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City University of New York, 1991

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GENETIC, STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE ALPHA-GLUCOSIDE TRANSPORTER ENCODED BY *MAL11-2*

by

Francis E. Cotty

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1991

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

GENETIC STRUCTURAL AND FUNCTIONAL ANALYSIS
OF THE ALPHA-GLUCOSIDE TRANSPORTER ENCODED BY *MAL11-2*

by

Francis E. Cotty

Advisor: Professor Corinne Michels

The determining factor in α-glucoside fermentation by *Saccharomyces* is the transport of this class of sugars into the cell. Different *Saccharomyces* strains ferment various α-glucosides including maltose, turanose, isomaltose, maltotriose and sucrose. Previous work in our laboratory has characterized the maltose permease encoded by the *MAL1* and *MAL6* loci. The *MAL1g* allele is a naturally occurring partially functional variant of *MAL1* proposed to be the result of a chromosomal rearrangement of the right telomere region of chromosome VII. Based upon restriction endonuclease mapping and Southern analysis, the genes encoding maltose permease at *MAL1* and *MAL1g* share no sequence homology. In this work we present a structural and functional characterization of the newly identified maltose permease encoded by the *MAL1g* allele of the *MAL1* locus of *Saccharomyces cerevisiae*, referred to as *MAL11-2*. *MAL11-2* encodes a highly hydrophobic proposed integral membrane protein with considerable structural homology to the *MAL61* encoded high-affinity maltose transporter, as well as to the *SNF3*, *HXT1* and *HXT2* encoded high affinity glucose transporters from *Saccharomyces*, and several transporters from a variety of organisms considered to

be members of the sugar transporter superfamily. The *MAL11-2* transporter is a high-affinity proton symporter with a Km for maltose of 4 to 5 mM. The sequence of the upstream region of the *MAL1g* allele is presented and compared to that of the *MAL6* locus. The 315 base pairs immediately upstream of *MAL11-2* show no significant homology to the *MAL6* locus. Starting at base pair -316 of the *MAL1g* allele the upstream sequence was found to be 100% identical to a 469 base pair region of the *MAL61-MAL62* intergenic region containing the UAS_{MAL} and extending beyond this into the *MAL12* open reading frame. The α-glucoside specificity of both the MAL11-1 permease and the MAL11-2 permease was determined using plasmid complementation in a genetically defined strain. We found the MAL11-1 maltose transporter is capable of transporting only maltose and turanose, while the MAL11-2 transporter is capable of transporting isomaltose, α-methylglucoside, maltotriose and palatinose as well as maltose and turanose. Neither is able to transport melezitose or trehalose.

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INTRODUCTION

The fermentation of α-glucosides by strains of *Saccharomyces* yeast is commercially important as an essential step in bread, beer, wine and champaign production. Naturally occurring strains of yeast ferment various α-glucosides found in flour and wort such as: maltose, maltotriose, isomaltose, turanose, melezitose, palatinose and sucrose. Transport of these sugars is the rate limiting step in their fermentation. As many as seven distinct α-glucoside transporters have been proposed based on biochemical and genetic analysis of various *Saccharomyces* strains (Lindegren and Lindegren, 1956; Barnett, 1981). Of these only one, the maltose permease encoded by the MAL loci, has been characterized in any detail (Cheng and Michels, 1989; Cheng and Michels, 1991). In this report we describe a second α-glucoside transporter.

I. Structure and function of the MAL loci

The *Saccharomyces cerevisiae* maltose fermentation phenotype is determined by a cluster of three genes referred to as the *MAL* locus. Five unlinked *MAL* loci have been identified and all are telomere-linked: *MAL1* (chromosome VII), *MAL2* (chromosome III), *MAL3* (chromosome II), *MAL4* (chromosome XI), and *MAL6* (chromosome VIII) (Charron et al., 1989, Mortimer and Schild, 1980). Fermenting strains may contain one or more of these loci (Charron et al., 1989, Chow et al.,

1983; Michels and Needleman, 1983, Michels and Needleman, 1984; Needleman and Michels, 1983; ten Berge, 1971). The three genes (GENE 1, GENE 2 AND GENE 3) which comprise a *MAL* locus are each required for the fermentation of maltose (Charron, Dubin and Michels, 1986; Cohen et al., 1984a, Cohen et al., 1984b; Michels and Needleman, 1984; Needleman et al., 1985). A nomenclature has been established to indicate both the GENE number and locus position; for example, *MAL61* represents GENE 1 at the *MAL6* locus (Needleman et al., 1985). The function of each gene in maltose fermentation has been determined.

GENE1 encodes maltose permease. Maltose transport is considered to be the rate limiting function in maltose fermentation (Cooper, 1982; Gortz, 1969; Sols and DelaFuente, 1971). The evidence that the GENE 1 homologues at each *MAL* locus encodes the maltose transporter is:

1. a mutant allele encoding a temperature sensitive maltose permease mapping to the *MAL1* locus has been identified (Goldenthal, Cohen and Marmur, 1983).
2. chemically induced maltose non-fermenting mutations of *MAL6* mapping to the *MAL61* gene lose the ability to transport maltose (Chang et al., 1989).
3. insertion of the yeast *URA3* gene into the N-terminal coding region of *MAL61* results in constitutive production of both the *MAL61* m-RNA and maltose permease (Chang et al., 1989).

4. transformation of yeast strains with high copy number plasmids containing the *MAL61* gene leads to a ten-fold increase in the level of maltose transport (Chang et al., 1989)
5. a deletion/disruption of *MAL61* completely abolishes maltose fermentation and maltose transport (Chang et al., 1989).
6. *MAL61* has been cloned and sequenced. It encodes a protein of 614 amino acids and approximate mass of 67,000 daltons (Cheng and Michels, 1989; Yao, Sollitti and Marmur, 1989).

The MAL61 protein displays considerable structural homology and approximately 25% sequence identity with known sugar transporters including: SNF3, a component of the high affinity glucose transporter of yeast; GLUT1, one of the five identified mammalian glucose transporters; and LAC12, the lactose transporter of *Kluyveromyces lactis*. Computer analysis of the deduced *MAL61* sequence predicts a protein composed of twelve hydrophobic domains in two blocks of six domains each separated by a hydrophilic region of approximately 70 residues. Therefore, *MAL61* appears to encode an integral membrane maltose transport protein (Cheng and Michels, 1989).

