Regulation of the Urea Active Transporter Gene (*DUR3*) in *Saccharomyces cerevisiae*

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The *DUR3* gene, which encodes a component required for active transport of urea in *Saccharomyces cerevisiae*, has been isolated, and its sequence has been determined. The deduced *DUR3* protein profile possesses alternating hydrophobic and hydrophilic regions characteristic of integral membrane proteins. Strong negative complementation observed during genetic analysis of the *DUR3* locus suggests that the *DUR3* product may polymerize to carry out its physiological function. Expression of *DUR3* is regulated in a manner similar to that of other genes in the allantoin pathway. High-level expression is inducer dependent, requiring functional *DAL81* and *DAL82* genes. Maintenance of *DUR3* mRNA at uninduced, nonrepressed basal levels requires the negatively acting *DAL80* gene product. *DUR3* expression is highly sensitive to nitrogen catabolite repression and also has a partial requirement for the *GLN3* product.

Urea uptake into *Saccharomyces cerevisiae* occurs via two systems. The first is an active transport system with an apparent Michaelis constant of 14 μ M and is sensitive to inhibitors of energy metabolism and uncoupling agents such as fluoride, arsenate, cyanide, and 2,4-dinitrophenol (9, 10, 13). Active transport is the predominant route of urea entry at extracellular concentrations below 0.25 mM. This characteristic made it possible to isolate mutant strains carrying defects in the active transport system; the mutant locus was designated *DUR3* (10, 29).

The second route of urea entry into the cell is via facilitated diffusion which occurs predominantly at extracellular concentrations of 0.5 mM and above (13, 29). Although only preliminarily characterized at present, this system appears to be insensitive to inhibitors of energy metabolism, distinguishing it from the active transport system (13, 29).

Production of components of the urea active transport system is induced by the allantoin pathway native or gratuitous inducers (allophanate and oxalurate [OXLU], respectively) (9, 31). Since active transport of urea does not occur in cells provided with readily used nitrogen sources such as asparagine, synthesis of this permease has also been suggested to be sensitive to nitrogen catabolite repression (NCR) (3, 13, 29). Little is known about whether regulatory mutations reported to participate in allantoin pathway gene expression (*DAL80*, *DAL81*, *DAL82*, and *GLN3*) affect global production of the urea active transport system because, except for a preliminary measurement with *dal81* and *dal82* mutants (20), the urea transport assays performed in allantoin pathway regulatory mutants have been performed in a genetic background containing a wild-type *DUR1,2* gene (14, 34). In such strains, urea transport is not dissociated from urea metabolism, and hence one cannot unambiguously interpret the results because *DUR1,2* product levels have clearly been shown to be negatively and positively regulated by the *DAL80* to -82 and *GLN3* products. Moreover, it is likely that the overall active transport system consists of multiple components (29). The regulation of their production

cannot, therefore, be rigorously studied until the individual components have been isolated.

The *DAL5* and *DAL7* genes of the allantoin pathway have been used as models to identify the *cis*- and *trans*-acting factors that regulate allantoin pathway gene expression and to elucidate their functions (24–26, 38). Activation of *DAL* gene transcription is mediated by multiple copies of the *cis*-acting element *UAS_{NTR}* which contains the sequence GATAA at its core (26). Operation of *UAS_{NTR}* is dependent upon the *GLN3* gene product (11). This is also the element through which NCR is exerted (12). This is the only positively acting element reported thus far for the inducer-independent *DAL* genes, *DAL3* and *DAL5* (26, 37).

Inducer-dependent *DAL* genes, such as *DAL7* and *DUR1,2*, also require a functional *GLN3* product for activation (11). However, they additionally require functional *DAL81* and *DAL82* gene products to respond to the presence of inducer (6, 7, 20, 22, 33). Inducible expression of *DAL7* and *DUR1,2* is maintained at a low level when inducer is absent by the *DAL80* product (15). In other words, *DAL80* product represses transcriptional activation mediated by *UAS_{NTR}*.

Our overall objective is to understand (i) the biochemical composition of the yeast urea uptake systems and (ii) the mechanisms that regulate urea permease component production in response to changing environments. We have isolated and sequenced the *DUR3* gene, determined its genomic location, and deduced the structure of its product. We have further studied the effects of mutating the positively and negatively acting allantoin pathway regulatory genes on this expression.

(A preliminary report of this work has already appeared [14a].)

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this work are listed in Table 1. Yeast cultures used for the urea amidolyase assays were grown in medium containing 0.17% yeast nitrogen base without amino acids and ammonium sulfate (YNB; Difco Laboratories) supplemented with glu-

