MW122</td>
<td>pMW16/pHOL1-1 (pMW13)</td>
</tr>
<tr>
<td>BJ2168</td>
<td>MATa ura3-52 trp1Δ leu2-3::411 trp1-1227 pep4-3 gag2</td>
</tr>
<tr>
<td>MW137</td>
<td>BJ2168/pCEN-HOL1-1-101, -6HA (pMW119)</td>
</tr>
<tr>
<td>MW150</td>
<td>BJ2168/p2α-HOL1-1-101,-6HA (pMW117)</td>
</tr>
<tr>
<td>MW147</td>
<td>BJ2168/pRS316</td>
</tr>
<tr>
<td>LH75</td>
<td>BJ2168/pCEN-HOL1-1-6HA (pLH18)</td>
</tr>
</tbody>
</table>

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

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

Isolation and characterization of new HOLL mutants. Two strategies were used to isolate spontaneous mutants that conferred uptake of histidinol. In the first, single colonies of a wild-type HOLL-1 strain (R757, Table 1) were allowed to develop on permissive medium (YPD) and then replica plated to YNB HA-25 mM histidinol. To ensure independent of the mutations, only a single Holl-1 mutant was picked from a replica-plated colony. Since all of the Holl-1 mutants were dominant, plasmid integration was used to determine which of them harbored alleles of the HOLL-1 locus. Plasmid pRG165-1 is an integrative plasmid that contains the 5' portion of HOLL-1 including the HOLL-1-101 mutation. For those Holl-1 mutants that harbored a mutation at the HOLL-1 locus, integration of pRG165-1 generated a HOLL-1X101 double mutant that was easily identified by its ability to grow on a very low concentration (0.25 mM) of histidinol. Seven independent Holl-1 mutants appeared to harbor mutations at HOLL-1 on the basis of this preliminary assessment. Plasmids harboring the entire HOLL region were recovered from genomic DNA prepared from pRG165-1 integrants by digestion with Xhol followed by circularization with T4 DNA ligase and transformation into E. coli. The DNA sequence of the entire HOLL region for each of the mutant alleles was determined.

In a second selection, plasmid-born mutant Holl-1 alleles were isolated from a holl1Δ2 strain (MW71) that harbored the HOLL-1-101-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-25 mM histidinol medium. Mutant Holl-1 alleles isolated by this selection were recovered directly by transformation of genomic DNA preparations into E. coli. The presence of a mutant Holl-1 plasmid on the alleles recovered by either of these selection strategies was confirmed by their ability to confer a Holl-1 phenotype to a holl1Δ2 recipient (strain MW71) upon transformation.

DNA sequence analysis. The DNA sequence of the 2.8-kb fragment containing the HOLL-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 HOLL-1-101-containing fragment was used to determine the DNA sequences of wild-type, Holl-1, and Holl-2 Holl-1 alleles. DNA sequencing reactions were performed by the dideoxy-chain termination method (26).

Construction of epitope-tagged Holl-1. To generate a Holl-1-101 allele containing multiple copies of the hemagglutinin (HA) epitope (21) a NotI site was inserted by oligonucleotide-directed mutagenesis immediately following the last codon in the HOLL open reading frame. A 18-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 hexaply-tagged HOLL-1-101 allele was recovered and subcloned into centromeric and high-copy plasmids, containing pRS316-HOLL-1-101,-6HA (pMW19) and pRS426-HOLL-1-101,-6HA (pMW117). Plasmid pH18, expressing an epitope-tagged HOLL-1 allele, was constructed by replacing the 1.3-kb EcoRI fragment in pMW19 (HOLL-1-101,-6HA) with that from pMW18 (HOLL-1).

**Immunoblot analysis.** s. cerevisiae protein was prepared as described previously (3) and HA-tagged proteins were detected by immunoblotting with monoclonal antibody 12CAS (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-32P]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.

**[Ca2+] uptake assays.** [Ca2+] was measured by incubating cells in the presence of CaCl2 at concentrations from 25 μM to 15 mM with a specific radioactivity of 0.5 μCi/pM of Ca2+. 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 CaCl2 concentration was prewarmed to 30°C. An aliquot of 106 cells was added to begin the assay. Samples of 105 cells were removed at various intervals, collected on 0.45-μm pore-size filters, and washed three times with 10 mM iced-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 CaCl2 concentration were averaged. Data are expressed as the initial rate of [Ca2+] uptake over the initial linear portion of uptake (picomoles per 106 cells per minute) versus the concentration of CaCl2 in the assay buffer.