GENE 2 encodes the enzyme maltase. The sequence of *MAL62* has been reported by Hong and Marmur (Hong and Marmur, 1986). This and other lines of research support the hypothesis that

this gene encodes maltase (Dubin et al., 1985; Federoff et al., 1982). Conclusive demonstration comes from genetic studies in which a temperature sensitive maltase variant was identified and shown to map to the *MAL12* gene of the *MAL1* locus (Cohen et al., 1984). Hydrolysis of maltose and other α-glucosides occurs only within the cell (Barnett, 1976, Cooper, 1982). Two α-glucosidases have been identified and characterized; they are maltase and isomaltase (α-methylglucosidase) (Barnett, 1976; Enzyme Nomenclature, 1978). These enzymes hydrolyze maltose, turanose, isomaltose, maltotriose, α-methylglucoside, melezitose and other α-glucosides, including sucrose, which contains both an α- and a β- glucoside linkage (Barnett, 1976). (For a detailed description of the structure of each of these sugars, see below). The intracellular presence of these α-glucosidases guarantees the fermentation of the α-glucoside so long as it is capable of being transported into the cell (Chang et al, 1989; Cooper, 1982; Dubin et al., 1988; Dubin et al., 1986).

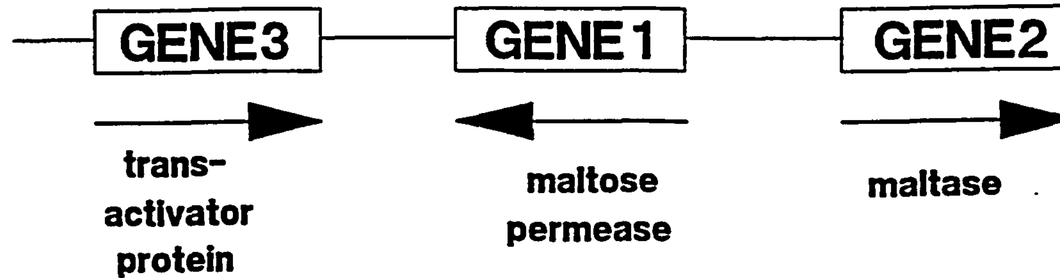
GENE 3 codes for the MAL activator. Deletion/ disruption of GENE 3 causes a non-inducible phenotype indicating that the GENE 3 product is a *trans*-acting protein required for the maltose inducible expression of GENE 1 and GENE 2 (Chang et al., 1988, Cohen et al., 1984a; Cohen et al., 1984b; Needleman et al., 1985). The MAL63 protein has been shown to bind within the upstream sequence of the *MAL61* and *MAL62* genes (Ni and Needleman, 1990). The GENE 3 of the *MAL2*, *MAL4* and *MAL6*

loci has been sequenced (Kim and Michels, 1988; Sollitti and Marmur, 1988; Wojciechowicz, Gibson and Michels, 1991). All are about 98% sequence homologous and have a cysteine rich DNA-binding domain (Kim and Michels, unpublished results). Thus, GENE 3 appears to encode a DNA binding transcriptional activator.

The MAL locus is a complex locus. Figure 1 summarizes the genetic organization of the *MAL* loci and the nomenclature used (Charron et al., 1989). All of the fully functional *MAL* loci exhibit functional, structural and sequence homology based on complementation analysis, restriction endonuclease mapping analysis and Southern analysis. The expression of the *MAL6* and *MAL1* loci is described in detail in Needleman et al. (1983) and Charron, Dubin and Michels (1986). Transcription of GENEs 1 and 2 is maltose inducible and glucose repressible. GENEs 1 and 2 are divergently transcribed from an intergenic region which, in *MAL6*, has been shown to contain a single UAS_{MAL} controlling expression of both genes (Hong and Marmur, 1987; Kim and Michels, 1988; Levine et al., 1991; Ni and Needleman, 1990). GENE3 is constitutively expressed (Needleman et al., 1985).

Figure 1. The organization and nomenclature of the *MAL* loci of *Saccharomyces*. The genomic organization of the *MAL* loci is indicated by GENE1, GENE2 and GENE3 within boxes. GENE3 is centromere proximal at all loci. The direction of transcription of each of these genes is indicated by arrows beneath the respective genes. Below the arrows is indicated the product of each gene. The genomic location of the *MAL* loci and the names of the GENEs at each locus are provided in the table.

The *MAL* Loci of *Saccharomyces* Yeasts



Locus	Chromosome	GENE3	GENE1	GENE2
MAL1	VII	MAL13	MAL11	MAL12
MAL2	III	MAL23	MAL21	MAL22
MAL3	II	MAL33	MAL31	MAL32
MAL4	XI	MAL43	MAL41	MAL42
MAL6	VIII	MAL63	MAL61	MAL62

II. The *MAL1g* allele

Early genetic studies of maltose fermentation in *Saccharomyces* species were hampered by the presence of multiple *MAL* loci in the yeast strains being analyzed (Michels and Needleman, 1983; Michels and Needleman, 1984). Attempts to isolate mutations in the genes encoding maltase or maltose permease failed, and the only class of non-fermenting mutations obtained fell into a single complementation group which, the results suggested, encoded a positive regulator (ten Berge et al., 1973). However, G. Naumov (1971) using natural isolates of maltose non-fermenting yeast strains was able to identify several strains carrying only a single, functionally mutant *MAL* locus (the *MAL1* locus). These mutant alleles fell into two complementation groups which he referred to as *MAL1p* and *MAL1g*. *MAL1p* strains complemented *MAL1g* strains to produce maltose fermenting diploids. In addition, he isolated a third class of non-fermenting strains which, when crossed with either *MALp* or *MALg* strains, did not produce fermenting diploids. This class he called mal^0. In a series of articles, Needleman and Michels and co-workers (Charron and Michels, 1988; Charron, Dubin and Michels, 1986; Michels and Needleman, 1983; Michels and Needleman, 1984; Needleman and Michels, 1983; Needleman et al., 1985) determined, using *MAL1p*, *MAL1g* and mal^0 strains obtained from Naumov, that the standard laboratory maltose fermenting strains contain 2 to 3

MAL loci. In each fermenting strain, one *MAL* locus is fully functional and contains all three GENEs required for fermentation. The other *MAL* loci are partially functional and fall into the *MALg* complementation group. *MALg* alleles of both *MAL1* and *MAL3* were identified from natural variants and these alleles are referred to as *MAL1g* and *MAL3g*, respectively. A DNA fragment, isolated from a *MAL6* strain and reported to contain the maltase gene, was shown to hybridize to DNA fragments tightly linked to all of the *MAL* loci, both functional and partially functional (Charron, Dubin and Michels, 1986; Michels and Needleman, 1983; Michels and Needleman, 1984; Needleman et al., 1985). Even the *mal*^{0} strains were shown to carry sequences homologous to the cloned fragment and linked to *MAL1*. Thus, *mal*^{0} strains contain another naturally occurring mutant allele of *MAL1*, referred to as *mal1*^{0} (Charron and Michels, 1988; Michels and Needleman, 1983).