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TABLE 1. Strains used

Strain	Genotype
<i>S. cerevisiae</i>	
M990-3C	<i>MATα his6 ura1 dur3</i>
K381-9D	<i>MATα spo11 ura3 ade6 arg4 aro7 asp5 met14 lys2 pet17 trp1-1</i>
M1433-10B	<i>MATα spo11 aro7 lys2 arg4 ade6 ura1 ura3</i>
M1433-8B	<i>MATα dur3 trp1-1 his6 ura3 met14</i>
K382-23A	<i>MATα spo11 ura3 can1 cyh2 ade2 his7 hom3</i>
K382-19D	<i>MATα spo11 ara3 can1 cyh2 ade2 his7 hom3 tyr1</i>
K398-4D	<i>MATα spo11 ura3 ade6 arg4 aro7 asp5 met14 lys2 pet17 trp1</i>
M1534-26D	<i>MATα ura3-52 dur3</i>
Σ 1278B	<i>MATα</i>
M406-11C	<i>MATα lys1 dur3-N154 N29</i>
M407-54A	<i>MATα ade6 lys1 dur3-N154 N29</i>
HEY 1	<i>MATα ade6 lys1 dur3-N154 N29 ura3</i>
RH218	<i>MATα trp1</i>
M384-22A	<i>MATα lys1 ade6</i>
HEY 2	<i>MATα lys1 ade6 ura3</i>
HEY 4	<i>MATα lys1 ade6 ura3 dur3Δ::hisG</i>
TCY 1	<i>MATα lys2 ura3</i>
RR91	<i>MATα lys2 ura3 gln3Δ::hisG</i>
HEY 6	<i>MATα lys2 ura3 dal81Δ::hisG</i>
M02	<i>MATα ura3 dal82-1</i>
M1-2B	<i>MATα ura3-52 trp1</i>
M1079	<i>MATα lys2</i> <i>MATα lys5</i>
M1081	<i>MATα lys2 dal80-1</i> <i>MATα lys5 dal80-1</i>
M1058	<i>MATα lys5 dur1</i> <i>MATα lys2 dur1</i>
M1059	<i>MATα lys5 dur1 $\pm$</i> <i>MATα lys2 dur1 dal80-1</i>
M1060	<i>MATα lys5 dur1 dal80-1</i> <i>MATα lys2 dur1 dal80-1</i>
M1410	<i>MATα lys5 dur1 dal81-2</i> <i>MATα lys2 dur1 dal81-2</i>
<i>E. coli</i>	
HB101	<i>hsdS20 (r_B⁻ m_B⁻) supE44 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>

cose at a final concentration of 2% and OXLU (0.25 mM) as the inducer. The poly(A)⁺ RNA preparations used for Northern (RNA) blot analyses were isolated from yeast cultures grown in Wickerham's minimal medium (36) supplemented with glucose to a final concentration of 2%. Nitrogen sources were provided at a concentration of 0.1%. Auxotrophies were supplemented where required, including the addition of L-glutamine at a concentration of 30 μ g/ml when strain RR91 was used (16). Media used for yeast transformation were yeast extract-peptone-dextrose (YEPD) for cell growth and YNB supplemented with 0.5% ammonium sulfate for selective growth following transformation. Casamino Acids, when used, were added to YNB medium at a concentration of 0.5%. Standard bacterial growth conditions were employed throughout this work. Yeast cells were transformed by the method of Ito et al. (19).

Determination of urea uptake by the assay of urea amidolysase activity. Yeast transformants were patched on YNB minimal agar plates and incubated overnight at 30°C. One hundred milliliters of YNB minimal medium, with proline (0.1%) as the sole nitrogen source and OXLU (0.25 mM) as the inducer, was inoculated from overnight patches and incubated at 30°C overnight with aeration. At a cell density of 30 Klett units, 2 ml of the culture was transferred at 1-min

intervals into 25-ml reaction flasks containing 1 μ Ci of [¹⁴C]urea (specific activity, 9.6 mCi/mM) and sufficient non-radioactive urea to yield a final concentration of 0.25 mM. The flasks were fitted with rubber caps, each supporting a plastic reaction cup containing 200 μ l of hyamine hydroxide (3). The flasks were preincubated for 5 min at 30°C with slow agitation. Reactions were terminated at 10-min intervals by the addition of 200 μ l of 70% perchloric acid with a 1-ml syringe. Forty-five minutes after the addition of acid, the cups were removed and dropped into 15 ml of liquid scintillation fluid. The radioactivity they contained was measured 24 h later to eliminate chemiluminescence.

Cloning of the *DUR3* gene. Strain M1534-26d was transformed with a YCp50-based yeast DNA library (27), and approximately 20,000 Ura⁺ clones were selected. These transformants were pooled and then spread on plates of selective medium (minimal glucose ureidoglycolate). Transformants that retained a stable Dur⁺ phenotype were purified and studied.

Northern blot analysis. Yeast strains used for Northern blot analyses were grown to the mid-log phase (40 to 60 Klett units) as measured on a Klett-Summerson photoelectric colorimeter (green filter). Total RNA was isolated by the method of Carlson and Botstein (8). Poly(A)⁺ RNA was isolated by using oligo(dT) cellulose (Pharmacia) columns and resolved on 1.4% agarose (Seakem; FMC Bioproducts) formaldehyde gels (30). The poly(A)⁺ RNAs were transferred to GeneScreen Plus nylon 66 (Dupont, NEN Research Products) membranes. The membranes were then hybridized to double-stranded DNA probes made radioactive by the randomly primed labelling method (Boehringer Mannheim). The procedures followed for capillary transfer of RNA and hybridization were those listed in the GeneScreen Plus protocol manual but without denaturation prior to RNA transfer.

Construction of the *DUR3* deletion strain. The *DUR3* deletion strain, HEY4, was constructed as depicted in Fig. 1. The 5.7-kb *Bam*HI-*Bam*HI fragment, containing the *URA3* selectable marker and genomic DNA flanking the *DUR3* gene, was isolated from plasmid pHE10 and used to transform wild-type yeast strain HEY2 (Table 1). Four stable ura⁺ transformants were selected and grown to saturation in YEPD medium. The cells were then washed with sterile water and plated onto 5-fluoroorotic acid medium (1). Genomic DNA was isolated from wild-type and 5-fluoroorotic acid-resistant colonies. Genomic Southern blot analysis was used to ascertain whether the *DUR3* gene had been replaced with a *hisG-URA3-hisG* fragment. Subsequently, we selected strains in which only the *hisG* fragment was retained (2). Southern blot analysis confirmed the structure of the desired deletion strain, HEY4.

Construction of the *DAL81* deletion strain. The *Bam*HI-*Pvu*II fragments from plasmid pPB48 (6) were used to construct strain HEY6 as mentioned above. Southern blot analyses were performed with strains TCY1 and HEY6 to confirm that the desired structure had been constructed.