For competition assays, the rate of [Ca2+] uptake in the presence of a fixed concentration of CaCl2 (0.5 mM) with 0.5, 2.0, or 5.0 mM CaCl2, MgCl2, or MnCl2, as competitor was determined. The amount of radioactivity contained in the cells after 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 the 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 HOLL mRNA from congenic HOLL-1 (R657) and HOLL-1-101 (R704) cells (Table 1) essentially as described by Ausubel et al. (5). 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 HOLL 1PE (5’-GGAAGATAGATGTT AAATGCGCAAGGTAATAGCGG-3’) corresponds to position +66 with respect to the HOLL coding sequence and was used to approximate the transcriptional start site. The 5'-most nucleotide of oligonucleotide HOLL 1PE 3’-TTTCCAGAGAGGCTCAATATACCTC CAGACATG-5’) corresponds to position +274 with respect to the HOLL coding sequence and was used to more accurately map the HOLL transcriptional start site.

**Nucleotide accession number.** The accession number for the HOLL-1-101 sequence is L42458.

**RESULTS**

HOLL mutants suppress the K+ requirement of trk1Δ trk2Δ cells. We previously showed that dominant mutations at HOLL conferred increased uptake of histidinol and sodium (12). To determine if mutations in HOLL might also confer increased K+ transport, the effects of holl1Δ2, HOLL-1-1 and HOLL-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 trk1Δ trk2Δ cells require approximately 30 to 50 mM potassium to support growth (20).

To determine first if the wild-type HOLL protein is involved in potassium transport, the potassium requirements of trk1Δ trk2Δ holl1Δ2 (MW15) cells expressing the wild-type HOLL 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 \( \text{HOL1} \) on the phenotype of \( \text{trk1}\Delta \text{trk2}\Delta \) cells. \( \text{trk1}\Delta \text{trk2}\Delta \) cells bearing vector sequences (pRS316) or the wild-type \( \text{HOL1} \); pMW18), single mutant \( \text{HOL1}\Delta 1 \); pMW13), or double mutant \( \text{HOL1}\Delta 1-\text{hol1}\Delta 2 \); pMW11) \( \text{HOL1} \) 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 \( \text{HOL1}\Delta 1 \) or \( \text{HOL1}\Delta 1-\text{hol1}\Delta 2 \) alleles were introduced into the \( \text{trk1}\Delta \text{trk2}\Delta \) recipient, they partially suppressed the \( \text{trk1}\Delta \text{trk2}\Delta \) phenotype (Fig. 1). Thus, the mutant hol1 transporter appears able to transport K⁺.

**Divalent ion uptake by mutant Hol1p.** High concentrations of Ca²⁺ added to the growth medium inhibited the ability of \( \text{HOL1}\Delta 1 \) and \( \text{HOL1}\Delta 1-\text{hol1}\Delta 2 \) cells to utilize histidinol and also suppressed their hypersensitivity to Li⁺ (unpublished results). These results suggested that Ca²⁺ might also be transported by the wild-type or mutant Hol1 proteins. Cells harboring the \( \text{hol1}\Delta 2 \) mutation or overexpressing the wild-type \( \text{HOL1} \) allele from a multicopy plasmid exhibited essentially indistinguishable rates of Ca²⁺ uptake, suggesting that the wild-type Hol1 protein does not normally transport Ca²⁺ (Fig. 2A). However, the rate of [⁴⁵Ca²⁺] uptake by \( \text{hol1}\Delta 2 \) cells expressing the \( \text{HOL1}\Delta 1-\text{hol1}\Delta 2 \) allele from a single-copy plasmid was significantly increased compared to \( \text{hol1}\Delta 2 \) cells harboring the vector alone (Fig. 2A). In separate experiments Mg²⁺ and Mn²⁺ were shown to be capable of inhibiting [⁴⁵Ca²⁺] uptake by \( \text{HOL1}\Delta 1-\text{hol1}\Delta 2 \) cells in a manner as efficient as isotopic Ca²⁺ (Fig. 2B). These results suggested that divalent cations in addition to Ca²⁺ are either transported by Hol1 mutants or can block the uptake of Ca²⁺.