III. *MAL1g* permease is different from the *MAL1* permease.

Charron and Michels (1988) reported the isolation and structural and functional analysis of all of the partially functional *MAL* alleles: *MAL1p*, *MAL1g* and *mal1*^{0}. They determined the *MAL1* alleles to be highly homologous to the *MAL1* locus but to have accumulated mutations and genetic rearrangements which led to the loss of function of one or more genes of the locus. Of particular interest was the

cloned 10.7 Kb fragment containing the *MAL1g*. They found that the restriction map of the *MAL1g* locus diverged significantly from that of the fully functional *MAL1* locus in the region encoding maltose permease. Homology to *MAL1* was limited to the maltase structural gene, *MAL12*, and approximately 2 Kb of DNA immediately to the right of *MAL12*. All sequence homology to GENE 1 and GENE 3 probes was lacking from the region. Despite the sequence divergence the 10.7 Kb DNA fragment containing the cloned *MAL1g* clearly encoded a functional maltose permease as determined by plasmid complementation. The *MAL1g* encoded permease is therefore functionally homologous to the *MAL1* encoded permease gene but of a distinctly different sequence. To differentiate these two alleles, the *MAL1* permease gene is referred to as the *MAL11-1* allele and the *MAL1g* permease gene is called the *MAL11-2* allele. The genotype of the *MAL1* locus is *MAL11-1 MAL12 MAL13*, and of the *MAL1g* locus is *MAL11-2 MAL12 mal13Δ*. The degree of divergence and lack of homology over such a long distance led Charron and Michels (1988) to present the hypothesis that the *MAL1g* locus resulted from a chromosomal rearrangement event. They suggest that the *MAL11-2* gene encodes a transporter capable of transporting maltose and other α-glucosides but its transcription is not normally induced by maltose or regulated by GENE 3. Additionally, they propose that this sequence is likely to be found on the centromere proximal side of the *MAL1* locus in the

un-rearranged chromosome VII. Given this, one possible rearrangement which could have produced the *MAL1g* allele is a large chromosomal deletion extending from the *MAL11-2* upstream sequence to the *MAL12* upstream sequences. Such a rearrangement would place the *MAL11-2* gene adjacent to the *MAL12* UAS_{MAL} making it maltose inducible and would result in the deletion of *MAL13* with a loss of the activator function.

Here we report the characterization of the structure and functional activity of the *MAL11-2* encoded maltose transporter. Like the *MAL11-1* encoded maltose permease this gene product is an α-glucoside transporter. The structure of the two transporters and their kinetic characteristics are similar; the substrate specificity of the two transporters is overlapping but distinct.

IV. Structure of the α-glucosides

Alpha-glucosides include a group of naturally occurring di- and tri- saccharides containing glucose. The D prefix refers to the configuration of the asymmetric carbon five. Figure 2a shows the three dimensional, or "chair", representation of D-glucose (Darnell, Lodish and Baltimore, 1986). Alpha and beta refer to the orientation of the hydroxyl group (-OH) about carbon one. In Figure 2a, the hydroxyl group is in the axial or alpha configuration. Figure 2b shows the Haworth projection of α-D-glucose. In forming an α-glucoside, the hydroxyl group of carbon one of an

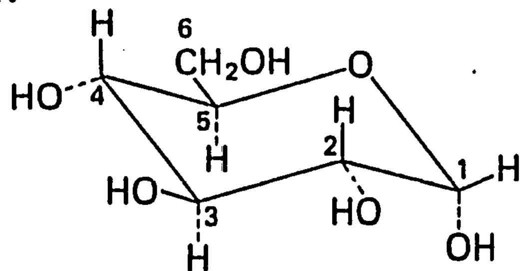
Figure 2.

a. Three dimensional representation of D-α-glucopyranose (Darnell et al., 1986)

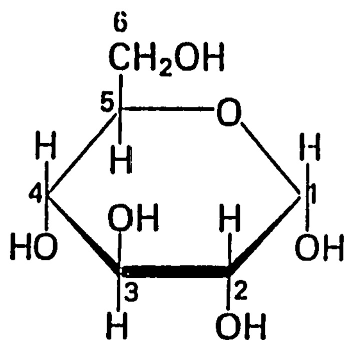
b. Haworth projection of α-D-glucospyranose (Darnell et al., 1986)

c. Maltose is 4-O-α-D-glucopyranosyl-D-glucopyranose and is formed by the dehydration synthesis from two α-D-glucopyranose units (Barnett, 1976)

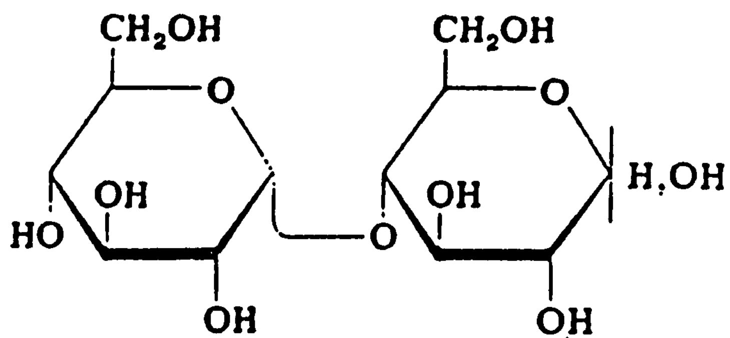
a.



b.



c.

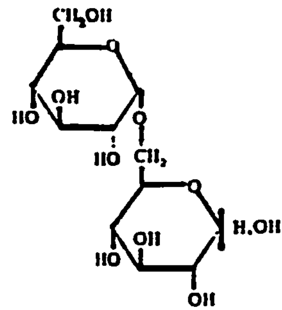


α-D-glucose molecule is reacted with a hydroxyl group of a second sugar (or methanol group) by dehydration synthesis to form an α-glucoside bond. This is seen in Figure 2c for maltose and in Figure 3 for most of the α-glucosides used in this study (Barnett, 1976). Maltose is a disaccharide of two α-D-glucose molecules. The complete chemical nomenclature for maltose is 4-O-α-D-glucopyranosyl-D-glucopyranose, and for palatinose is 6-O-α-D-glucopyranosyl-D-fructofuranose.

VI. Alpha-glucoside transporters

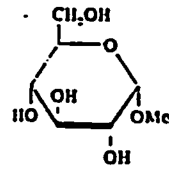
The maltose transporter. The maltose permease of *Saccharomyces* is a proton symporter (Serrano, 1977). Genetic analysis of the GENE 1 of *MAL6* and *MAL1* (described above) strongly suggests that GENE 1 encodes the maltose permease or a component of the permease. In a *MAL1* strain containing a single copy of GENE 1, the *MAL11-1* gene, Cheng and Michels (1991) demonstrated the presence of two kinetically distinguishable maltose transporters: a high-affinity transporter with a K_M of 4 mM and a low-affinity transporter with a K_M of 75 to 80 mM. Similar results have been obtained by Busteria and Lagunas (1985) using genetically undefined strains. Cheng and Michels (1991) went on to show that the high-affinity transporter is inducible by maltose and that in induced conditions only the low-affinity transporter is present. Additionally, deletion of the *MAL11-1* gene led to both a loss of the ability to ferment maltose and the