DNA sequence analysis. The nucleotide sequence of the entire 3.8-kb *Bam*HI fragment of plasmid pHE9 was determined by using the Sequenase II protocol of U.S. Biochemical Corp. (32) and synthetic oligonucleotides as primers. Both strands were completely sequenced.

Hybridization and primer extension reactions. The procedure employed for hybridization and primer extension reactions was basically that of Frank et al. (17).

Nucleotide sequence accession numbers. The nucleotide sequence of the *DUR3* gene has been deposited in the GenBank data base under accession number L19875.

FIG. 1. Strategy used to disrupt the *DUR3* gene. Plasmid pHE10 was digested with *Bam*HI, and the desired 5.7-kb fragment was used for yeast transformation and replacement of the chromosomal *DUR3* gene.

RESULTS

Control of the urea active transport permease production. We first verified and extended the preliminary data in the literature concerning whether production of overall urea active transport activity was controlled by the allantoin pathway regulatory genes *DAL80* and *DAL81*. This was accomplished by constructing *dur1*, *dal80* and *dur1*, *dal81* double mutants. *dur1* mutants lack urea carboxylase activity and hence effectively dissociate the active transport of urea from its metabolism (13). The active transport of urea was then measured in these strains by assaying the intracellular accumulation of [¹⁴C]urea after growth in the presence or absence of inducer (i.e., OXLU). Urea active transport was low in cells grown without inducer and markedly increased when cultures were provided with OXLU (Fig. 2A, open and closed circles). In a *dal80* mutant strain grown without inducer, urea active transport levels were much greater than they were in wild-type cells provided with inducer (Fig. 2A, triangles). In contrast, *dal81* cultures exhibited only minimal urea active transport whether or not OXLU was provided (Fig. 2B, open and closed squares). These observations suggested that production of the overall urea active transport system was regulated in a manner similar to that of other allantoin pathway genes such as *DUR1*, *2* or *DAL7* (16, 18, 38).

FIG. 2. Assay of active transport of urea in wild-type and *dal80* and *dal81* mutant strains grown in the presence (+) or absence (–) of inducer (OXLU). The strains used in this experiment were M1060, M1058, M1059, and M1410. The pertinent portion of the genotype of each strain appears in the figure. Standard assay conditions were used (11, 28).

Chromosomal location of the *DUR3* gene. The *dur3* chromosomal location was determined by using standard genetic methods (23) involving a diploid strain (M1434) that was heterozygous for a *dur3* mutation as well as an array of marker mutations. As shown in Table 2, few nonparental ditype asci were observed for the *dur3-arg4*, *dur3-spo11*, or *arg4-spo11* gene pairs, indicating linkage of the three loci.

TABLE 2. Linkage of the *dur3*, *spo11*, and *arg4* CEN VIII loci

Cross	Gene pair	No. of tetrads analyzed	No. of parental ditypes	No. of non-parental ditypes	No. of tetra-types	Calculated genetic distance (cM) ^a	
						King and Mortimer ^b	Snow ^c
M1434	<i>arg4-dur3</i>	248	130	3	115	27.6	27.1
	<i>spo11-dur3</i>	248	185	1	62	13.5	13.8
	<i>arg4-spo11</i>	248	90	4	154	35.7	36.2

^a cM, centimorgans.

^b The formula used to obtain the reported values was from King and Mortimer (20a).

^c The formula used to obtain the reported values was from Snow (28).

TABLE 3. Analysis of recombinant asci from cross M1434

A horizontal line represents a genetic map. Above the line, from left to right, are markers: mak7, spo11, dur3, arg4, cr15, and put2. Below the line, a vertical tick mark is positioned between spo11 and dur3, labeled with a bold 'I'. Another vertical tick mark is positioned between dur3 and arg4, labeled with a bold 'II'. A solid black dot is located on the line between dur3 and arg4, slightly closer to dur3.

Rank *	Tetrad Region	Number of Asci	Strand Relations of Multiple Exchanges		
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2ST	3ST	4ST
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0	—	86			
1	I	44			
1	II	97			
2	I + II	17	4	11	2
2	II	2	0	0	2
3	I + II	2	0	0	2

* Indicates the number of exchanges that occurred.

Genetic distances derived from this three-point cross, calculated by the method of either Snow (28) or King and Mortimer (20a), suggested a gene order of *spo11-dur3-arg4* (Table 2). This order of loci was also supported by the

segregation patterns of the markers in the cross (Table 3). The above data placed the *dur3* locus about 14 centimorgans proximal to *spo11* on the left arm of chromosome VIII (Table 3, genetic map).

Isolation of the *DUR3* gene. Recombinant plasmids putatively containing the *DUR3* gene were isolated by transformation complementation procedures as described in Materials and Methods. We isolated plasmids from eight transformants that complemented the growth defect (poor growth on minimal glucose-ureidoglycollate medium) of a urea permease-deficient mutant strain, M1534-26d. We analyzed three of these plasmids, which turned out to be the same, and chose one (plasmid pTSC239) for more detailed analysis. The restriction map of plasmid pTSC239, along with those of a number of relevant subclones derived from it, appears in Fig. 3.

To obtain evidence that these plasmids contained the desired gene, we subcloned the 3.5-kb *EcoRI* fragment derived from plasmid pTSC239 into vector YIp5 and used the resulting plasmid (pRH1) as the source of DNA to transform strain M1-2b. We selected a stable *Ura*⁺ transformant after growth to saturation in YEPD medium. This transformant was then crossed to mapping strain K398-4D, the resulting diploid strain was sporulated, and the meiotic

FIG. 3. Restriction map of and DNA sequencing strategy for DNA fragments containing the *DUR3* locus. The bottom portion of the figure depicts the results of experiments testing the abilities of various DNA fragments derived from the insert of plasmid pTSC239 to complement the growth defect of the *dur3* mutation. Ureidoglycollate (UG) was used as the diagnostic nitrogen source.