**\( \text{HOL1} \) encodes a predicted membrane protein.** The DNA sequence of the 2.8-kb fragment that contains the \( \text{HOL1}\Delta 1-\text{hol1}\Delta 2 \) 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 \( \text{HOL1} \) 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 ber 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 yh18) 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* ber 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 \( \text{hol1}\Delta \) null alleles.** The DNA sequence of \( \text{HOL1} \) revealed that the \( \text{hol1}\Delta \) 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 \( \text{HOL1} \) is a nonessential gene, a deletion-disruption allele, \( \text{hol1}\Delta 2 \), was constructed; this replaced 850 nucleotides of the \( \text{HOL1} \) open reading frame with the \( \text{URA3} \) gene (see Materials and Methods). The \( \text{hol1}\Delta 2 \) mutation was
generated in cells that harbored the HOL1-1.101 allele and resulted in the loss of the Hol1 phenotype in these cells. Haploid cells containing the hol1D2 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 hol1D2 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-

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.

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).
The observation that the HOLL-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 HolI protein that may be similarly involved in transport and thus begin to establish a putative map of the transport pathway through HolI, a collection of spontaneous independent HOLL-1 mutants was obtained by selecting for the growth of a wild-type HOLL strain (R757) on medium lacking histidinol and supplemented with 2.5 mM histidinol. Seven mutant HOLL alleles were cloned, and their sequences were determined. Two of the HOLL-1 mutations were identical to HOLL-1, while another resulted in a G-to-T transversion 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).

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The sequences of wild-type and HOLL-1 alleles carried on plasmids pMW18 and pMW13, respectively, were determined and compared to the HOLL-1,1,101 sequence. These comparisons revealed that the HOLL-1 mutation is a single C-to-T transition at position +1530, resulting in a G-to-T transversion 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 HolI mutants capable of growth on 2.5 mM histidinol were selected from holl12 alleles that harbored a centromeric plasmid expressing the HOLL-1 allele upstream of an otherwise wild-type HOLL gene (strain MW71). Since the HOLL-1 mutation increases the efficiency of HOLL translation (described below), this construct allowed the identification of potentially weaker HOLL alleles.

The DNA sequences of 13 plasmid-borne HOLL mutants isolated by this selection (designated HOLL-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). L191F is predicted to lie within the fifth membrane-spanning domain (Fig. 3). Consistent with the hypothesis that this selection scheme might identify weaker HOLL alleles, the L191F mutants are unable to confer growth on histidinol in the absence of the HOLL-1 mutation (unpublished results).

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

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

Inspection of the HOLL-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 HOLL-101 phenotype, additional independent mutants similar to HOLL-101 were isolated (see Materials and Methods). DNA sequence analysis of two additional alleles revealed that one, HOLL-102, resulted in a single nucleotide change of A to G at position −215 and the other, HOLL-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 HolI phenotype of HOLL-1 cells abolished the putative translational start site (5′ ATG 3′) located upstream of the presumed authentic HOLL initiation codon.

If mutations in the upstream ATG (uATG) affect translation of HOLL, this site should reside within the DNA 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 HOLL 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. 5. Detection of HOLL transcripts. Poly(A) RNA was isolated from BJ2168 HOLL cells containing either vector (pRS316; strain MW71), HOLL-1,6HA (pLH18; strain LH75), or HOLL-1,101,6HA (pMW119; strain MW137) plasmids and probed with a 32P-labeled DNA fragment contained within the coding region of the HOLL gene (615-bp KpnI-KpnI; see Fig. 1). HOLL, 2.5-kb chromosomal HOLL transcript; HOLL (6HA), larger transcript from plasmid-borne HOLL-1,6HA or HOLL-1,101,6HA alleles; 6HA, hextuply tagged with influenza HA epitope. See Table 1 for complete genotypes.](https://journals.asm.org/journal/jb)
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 HOL1-105 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 HOL1-1-101 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
single-copy *HOL1*-6×HA-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 for a regulator of transport. In contrast, the hol1Δ2 mutation does not lead to any of the Hol1 phenotypes. Furthermore, allelic differences in the severity of the Hol1 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 Ca²⁺ (unpublished results) and the increased Ca²⁺ uptake could be inhibited by Mg²⁺ or Mn²⁺. 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 Hol1 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 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 antiporers, 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/Δ trk2Δ/Δ* 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 H01-mediated transport each resulted in the disruption of the putative translational initiation codon of the uORF. One such mutation, H01-101, confers a 5- to 10-fold increase in steady-state levels of epitope-tagged Hol1 protein. Since H01 mRNA levels are not increased in the H01-101 mutants, this strongly suggests that the uORF confers translational repression upon H01.

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 the first example of spontaneous mutations that relieve translational repression by eliminating the putative start codon of an uORF. In addition, if H01 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 H01 is released from this potential translational repression may provide important clues regarding the identity of its normal substrate(s).

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