Figure 3. The structure of some of the α-glucosides used in this study. Haworth projections of isomaltose, α-methylglucoside, sucrose, maltotriose, trehalose, melezitose and turanose. (Barnett, 1976)

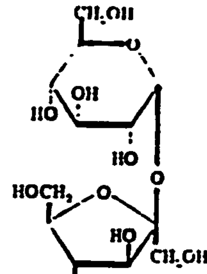


Isomaltose

[illegible] O-[illegible]-D-Glucopyranosyl-
D-glucopyranose

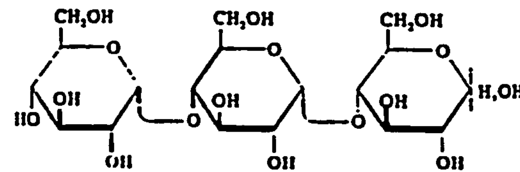


Methyl α-D-glucopyranoside



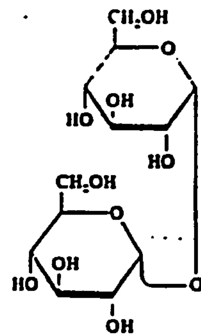
Sucrose

β-D-Fructofuranosyl
α-D-glucopyranoside



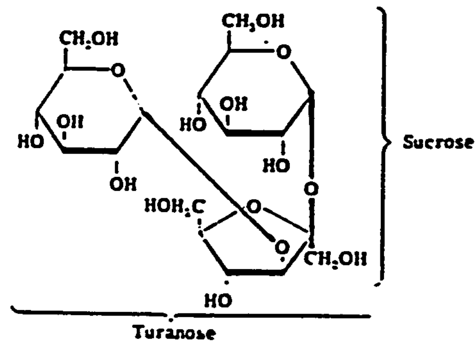
Maltotriose

O-α-D-Glucopyranosyl-(1→4)-
O-α-D-glucopyranosyl-(1→4)-
D-glucopyranose



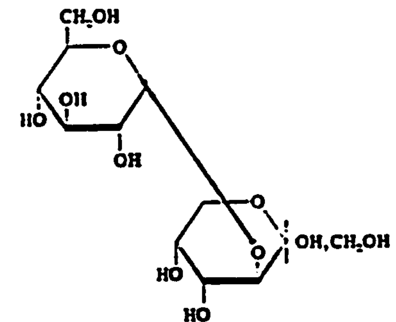
[illegible] Trehalose

[illegible] Glucopyranosyl
[illegible] D-glucopyranoside



Melezitose

O-α-D-Glucopyranosyl-(1→3)-
O-β-D-fructofuranosyl
α-D-glucopyranoside



Turanose

3-O-α-D-Glucopyranosyl-
D-fructopyranose

high-affinity transport, indicating that *MAL11-1* encodes the high-affinity transporter or a component of this transporter. The gene(s) encoding the low-affinity transporter has not been identified.

Using a genetically defined strain, Chang et al. (1989) report that the *MAL61* encoded transporter is able to transport (as assessed by fermentation) maltose and turanose. This is based on the finding that the parent *MAL61* strain is able to ferment both α-glucosides but that *mal61* mutants are unable to ferment either sugar. However, these results do not allow us to conclude that the *MAL61* permease can not transport the other α-glucosides. It is possible that it is capable of transporting these sugars but that these sugars are not able to induce the expression of the gene.

The α-methylglucoside transporter. The natural substrate for the α-methylglucoside transporter has not been determined, although Barnett has assumed it to be isomaltose (1976). Studies of the α-methylglucoside transporter have been hampered by the fact that the *MGL* gene functions are not well defined. The several laboratories studying α-methylglucoside fermentation have used different nomenclatures and little effort has been made to correlate the functional or genetic equivalence of the different gene systems. In summary, an unlinked series of interacting genes essential for α-methylglucoside fermentation have been defined. These are referred to as: *MGL1*, *MGL2*, *MGL3*, *MGL4* (Hawthorne, 1958); or

X, Y1, Y2 (Terue, Okada and Oshima, 1959); or *MGLa, MGLb, MGLc, MGLd, MGLe* (ten Berge, 1971, 1973; Naumov and Bashkirova, 1985) depending on the nomenclature system. The clearest analysis of these genes is described by Naumov and Bashkirova (1985) where it is shown that mutant alleles of the *MGLa, MGLb* and *MGLc* genes are all complemented by the *MAL6-C2* constitutive regulator indicating that these genes encode the MGL-activator and that *MGLd* and *MGLe* encode the structural genes. The *MGL* series of genes are required in pairs in order to ferment α-methylglucoside (*MGL1 MGL2; MGL3 MGL2*) (Hawthorne, 1958). Constitutive *MAL* mutations also interact to allow for fermentation (*MGL1 MAL4-C; MGL3 MAL4-C*) indicating common features to the regulation of the *MAL* and *MGL* genes and that *MGL1* and *MGL3* are structural genes (Khan and Eaton, 1968; Naumov and Bashkirova, 1984a). The *MGL2* gene reportedly encodes the α-methylglucoside transporter but this may not be consistent with some of the genetic results (Naumov and Bashkirova, 1984b). *MGL2* is *MAL3* linked (Mortimer and Schild, 1980).

Two different α-methylglucoside transporters have been reported: a constitutive, facilitated diffusion transporter with a K_M of 50 mM and an inducible, active transporter with a K_M of 1.8 mM (Okada and Halvorson, 1964a; 1964b). In α-methylglucoside induced cells, α-thioethylglucoside (a non-metabolizable α-glucoside analog) is transported by the α-methylglucoside active transporter and this transport is

competed by α-methylglucoside, maltose and trehalose indicating that this transport system is capable of transporting all of these sugars (Harris and Thompson, 1960; Okada and Halvorson, 1964b).

The other α-glucoside transporters. Palleroni and Lindegren (1952) isolated melezitose non-fermenting strains which were able to ferment melezitose on induction by maltose, a phenomenon known as heterologous adaptation (induction) suggesting that maltose acts as an inducer of the melezitose fermentation enzymes. Consistent with this are the findings of Perkins and Needleman (1988) and Needleman and Eaton (1974) that constitutive *MAL* alleles acquire the ability to transport melezitose and sucrose as well as α-methylglucoside. Perkins and Needleman (1988) show that the gene encoding the melezitose transporter (*MTP1*) is *MAL1*-linked.

Sucrose transport appears to occur by means of a high-affinity, proton symporter (Santos et al., 1982). Many of the kinetic characteristics are similar to the maltose transporter and, in fact, the transport of sucrose by this system is competed by maltose. This finding, along with the results of Perkins and Needleman (1988) suggests that the same transporter is capable of transporting maltose, α-methylglucoside, melezitose and sucrose.

It has been reported that maltotriose is transported by yet another permease having a high substrate specificity (Barnett, 1981). Little else is known about this transporter.