TABLE 4. Linkage between the *spo11*, *arg4*, and integrated *URA3* loci

Cross	Gene pair	No. of parental ditypes	No. of nonparental ditypes	No. of tetratypes	Genetic distance (cM) ^a
M1672	<i>arg4-URA3</i>	31	0	16	17
	<i>spo11-URA3</i>	25	0	22	22
	<i>arg4-spo11</i>	18	0	29	31

^a These values were obtained by using the formula of Perkins (23). cM, centimorgans.

products were analyzed. As shown in Table 4, strong linkage was observed between the *URA3*, *arg4*, and *spo11* markers. The only unsettling aspect of these data was that the observed distance between the *arg4* and *URA3* markers was smaller than that between *URA3* and *spo11* (Table 4). The reason underlying this observation is not clear. It may have derived from using completely different strains in the two genetic crosses or from analyzing a limited number of tetrads. In any case, this result made us more cautious as we evaluated subsequent data.

Localization of the *DUR3* gene. We localized the sequences of plasmid pTSC239 responsible for complementation of the *dur3* growth phenotype (poor growth on ureidoglycollate) by subcloning various DNA fragments from this plasmid and testing them for their ability to complement *dur3* mutations in a fashion similar to pTSC239. As shown in Fig. 3 (bottom), the smallest complementing DNA fragment (3.8 kb) was

contained in plasmid pTSC316. The insert of plasmid pTSC311 is identical to that contained in plasmid pTSC309; however, the vectors used for plasmids pTSC309 and pTSC311 were pBR322 and YCp50, respectively.

A transcription map of the insert of plasmid pTSC309, which was able to complement the *dur3* mutant phenotype, was constructed. To generate this map, the insert of plasmid pTSC309 was divided into six DNA fragments (Fig. 4, fragments A to F). The isolated fragments were labeled and hybridized to poly(A)⁺ RNA preparations derived from strain M1079 grown in minimal glucose-proline medium containing 0.25 mM OXLU. As shown in Fig. 4, two RNA species approximately 2.1 and 0.7 kb in length hybridized to these probes. Fragment A showed strong hybridization to the 0.7-kb mRNA but not to the 2.1-kb species. Fragment B behaved similarly, except that very weak hybridization to the 2.1-kb species could be detected in overexposures of the autoradiograph. Fragment C exhibited strong hybridization to both RNA species. Fragments D, E, and F both hybridized strongly to the 2.1-kb species. On the basis of this preliminary transcript map, we subcloned the 2.4-kb *EcoRI* fragment from plasmid pTSC309 to yield plasmid pHE8. Plasmids pHE8 and pTSC316 were used to transform mutant strain M1534-26d, but only the larger of the two plasmids (pTSC316) was able to complement the mutant defect.

Our next objective was to ascertain whether DNA sequences carried on plasmid pTSC239, the original isolate, and subcloned plasmids pTSC311 and pTSC316 were able to restore urea transport to an uptake-defective *dur3* strain. Since we were not studying the quantitative kinetic details of

FIG. 4. Transcript map of plasmid pTSC309. The boxes below the restriction map of plasmid pTSC309 represent the fragments used as radioactive probes. RNA was derived from wild-type (strain M1079) cells grown in 0.1% minimal glucose-proline medium in the presence of OXLU (0.25 mM). Poly(A)⁺ RNA samples (10 µg per lane) were resolved in a 1.4% formaldehyde-agarose gel and transferred to GeneScreen Plus membrane. The Northern blots were hybridized to the indicated DNA fragments. The fragments used for hybridization are indicated by the letters in the boxes and along the top of the autoradiograms.

FIG. 5. (A) Differential rate of urea amidolase activity in *dur3* mutant strain HEY1 transformed with plasmids YCp50, pTSC316, pTSC311, and pTSC239 and wild-type strain M384-22A (wt). Cultures were grown to a cell density of 30 Klett units in minimal YNB medium with 0.1% proline as the sole nitrogen source and 0.25 mM OXLU as the inducer. Urea amidolase activity was assayed at 10-min intervals over 90 min. The reactions were terminated by the addition of 70% perchloric acid, and ¹⁴CO₂ was collected in hyamine hydroxide (35). (B) Differential rate of urea amidolase production in *dur3* deletion strain HEY4 transformed with plasmids YCp50, pTSC316, and pTSC311 and the wild-type strain HEB2 (wt) transformed with YCP50. Growth and assay procedures were the same as those described for panel A.

urea transport but rather were qualitatively determining whether the biochemical defect of a *dur3* mutant strain could be complemented by the cloned DNA, we used an indirect assay of urea uptake employing an easily transformable *dur3* mutant. For this assay, *dur3* mutant cultures were grown in the presence of OXLU, which induces high levels of urea amidolase (the *DUR1,2* gene product), which degrades urea to carbon dioxide and ammonia (4, 31, 35). Therefore, if exogenously provided radioactive urea entered the cell, it would be rapidly degraded to carbon dioxide, which we could easily measure by standard assay methods (5). However, urea could only enter the cell if the *dur3* mutation was complemented. Therefore, we incubated cultures of strain HEY1, transformed with plasmids YCp50 (the vector), pTSC239, pTSC311, and pTSC316, with [¹⁴C]urea and monitored the amount of radioactive carbon dioxide evolved. As shown in Fig. 5A, when plasmid YCp50 was used as the source of transforming DNA, no radioactive carbon dioxide was detected (open circles). In contrast, when any one of the three plasmids mentioned above were used in place of plasmid YCp50, clearly measurable quantities of carbon dioxide evolved from the cultures. However, the level of CO₂ evolution observed in the transformants was only a small fraction of the levels observed in the wild type. Such a result could derive from (i) a defect in the assay, (ii) failure of the transforming plasmids to contain all of the DNA sequences necessary for complementation of the *dur3* mutations, or (iii) negative complementation of the plasmid-borne wild-type gene by the defective chromosomal copy. We did not consider either of the first two explanations as likely possibilities. The only means of effectively evaluating the third possibility was to repeat the experiment with a *dur3* deletion strain.