In summary, several interacting phenomena are at work here to produce the α-glucoside fermenting phenotype of different *Saccharomyces* strains. First, there are at least two maltose transporters, each capable of transporting maltose and at least one or more other α-glucoside sugars. Second, these transporters are inducible, but each sugar may not be capable of inducing the expression of all of the transporters that are capable of transporting it. Finally, strains are likely to contain some but not all of the genes encoding these different transporters since both the *MAL* and *MGL* loci are known to be telomere-linked polygenic systems (Mortimer and Schild, 1980).

In this work we report the sequence of the *MAL11-2* gene. The *MAL11-2* gene consists of an 1858 base pair open reading frame. The deduced amino acid sequence of the open reading frame is 617 residues long, with considerable hydrophobicity. Hydrophobicity plots of the deduced amino acid sequence suggest that it encodes an integral membrane protein. The proposed MAL11-2 transporter shares structural and sequence characteristics of members of the sugar transporter superfamily but exhibits greatest similarity to the MAL61 maltose transporter. The 785 bp region upstream of *MAL11-2* contains a 469 base pair sequence with almost 100% identity to the *MAL61-MAL62* intergenic region containing the UAS_{MAL}. The 316 bases immediately upstream of *MAL11-2* share only 35.8% homology with the *MAL6* intergenic region which is 89 bases

longer. Like the *MAL1* and *MAL6* encoded maltose transporters, the MAL11-2 transporter is a high-affinity maltose/proton symporter. The MAL11-1 and MAL11-2 transporters have different substrate specificities. We show conclusively that the *MAL11-1* encoded transporter is capable of transporting only maltose and turanose; and the *MAL11-2* encoded transporter is capable of transporting not only maltose and turanose but isomaltose, α-methylglucoside, maltotriose and palatinose. Neither the MAL11-2 transporter nor the MAL11-1 transporter are able to transport melezitose or trehalose. The evolutionary events leading to formation of the *MAL1g* allele have not been determined.

MATERIALS AND METHODS

Strains. Yeast strain 600-1B (*MATa ura3-52 leu2-3,112 MAL1 SUC1*) was described in detail in Charron, Dubin and Michels (1986). It contains a single *MAL1* locus including the *MAL11-1* gene and is highly sequence homologous to the other four dominant *MAL* loci over an approximately 9.0 kbp region (Charron and Michels, 1988). Strain 100-1A is isogenic to 600-1B but contains a deletion disruption of the *MAL11-1* gene (Charron and Michels, 1988). The genotype of 100-1A is *MATa mal11Δ::URA3 MAL12 MAL13 ura3-52 leu2-3,112*. A *ura3* mutation was selected in the *URA3* gene integrated at *MAL1* of 100-1A using 5-fluoro-orotic acid (5-FOA) (Boeke, LaCroute and Fink, 1984). Several uracil$^-$ mutant strains were tested and strain 100-1Au was selected because it reverted to *URA* at a frequency of less than 1 in 10^6 cells. Strains 340-2A (*MATα MAL11-2 MAL12 mal13Δ ura3-52 ade*) and 340-FCB (*MATa MAL11-2 MAL12 mal13Δ ura3-52 leu2,3-112 trp*) both contain the *MAL1g* allele, the naturally occurring partially functional *MAL1* allele. Strain 340-FCB was obtained from a diploid of strains 340-3B (*MATα ura3-52 trp met ade*) and 600-1B by random spore analysis with selection for the uracil$^-$, leucine$^-$, maltose$^-$ phenotype. Strains 340-3B and 340-2A have been previously described (Charron and Michels, 1988).

Growth conditions. Media was described in detail in Sherman et al. (1970). Yeast strains were cultured at 30^{0}C in

complete media, YP (1% yeast extract, 2% yeast peptone) plus the indicated amount of sugar, or in selective media (0.67% yeast nitrogen base without amino acids) plus the indicated amount of sugar and either uracil, leucine or other amino acids as required for selection.

Plasmid construction and yeast transformation. Plasmid pFE52 was obtained from M. Carlson and has been described in detail in Charron and Michels (1988). Plasmid pFQ19 was constructed by insertion of the 5.8 Kb *Eco*RI fragment of pFE52 containing the *MAL11-2* gene into pBR325. Plasmid pMAL11-2 was derived from pFQ19 by insertion of the 2.2 Kb *Bgl*II fragment containing the *LEU2* gene of YEp13 into the *Bam*HI site of pFQ19. Plasmid pMAL64-R10 contains the *MAL64-R10* gene encoding a constitutive MAL activator in the integrating yeast shuttle vector YIp5 (Wojciechowicz et al., 1991). Plasmid YCpMAL43-C contains the constitutive MAL-activator allele from *MAL4*, referred to as *MAL43-C* (Charron and Michels, 1987; Wojciechowicz et al. 1991).

Transformations were performed by the method of Ito et al.(1983). Stability of the selective marker in transformants was determined following growth in non-selective media (YP + 2% glucose). Stable transformants were verified by Southern analysis. Plasmid pMAL11-2 was judged to be a highly stable episomal plasmid by these methods.

Recombinant DNA techniques. Restriction digestion, ligation, DNA electrophoresis and other nucleic acid

manipulations were performed as previously described (Maniatis et al. 1982; Ausubel et al. 1989) and/or as recommended by the suppliers. Southern analysis (Southern, 1975) was performed using nylon 66 plus membranes (TM NYP4 - RL15, Hoeffer Scientific Instruments) following conditions described by Reed and Mann (1985). Overnight hybridization was performed in a hybridization oven at 42^{0}C. Following overnight hybridization in formamide (Ausubel et al. 1989), the membranes were washed with 2 X SSC, 0.1% SDS for 5 minutes and for 15 minutes followed by 0.5 X SSC, 0.1% SDS for 15 minutes, 0.1 X SSC, 0.1% SDS for 15 minutes and 0.1 X SSC, 0.1% SDS for 30 minutes. All washes were performed at 42^{0}C.

DNA sequence analysis. Sequencing of both single and double stranded DNA was done using the method of Sanger et al. (1977). The yeast genomic DNA contained in the *Sal*I-*Xho*I fragment of pFQ19 was inserted into the *Sal*I site of sequencing vector M13mp19 in both orientations for sequencing of both strands. The *Bgl*II-*Xho*I fragment of pFQ19 was inserted into the *Bam*HI-*Sal*I site of M13mp19 which allowed sequencing of one strand using single stranded sequencing methods. The other strand was sequenced in plasmid pFQ19 using double stranded sequencing methods (Chen and Seeburg, 1985). A series of overlapping fragments in both directions were generated using synthetic oligo-nucleotide primers. Primers were synthesized with the Applied Biosystems DNA synthesizer (Model 381A).

Computer analysis. Nucleic acid and protein sequence data were manipulated and analyzed with the University of Wisconsin Genetics Computer Group software version 7.0 (Devereux, Haeberli and Smithies, 1984) running on the VAX 6000 computer at the Computer Center, Queens College, The City University of New York, Flushing, New York. The programs used are cited in the text. Unless otherwise stated, default settings were used for the analysis.