A deletion constructed *in vitro* could also be used directly to assess whether our cloned sequences contained the *DUR3* gene. If the 2.1-kb transcript encoded the *DUR3* gene, then deletion of the 1.9-kb *Bgl*III-*Bgl*III fragment (and its replacement with a selectable marker) should generate a phenotype similar to that of the *dur3* mutant isolated by classical genetic methods (29). We constructed such a deletion as described in Materials and Methods (Fig. 1) and replaced the wild-type allele encoding the 2.1-kb transcript. After verification of the genomic structure in the area of the deletion by Southern blot analysis (data not shown), we streaked several independently isolated deletion mutants and a wild-type positive control strain on minimal media containing either urea, ureidoglycollate, or ammonium sulfate as the sole nitrogen source. All of the deletion-disruption mutants failed to grow on urea- or ureidoglycollate-containing plates (16a). These deletion-disruption mutations, like the previously reported ethyl methanesulfonate (EMS)-generated *dur3* mutations, were recessive to the wild-type allele. Only parental asci were observed to emanate from a cross of the deletion-disruption mutant to a strain containing an EMS-generated *dur3* mutation (data not shown).

These results were consistent with the suggestion that the gene encoding the 2.1-kb transcript was the same one mutated in the *dur3* mutant. We proceeded on this basis and repeated the biochemical measurements described in the legend to Fig. 5A. As shown in Fig. 5B, quite different results were observed compared with those observed with the EMS-generated *dur3* mutant. As before, no demonstrable CO₂ evolution was observed when the deletion mutant was transformed with vector plasmid YCp50. However, when plasmid pTSC311 or pTSC316 was used, high-level CO₂ evolution occurred. In this case, CO₂ evolution was the

										-150*					-130*						
										TGCATGAAGATACGCTCTTGTTCGCCAAGTATATAAAAT											
-110*					-90*					-70*					-50*						
ATAAAATGAAGCAGTAGTATAGCCGTCAGCTTTACTATTGTTAGCAGTTTCCCTTGTCTAGATTCTTAAATTTGGGTTTCAT																					
-30*					-10*					10*					30*						
CCAAGAAAAAGTAATAACATAAATTTGTCATTGTTATAGTATG										GGA	GAA	TTT	AAA	CCT	CCG	CTA	CCT	CAA			
										Met	Gly	Glu	Phe	Lys	Pro	Pro	Leu	Pro	Gln		
50*										70*										90*	
GGC	GCT	GGG	TAC	GCT	ATT	GTA	TTG	GGC	CTA	GGG	GCC	GTA	TTT	GCA	GGA	ATG	ATG	GTT	TTG		
Gly	Ala	Gly	Tyr	Ala	Ile	Val	Leu	Gly	Leu	Gly	Ala	Val	Phe	Ala	Gly	Met	Met	Val	Leu		
110*										130*										150*	
ACC	ACT	TAT	TTA	CTG	AAA	CGT	TAT	CAA	AAG	GAA	ATC	ATC	ACA	GCA	GAA	GAA	TTC	ACC	ACC		
Thr	Thr	Tyr	Leu	Leu	Lys	Arg	Tyr	Gln	Lys	Glu	Ile	Ile	Thr	Ala	Glu	Glu	Phe	Thr	Thr		
170*										190*										210*	
GCC	GGT	AGA	TCT	GTA	AAA	ACC	CGG	CTT	AGT	GGC	TGC	AGC	GTG	GTT	TCT	AGT	TGG	ATC	TGG		
Ala	Gly	Arg	Ser	Val	Lys	Thr	Arg	Leu	Ser	Gly	Cys	Ser	Val	Val	Ser	Ser	Trp	Ile	Trp		
230*										250*										270*	
TGT	TCT	ACA	TTG	TTA	ACG	TCG	TCA	ACA	AAG	GAA	TAT	GCA	GAC	GGT	ATA	TTT	GGC	GGT	TAT		
Cys	Ser	Thr	Leu	Leu	Thr	Ser	Ser	Thr	Lys	Glu	Tyr	Ala	Asp	Gly	Ile	Phe	Gly	Gly	Tyr		
290*										310*										330*	
GCG	TAC	GCT	GCT	GGC	GCA	TGC	TTC	CAA	ATT	ATT	GCA	TTC	GCA	ATT	TTG	GCA	ATT	AAA	ACC		
Ala	Tyr	Ala	Ala	Gly	Ala	Cys	Phe	Gln	Ile	Ile	Ala	Phe	Ala	Ile	Leu	Ala	Ile	Lys	Thr		
350*										370*										390*	
AAG	CAA	ATG	GCT	CCC	AAT	CGC	CAC	ACA	TAT	TTA	GAA	TTA	GTG	AGA	ACA	AGA	TAT	GGT	AAG		
Lys	Gln	Met	Ala	Pro	Asn	Ala	His	Thr	Tyr	Leu	Glu	Leu	Val	Arg	Thr	Arg	Tyr	Gly	Lys		
410*										430*										450*	
ATC	GGC	CAT	GGT	TGC	TAC	TTG	TTT	TAT	GCC	ATC	GCG	ACG	AAT	ATT	TTA	GTC	ACT	TCA	ATG		
Ile	Gly	His	Gly	Cys	Tyr	Leu	Phe	Tyr	Ala	Ile	Ala	Thr	Asn	Ile	Leu	Val	Thr	Ser	Met		
470*										490*										510*	
CTT	TTA	ACT	TCA	GGT	TCT	GCT	GTC	TTT	AGT	GAT	TTA	ACC	GGG	ATG	AAC	ACT	ATC	GCA	TCA		
Leu	Leu	Thr	Ser	Gly	Ser	Ala	Val	Phe	Ser	Asp	Leu	Thr	Gly	Met	Asn	Thr	Ile	Ala	Ser		
530*										550*										570*	
TGT	TTT	TTA	CTG	CCT	GTG	GGT	GTT	GTT	GTT	TAT	ACT	CTA	TTT	GGT	GGG	ATT	AAA	GCA	ACT		
Cys	Phe	Leu	Leu	Pro	Val	Gly	Val	Val	Val	Tyr	Thr	Leu	Phe	Gly	Gly	Ile	Lys	Ala	Thr		
590*										610*										630*	
TTC	TTA	ACG	GAC	TAT	ATG	CAC	ACA	TGT	GTC	ATT	ATC	ATC	ATT	GTC	CTC	GTA	TTT	GCC	TTT		
Phe	Leu	Thr	Asp	Tyr	Met	His	Thr	Cys	Val	Ile	Ile	Ile	Ile	Val	Leu	Val	Phe	Ala	Phe		
650*										670*										690*	
AAA	GTT	TAT	GCG	ACT	AGT	GAT	GTT	TTA	GGC	TCA	CCG	GGA	AAA	GTT	TAT	GAC	TTA	GTT	CGT		
Lys	Val	Tyr	Ala	Thr	Ser	Asp	Val	Leu	Gly	Ser	Pro	Gly	Lys	Val	Tyr	Asp	Leu	Val	Arg		
710*										730*										750*	
GAA	GCC	GCC	AAG	AGG	CAT	CCA	GTA	GAC	GGT	AAC	TAT	CAG	GGT	GAA	TAT	ATG	ACC	ATG	ACA		
Glu	Ala	Ala	Lys	Arg	His	Pro	Val	Asp	Gly	Asn	Tyr	Gln	Gly	Glu	Tyr	Met	Thr	Met	Thr		
770*										790*										810*	
TCC	AAA	TCC	GCT	GGT	ATT	TTA	TTA	ATT	ATT	AAC											