Maltose transport assay. The method used to measure the uptake of radiolabeled sugar was described by Kruckeberg and Bisson (1990) with modifications. This method of determining maltose transport kinetics measured zero trans-influx and has been fully described (Cheng and Michels, 1991). Briefly, a 5 hr culture of cells was used to inoculate 120 ml of YP plus 2% maltose and cultured to an $O.D._{600}$ of 0.9 to 1.0. Cells were resuspended in 0.1 M tartaric acid-TRIS (pH 4.2) buffer. Samples (80μl) of cells were incubated for 0 and 10 seconds with radiolabeled maltose (α-D-^{14}C-maltose; Amersham) at 20^{0}C. Transport was rapidly quenched by addition of 10 ml of ice-cold water. The cells were collected on glass fiber filters under vacuum and washed three times with 10 ml ice-cold water. The amount of radioactivity taken up was determined by liquid scintillation counting using Ecosinct A scintillation fluid. The initial velocity of maltose uptake over a range of substrate concentrations from 0.2 to 200 mM was determined. The data was plotted as velocity (V) versus

velocity/substrate concentrations (V/S), using the Eadie-Hofstee transformation. (Eadie, 1952; Hofstee, 1952).

Sugar fermentation. Alpha-glucoside fermentation was determined as production of gas following 3 days incubation at 30°C in Durham tubes containing 2% filter sterilized sugar in YP. Since melezitose is a poor substrate for maltose, fermentation of melezitose was determined by acid production as seen upon the addition of 3 drops of 0.002% bromocresol purple on day five (Perkins and Needleman, 1988).

Results

The structure of the *MAL11-2* permease. Figure 4 presents the restriction map of the 5.8 kb *Eco*RI fragment from plasmid pFE52. Plasmid pFE52 was characterized by Charron and Michels (1988) and shown to contain the *MAL11-2* and *MAL12* genes of the *MAL1g* locus. Plasmid pMAL11-2, containing the 5.8 kb *Eco*RI fragment from pFE52 complements strain 100-1A, a maltose nonfermenting strain carrying a deletion disruption of the *MAL11-1* gene of the *MAL1* locus. Therefore, the *Eco*RI fragment of pMAL11-2 contains the *MAL11-2* gene. Based on the results of restriction endonuclease mapping, Southern analysis using *MAL6*-derived probes and complementation analysis, the approximately 2 kbp region at the right end of this fragment is *MAL12* and encodes maltase (Charron and Michels, 1988). Thus, our initial sequencing efforts concentrated on the *Sal*I-*Xho*I fragment.

All 2286 bases of the *Sal*I-*Xho*I fragment have been sequenced and shown to contain a single 1851 base pair open reading frame (see Figure 5). Computer translation yields a deduced protein of 617 amino acids (molecular weight of 68,178 daltons) which consists of 37% hydrophobic residues. The GES hydrophobicity scale was used with a window of 20 residues to determine regions of the protein which are highly hydrophobic and could potentially lie within the lipid layer of the plasma membrane (Engleman, Goldman and

Figure 4. Restriction endonuclease map of the *MAL11-2* gene of *S. cerevisiae*. The restriction endonuclease map of the 5.8 kb *EcoR*I fragment of pFE52 containing the *MAL11-2* gene and its flanking sequence is shown. Approximate locations of the coding regions of *MAL11-2* and *MAL12* are indicated above the map and the direction of transcription of *MAL11-2* is shown by the arrow below the map. The telomere of chromosome VII located to the right in this diagram. Recognition sites for restriction endonucleases are abbreviated as follows: A, *Acc*I; Ba, *Bal*I; B, *Bgl*II; C, *Cla*I; D, *Dra*I; H, *Hinc*II; Ha, *Hae*I; Hp, *Hpa*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I; Sc, *Sca*I; Ss, *Ssp*I; X, *Xho*I. *Bgl*II, *Cla*I, *Eco*RI, *Hpa*I, *Sal*I and *Xho*I sites were determined by restriction digest. *Acc*I, *Bal*I, *Dra*I, *Hae*I, *Hinc*II, *Pvu*II, *Sca*I and *Ssp*I were determined using the GCG program MAP.

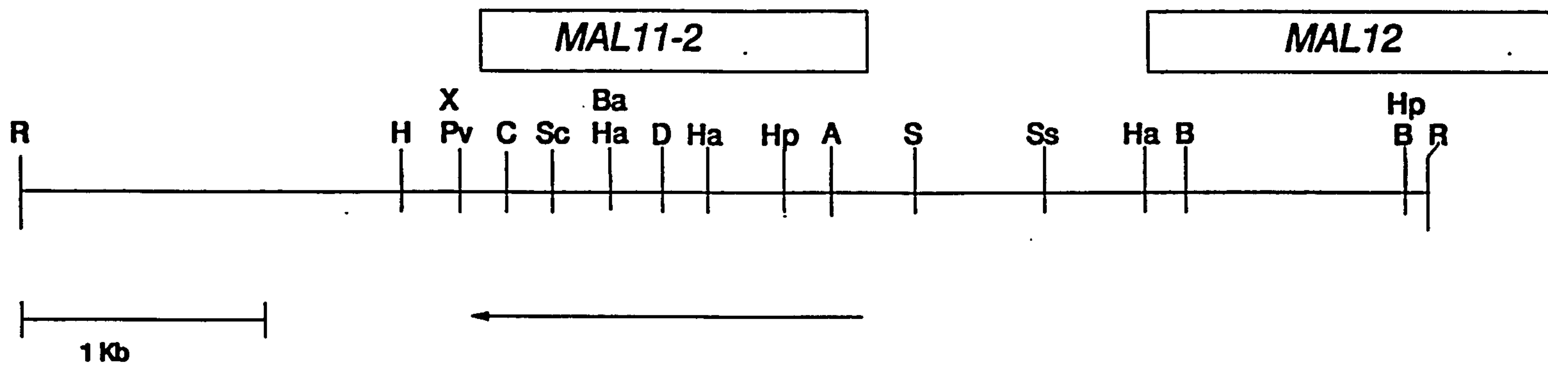

MAL11-2
MAL12
X
Ba
Hp
R
H
Pv
C
Sc
Ha
D
Ha
Hp
A
S
Ss
Ha
B
B
R
1 Kb

Figure 5. Nucleotide sequence of the *MAL11-2* gene and predicted amino acid sequence of the gene product. The nucleotide sequence of the *MAL11-2* gene is given starting at the upstream *Sal*I site. Nucleotide numbers are on the left with the first base of the initiation codon as nucleotide +1. The amino acid residue numbers are shown to the right. Putative 21 residue transmembrane domains are underlined. The location of these is based hydrophobicity plots using the GES hydrophobicity values and the GCG program PEPPLOT. Charged residues within transmembrane domains are circled.

```
-123                                                                      AGGTCGACATCTTAATTTTTTATATAAAGCGGT
 -90  TTGGTATTGATTGTTTGAAGAATTTTCGGGTTGGTGTTTCTTTCTGATGCTACATAGAAGAACATCAAACAACTAAAAAAATAGTATAAT

   1  ATGAAAAATATCATTTCATTGGTAAGCAAGAAGAAGGCTGCCTCAAAAAATGAGGATAAAAACATTTCTGAGTCTTCAAGAGATATTGTA
       M  K  N  I  I  S  L  V  S  K  K  K  A  A  S  K  N  E  D  K  N  I  S  E  S  S  R  D  I  V     30

  91  AACCAACAGGAGGTTTTCAATACTGAAGATTTTGAAGAAGGGAAAAAGGATAGTGCCTTTGAGCTAGACCACTTAGAGTTCACCACCAAT
       N  Q  Q  E  V  F  N  T  E  D  F  E  E  G  K  K  D  S  A  F  E  L  D  H  L  E  F  T  T  N     60

 181  TCAGCCCAGTTAGGAGATTCTGACGAAGATAACGAGAATGTGATTAATGAGATGAACGCTACTGATGATGCAAATGAAGCTAACAGCGAG
       S  A  Q  L  G  D  S  D  E  D  N  E  N  V  I  N  E  M  N  A  T  D  D  A  N  E  A  N  S  E     90

 271  GAAAAAAGCATGACTTTGAAGCAGGCGTTGCTAAAATATCCAAAAGCAGCCCTGTGGTCCATATTAGTGTCTACTACCCTGGTTATGGAA
       E  K  S  M  T  L  K  Q  A  L  L  K  Y  P  K  A  A  L  W  S  I  L  V  S  T  T  L  V  M  E    120

 361  GGTTATGATACCGCACTACTGAGCGCACTGTATGCCCTGCCAGTTTTTCAGAGAAAATTCGGTACTTTGAACGGGGAGGGTTCTTACGAA
       G  Y  D  T  A  L  L  S  A  L  Y  A  L  P  V  F  Q  R  K  F  G  T  L  N  G  E  G  S  Y  E    150

 451  ATTACTTCCCAATGGCAGATTGGTTTAAACATGTGTGTCCTTTGTGGTGAGATGATTGGTTTGCAAATCACGACTTATATGGTTGAATTT
       I  T  S  Q  W  Q  I  G  L  N  M  C  V  L  C  G  E  M  I  G  L  Q  I  T  T  Y  M  V  E  F    180

 541  ATGGGGAATCGTTATACGATGATTACAGCACTTGGTTTGTTAACTGCTTATATCTTTATCCTCTACTACTGTAAAAGTTTAGCTATGATT
       M  G  N  R  Y  T  M  I  T  A  L  G  L  L  T  A  Y  I  F  I  L  Y  Y  C  K  S  L  A  M  I    210

 631  GCTGTGGGACAAATTCTCTCAGCTATACCATGGGGTTGTTTCCAAAGTTTGGCTGTTACTTATGCTTCGGAAGTTTGCCCTTTAGCATTA
       A  V  G  Q  I  L  S  A  I  P  W  G  C  F  Q  S  L  A  V  T  Y  A  S  E  V  C  P  L  A  L    240

 721  AGATATTACATGACCAGTTACTCCAACATTTGTTGGTTATTTGGTCAAATCTTCGCCTCTGGTATTATGAAAAACTCACAAGAGAATTTA
       R  Y  Y  M  T  S  Y  S  N  I  C  W  L  F  G  Q  I  F  A  S  G  I  M  K  N  S  Q  E  N  L    270

 811  GGGAACTCCGACTTGGGCTATAAATTGCCATTTGCTTTACAATGGATTTGGCCTGCTCCTTTAATGATCGGTATCTTTTTCGCTCCTGAG
       G  N  S  D  L  G  Y  K  L  P  F  A  L  Q  W  I  W  P  A  P  L  M  I  G  I  F  F  A  P  E    300

 901  TCGCCCTGGTGGTTGGTGAGAAAGGATAGGGTCGCTGAGGCAAGAAAATCTTTAAGCAGAATTTTGAGTGGTAAAGGCGCCGAGAAGGAC
       S  P  W  W  L  V  R  K  D  R  V  A  E  A  R  K  S  L  S  R  I  L  S  G  K  G  A  E  K  D    330

 991  ATTCAAGTTGATCTTACTTTAAAGCAGATTGAATTGACTATTGAAAAAGAAAGACTTTTAGCATCTAAATCAGGATCATTCTTTAATTGT
       I  Q  V  D  L  T  L  K  Q  I  E  L  T  I  E  K  E  R  L  L  A  S  K  S  G  S  F  F  N  C    360

1081  TTCAAGGGAGTTAATGGAAGAAGAACGAGACTTGCATGTTTAACTTGGGTAGCTCAAAATAGTAGCGGTGCCGTTTTACTTGGTTACTCG
       F  K  G  V  N  G  R  R  T  R  L  A  C  L  T  W  V  A  Q  N  S  S  G  A  V  L  L  G  Y  S    390

1171  ACATATTTTTTTGAAAAGAAGCAGGTAATGGCCACCGACAAGGCGTTTACTTTTTCTCTAATTCAGTACTGTCTTGGGTTAGCGGGTACA
       T  Y  F  F  E  K  K  Q  V  M  A  T  D  K  A  F  T  F  S  L  I  Q  Y  C  L  G  L  A  G  T    420

1261  CTTTGCTCCTGGGTAATATCTGGCCGTGTTGGTAGATGGACAATACTGACCTATGGTCTTGCATTTCAAATGGTCTGCTTATTTATTATT
       L  C  S  W  V  I  S  G  R  V  G  R  W  T  I  L  T  Y  G  L  A  F  Q  M  V  C  L  F  I  I    450

1351  GGTGGAATGGGTTTTGGTTCTGGAAGCAGCGCTAGTAATGGTGCCGGTGGTTTATTGCTGGCTTTATCATTCTTTTACAATGCTGGTATC