FIG. 6. Nucleotide sequence of the *DUR3* gene. Arrows under the sequence indicate the positions of major and minor extension fragment termini.

1130* 1150* 1170*
 GAA CTA ATT GCC GTT TCA TCT GTT TTC ACT TAC GAT ATC TAT AGA GAA TAT ATT GAT CCT
 Glu Leu Ile Ala Val Ser Ser Val Phe Thr Tyr Asp Ile Tyr Arg Glu Tyr Ile Asp Pro
 1190* 1210* 1230*
 CGT GCA AGC GGT AAG AAA TTG ATT TAC ACA TCA CAC GTT GCT TGT ATC TTT TTT GGT CTT
 Arg Ala Ser Gly Lys Lys Leu Ile Tyr Thr Ser His Val Ala Cys Ile Phe Phe Gly Leu
 1250* 1270* 1290*
 GCC ATG AGT GGA TTT TCG GTT GGT TTA TAC TAT GGT GGT ATT TCT ATG GGT TAT ATC TAT
 Ala Met Ser Gly Phe Ser Val Gly Leu Tyr Tyr Gly Gly Ile Ser Met Gly Tyr Ile Tyr
 1310* 1330* 1350*
 GAA ATG ATG GGT ATA ATT ATT AGT AGT GCA GTA TTA CCT GTC GTT TTG ACC TTA TGT TCC
 Glu Met Met Gly Ile Ile Ile Ser Ser Ala Val Leu Pro Val Val Leu Thr Leu Cys Ser
 1370* 1390* 1410*
 AAA GAC ATG AAT TTG GTG GCC GCT GTA GTG TCG CCT ATT TTG GGC ACA GGA CTG GCT ATA
 Lys Asp Met Asn Leu Val Ala Val Val Ser Pro Ile Leu Gly Thr Gly Leu Ala Ile
 1430* 1450* 1470*
 ATG TCA TGG CTT GTC TGT ACC AAA TCC CTT TAT AAA GAA TTG ACC GTG GAT ACT ACG TTC
 Met Ser Trp Leu Val Cys Thr Lys Ser Leu Tyr Lys Glu Leu Thr Val Asp Thr Thr Phe
 1490* 1510* 1530*
 ATG GAT TAT CCA ATG TTA ACA GGT AAC TTG GTG GCT TTG CTA TCA CCA GCC ATT TTT ATT
 Met Asp Tyr Pro Met Leu Thr Gly Asn Leu Val Ala Leu Leu Ser Pro Ala Ile Phe Ile
 1550* 1570* 1590*
 CCT ATT TTA ACG TAT GTG TTT AAG CCA CAA AAT TTT GAC TGG GAG AAA ATG AAA GAT ATT
 Pro Ile Leu Thr Tyr Val Phe Lys Pro Gln Asn Phe Asp Trp Glu Lys Met Lys Asp Ile
 1610* 1630* 1650*
 ACT AGA GTT GAC GAA ACT GCA GAG TTA GTT CAG GCT GAC CCT GAT ATC CAG CTT TAC GAT
 Thr Arg Val Asp Glu Thr Ala Glu Leu Val Gln Ala Asp Pro Asp Ile Gln Leu Tyr Asp
 1670* 1690* 1710*
 GCT GAA GCT AAC GAT AAG GAA CAA GAA GAA GAA ACA AAT TCT CTG GTC TCA GAT AGT GAA
 Ala Glu Ala Asn Asp Lys Glu Gln Glu Glu Glu Thr Asn Ser Leu Val Ser Asp Ser Glu
 1730* 1750* 1770*
 AAA AAC GAT GTT AGA GTA AAT AAT GAA AAA TTG ATT GAG CCT AAC CTT GGT GTT GTA ATA
 Lys Asn Asp Val Arg Val Asn Asn Glu Lys Leu Ile Glu Pro Asn Leu Gly Val Val Ile
 1790* 1810* 1830*
 AGT AAT GCC ATT TTT CAA GAA GAT GAC ACA CAG TTA CAA AAT GAA TTA GAC GAA GAA CAA
 Ser Asn Ala Ile Phe Gln Glu Asp Asp Thr Gln Leu Gln Asn Glu Leu Asp Glu Glu Gln
 1850* 1870* 1890*
 AGA GAA CTA GCA CGT GGT TTA AAA ATT GCA TAC TTC CTA TGT GTT TTT TTC GCT TTG GCA
 Arg Glu Leu Ala Arg Gly Leu Lys Ile Ala Tyr Phe Leu Cys Val Phe Phe Ala Leu Ala
 1910* 1930* 1950*
 TTT TTG GTA GTT TGG CCC ATG CCC ATG TAT GGT TCC AAA TAT ATC TTC AGT AAA AAA TTC
 Phe Leu Val Val Trp Pro Met Pro Met Tyr Gly Ser Lys Tyr Ile Phe Ser Lys Lys Phe
 1970* 1990* 2010*
 TTT ACC GGT TGG GTT GTT GTG ATG ATC ATC TGG CTT TTT TTC AGT GCG TTT GCC GTT TGT
 Phe Thr Gly Trp Val Val Val Met Ile Ile Trp Leu Phe Phe Ser Ala Phe Ala Val Cys
 2030* 2050* 2070*
 ATT TAT CCA CTC TGG GAA GGT AGG CAT GGT ATA TAT ACC ACT TTG CGA GGC CTT TAC TGG
 Ile Tyr Pro Leu Trp Glu Gly Arg His Gly Ile Tyr Thr Thr Leu Arg Gly Leu Tyr Trp
 2090* 2110* 2130*
 GAT CTA TCT GGT CAA ACT TAT AAA TTA AGG GAA TGG CAA AAT TCG AAC CCA CAA GAT CTG
 Asp Leu Ser Gly Gln Thr Tyr Lys Leu Arg Glu Trp Gln Asn Ser Asn Pro Gln Asp Leu
 2150* 2170* 2190*
 CAT GTA GTA ACA AGC CAA ATT AGT GCG AGA GCA CAT AGA CAA TCA TCA CAT TTC GGA CAA
 His Val Val Thr Ser Gln Ile Ser Ala Arg Ala His Arg Gln Ser Ser His Phe Gly Gln
 2210* 2230* 2250*
 GTT GAT GAA ATA ATTTAGCTTTCAAAATATAATATTCTATCCACTCATCAACCTCATTAATAATTTTAAATAGC
 Val Asp Glu Ile Ile
 2270* 2290* 2310* 2330*
 TCTAATTATAAAATAGTCTTTTATTATAGGTACATAAAAGAAAAGTCAAGGGGAAAAAAGGAAAT
 2350* 2370* 2390* 2410*
 TTCTAAATTTA

FIG. 6—Continued.

FIG. 7. Hydropathy plot of *DUR3* protein constructed by the method of Kyte and Doolittle (21). A window of seven amino acids was used in this analysis.

same as that of the wild-type positive control. These data suggest that strong negative complementation was the reason for the poor uptake observed with the EMS-generated *dur3* mutant (Fig. 5A).

Nucleotide sequence of the *DUR3* gene. By using the strategy depicted in Fig. 3, we determined the nucleotide sequence of the *DUR3* gene and its flanking regions. Both strands were completely sequenced. The gene possessed an open reading frame of 2,205 bp putatively encoding a 735-amino-acid protein with a calculated M_r of 80,737 and a pI of 5.59 (Fig. 6). Hydropathy analysis (21) of the deduced *DUR3* protein sequence indicated that overall it was very hydrophobic and possessed alternating hydrophilic and hydrophobic regions characteristic of other permease components whose structures have been reported (Fig. 7) (25).

Regulated expression of *DUR3*. We showed in the results

FIG. 8. Northern blot analysis of poly(A)⁺ RNAs derived from wild-type (M1079) and *dal80* mutant (M1081) strains grown in the presence or absence of inducer or in the presence of the repressing nitrogen source asparagine. Lanes A, B, and E contain poly(A)⁺ from cells grown on proline (PRO, 0.1%). Culture medium for cells used in lane B contained 0.25 mM oxalurate as inducer. Lanes C and D derived from cells grown on asparagine (ASN, 0.1%). The RNA species (10 µg per lane) were hybridized to the 5.7-kb *Bam*HI-*Bam*HI fragment of plasmid pTSC309 and plasmid TCM3.1 (*TCM1* gene) labeled by the randomly primed DNA synthetic method. The 0.7-kb species is an unknown message.

FIG. 9. Expression of the *DUR3* gene in wild-type, *dal81* mutant (left), and *dal82* mutant (right) cultures grown in minimal glucose-proline medium (PRO). OXLU was provided at a final concentration of 0.25 mM where indicated. The mRNA species (7 µg) were hybridized to the 3.8-kb *Bam*HI-*Bam*HI fragment of plasmid pTSC316, and plasmid TCM 3.1 was labeled by the randomly primed method. Lanes A, B, E, and F contain poly(A)⁺ RNA prepared from wild-type strain TCY1, lanes C and D were derived from *dal81* deletion strain HEY6, and lanes G and H were derived from *dal82* mutant strain M02.

FIG. 10. Effect of the disruption of the *GLN3* gene on expression of *DUR3*. The experimental conditions for this experiment were as described in the legend to Fig. 9 except that glutamine (34 µg/ml) was added to both wild-type and *gln3* mutant cultures to supplement the glutamine auxotrophy that resulted from the *gln3* mutation. We have previously shown that glutamine addition at this low level does not elicit NCR. The wild-type and *gln3* disruption strains were TCY1 and RR91, respectively.

depicted in Fig. 2 that overall urea active transport, a component of which is encoded by *DUR3*, was an allophanate- or OXLU-inducible process controlled by the allantoin pathway regulatory genes. Therefore, we expected the 2.1-kb transcript observed in Fig. 4 to be inducible, responsive to the *DAL* regulatory genes, and sensitive to NCR (13). To assess these characteristics, we isolated poly(A)⁺ RNA from wild-type strain M1079 grown in minimal proline medium with or without OXLU. In addition, the strain was grown in medium containing asparagine as the sole nitrogen source, a condition that elicits strong NCR (9, 16). The 0.7-kb transcript was insensitive to the presence of OXLU or to NCR (Fig. 8, lanes A to C). In contrast, the steady-state amount of 2.1-kb transcript increased about 10-fold (determined by densitometric scanning of the autoradiogram) when OXLU was added to the culture medium (Fig. 8, lanes A to C). The above experiment was repeated in a *dal80* mutant strain, M1081, grown either in glucose-proline or glucose-asparagine medium. The 0.7-kb transcript was again refractile to physiological or genetic manipulation, whereas the amount of the 2.1-kb transcript increased approximately 45-fold in the *dal80* mutant strain M1081 compared with that in the wild type (Fig. 8, compare lanes A and E). Expression of the 2.1-kb transcript in the *dal80* mutant was also very sensitive to NCR, decreasing by approximately 90-fold in glucose-asparagine-grown cells compared with those grown in glucose-proline medium (Fig. 8, compare lanes E and D).

The transcription factors required for *DUR3* response to inducer were identified by assaying steady-state levels of *DUR3* mRNA in wild-type and mutant strains grown with or without inducer. As shown in Fig. 9 (lanes A, B, E, and F), a wild-type strain yielded an inducer response similar to the one described in Fig. 8. When the experiment was repeated in strains carrying either a deletion of *dal81* or a mutation of *dal82*, the response to the addition of inducer to the medium was minimal (Fig. 9, lanes C, D, G, and H). To assess the extent of *GLN3* requirement for *DUR3* expression, we compared the levels of *DUR3* mRNA in uninduced cultures of wild-type and *gln3* mutant strains. There is a *GLN3* requirement for *DUR3* expression, but it is not as strong as that observed for the *DAL5* gene (compare Fig. 4, lanes A and C, of reference 11 with Fig. 10, lanes A and C, of this work). We did not measure *DUR3* mRNA levels in the *gln3* mutant strain grown in medium containing OXLU because in some strains OXLU uptake requires the *GLN3* product (23a).

5' Terminus of *DUR3* RNA. To determine the approximate position of the *DUR3* 5' terminus, we used a standard primer extension method (17). Poly(A)⁺ RNA preparations were prepared from cultures of wild-type strain M1079 grown in minimal glucose-proline medium with and without 0.25 mM OXLU (final concentration). Products of the primer extension reaction, performed as described in Materials and Methods, were resolved on an acrylamide sequencing gel along with the products of sequence reactions. The complete reaction mixture with RNA derived from induced cultures yielded two major extension fragments which terminated at positions -29 and -39 (data not shown). Additional minor species terminated at positions -26, -28, -43, -54, -55, and -56 (Fig. 6). None of the above primer extension products were observed when RNA from uninduced cultures was used in place of that from induced cells. Similarly, no extension products were observed if RNA, primer, or reverse transcriptase was omitted from the reaction mixture.

DISCUSSION

This work describes isolation and structural characterization of the first gene whose product participates in the active transport of urea in *S. cerevisiae*, *DUR3*. As expected, the protein encoded by *DUR3* exhibits a hydropathy profile consistent with that expected of an integral membrane protein. During experiments to verify the nature of the cloned DNA fragment, we found that an EMS-generated *DUR3* mutation (presumed to be a point mutation) exhibited a remarkably strong ability to negatively complement a plasmid-borne wild-type allele. Such negative complementation usually derives from the formation of polymeric protein complexes consisting of functional and nonfunctional protein monomers. If this interpretation applies in the case of *DUR3*, it suggests that *DUR3* protein polymerizes into its physiologically functional state.

DUR3 expression is regulated in a fashion similar to that of the other allantoin pathway genes; it is highly inducible when allophanate or OXLU is present in the cell, and induction requires participation of the *DAL81* and *DAL82* genes (6, 22). Low-level *DUR3* expression is maintained by a functional *DAL80* gene product when inducer is absent. These characteristics are similar to those observed when overall urea active transport was measured (29; and this work).

These data raise a question concerning the relationship of proteins that participate in urea active transport and facilitated diffusion. The latter process appears to occur in an inducer-independent fashion and is not sensitive to NCR (13, 29). Therefore, the urea-binding protein that mediates its facilitated diffusion must be present in an inducer-independent, NCR-insensitive manner. These observations prompt several questions about the function of the *DUR3* product. Is the *DUR3* product the urea-binding protein required for active transport? If it is, then one must suggest the existence of a second urea-binding protein to participate in facilitated diffusion. Alternatively, it might be a protein that participates in coupling a single urea-binding protein to the energy transduction complex needed for active transport to occur. Distinguishing these possibilities will require considerably more information about the urea-facilitated diffusion system. This would include identification and isolation of the additional genes and cognate proteins that mediate the active transport of urea in this system.

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