       G  G  M  G  F  G  S  G  S  S  A  S  N  G  A  G  G  L  L  L  A  L  S  F  F  Y  N  A  G  I    480

1441  GGTGCAGTTGTTTACTGTATCGTTGCTGAAATTCCATCAGCGGAGTTGAGAACTAAGACTATAGTGCTGGCCCGTATTTGCTACAATCTC
       G  A  V  V  Y  C  I  V  A  E  I  P  S  A  E  L  R  T  K  T  I  V  L  A  R  I  C  Y  N  L    510

1531  ATGGCCGTTATTAACGCTATATTAACGCCCTATATGCTAAACGTGAGCGATTGGAACTGGGGTGCCAAAACTGGTCTATACTGGGGTGGT
       M  A  V  I  N  A  I  L  T  P  Y  M  L  N  V  S  D  W  N  W  G  A  K  T  G  L  Y  W  G  G    540

1621  TTCACAGCAGTCACTTTAGCTTGGGTCATCATCGATCTGCCTGAGACAACTGGTAGAACCTTCAGTGAAATTAATGAACTTTTCAACCAA
       F  T  A  V  T  L  A  W  V  I  I  D  L  P  E  T  T  G  R  T  F  S  E  I  N  E  L  F  N  Q    570

1711  GGGGTTCCTGCCAGAAAATTTGCATCTACTGTGGTTGATCCATTCGGAAAGGGAAAAACTCAACATGATTCGCTAGCTGATGAGAGTATC
       G  V  P  A  R  K  F  A  S  T  V  V  D  P  F  G  K  G  K  T  Q  H  D  S  L  A  D  E  S  I    600

1801  AGTCAGTCCTCAAGCATAAAACAGCGAGAATTAAATGCAGCTGATAAATGTTAAGTAAAAGGGTTGTTTTTTTTTTTTTGGAAGAAATAA
       S  Q  S  S  S  I  K  Q  R  E  L  N  A  A  D  K  C                                           617

1891  GGAATCCCTTTGACTGCTCCCAAAACCCTCAGCTAGCTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAAGGCA
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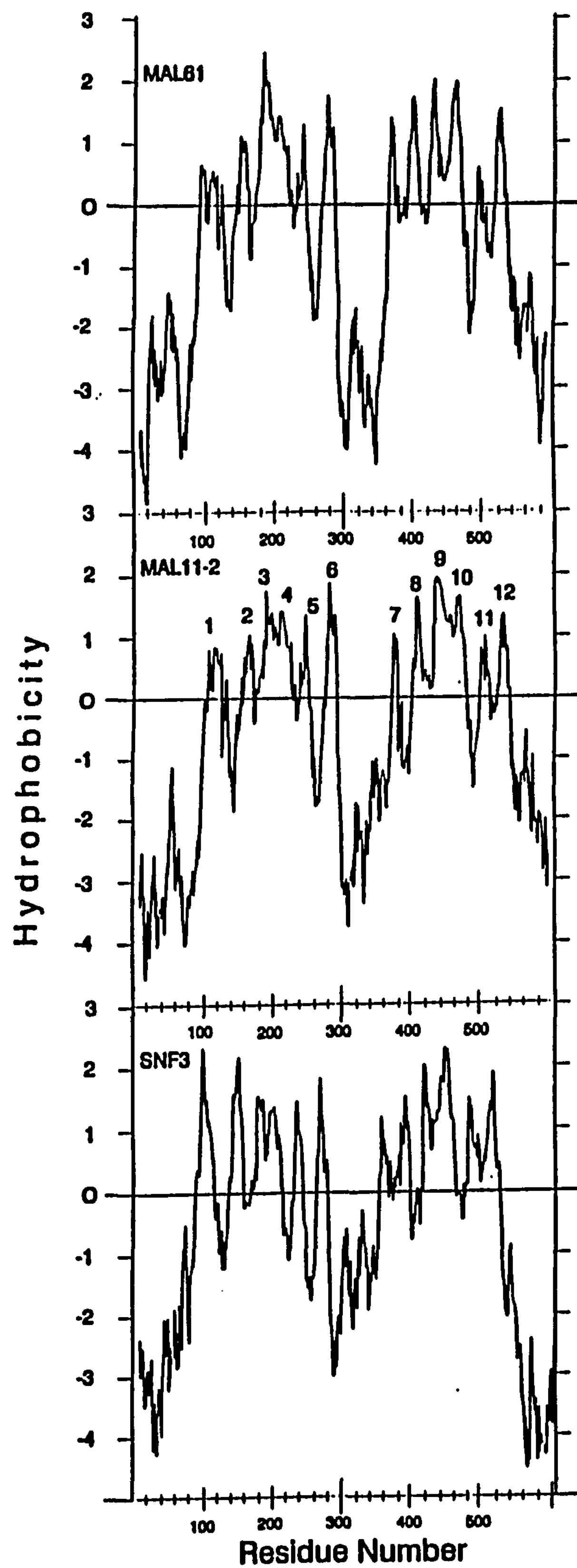
Steitz, 1989). The results are shown in Figure 6 and indicate that the MAL11-2 protein contains 12 potential transmembrane domains supporting the conclusion that this gene encodes an integral membrane protein.

MAL11-2 as a member of the sugar transporter superfamily. Figure 6 presents a hydrophobicity analysis of MAL11-2 and compares it to MAL61 and SNF3. The MAL11-2 transporter shares significant structural homology with these two transporters and with other members of this superfamily of transporters. This group includes: the *Saccharomyces cerevisiae* high-affinity maltose transporter encoded by *MAL61* (Cheng and Michels, 1989), and the high-affinity glucose transporters encoded by *HXT1* (Lewis and Bisson, 1991), *HXT2* (Kruckeberg and Bisson, 1990) and *SNF3* (Celenza et al., 1985); the high-affinity galactose transporter encoded by *GAL2* (Nehlin, Carlberg and Ronne, 1989; Szkutnicka et al., 1989); the mammalian glucose transporters encoded by *GLUT1*, *GLUT2*, *GLUT3*, *GLUT4* and *GLUT5* (Gould and Bell, 1990); and the *E. coli* arabinose and xylose transporters encoded by *AraE* and *XylE* (Maiden et al., 1987). Twelve hydrophobic proposed transmembrane domains, arranged as two blocks of six domains each, separated by approximately 70 residue hydrophilic loop is clearly shown for MAL11-2 in Figure 6 and is characteristic of all of the members of this superfamily. Hydrophobicity plots of MAL11-2 and MAL61 are so similar they are almost super-imposable. The overall distribution and spacing of the

Figure 6. Hydrophobicity profile of the predicted MAL61, MAL11-2 and SNF3 proteins. Plots were generated by the GCG program PEPPLOT using the GES hydrophobicity scale. Potential transmembrane domains are numbered for MAL11-2. Also seen in this figure are the hydrophilic C-terminal and N-terminal domains as well as the 70 residue hydrophilic domain which separates the two blocks of 6 hydrophobic domains. All proteins were analyzed over 617 residues starting from the N-terminus.



MAL61
MAL11-2
SNF3
1
2
3
4
5
6
7
8
9
10
11
12
Hydrophobicity
Residue Number
100
200
300
400
500
3
2
1
0
-1
-2
-3
-4

transmembrane domains is conserved. A proposed long extracellular loop between domains 1 and 2 is also a common feature. Domains 3 and 4 as well as 9 and 10 are not well separated. Each of the yeast transporters has an approximately 115 residue N-terminal and 65 residue C-terminal hydrophilic proposed intracellular region with one exception, the *SNF3* C-terminal hydrophilic region is approximately 300 residues long.

MAL11-2 exhibits approximately 20-25% sequence identity, overall, to other members of the sugar transporter superfamily. The greatest homology lies in motifs identified as characteristic of the sugar transporter superfamily. Figure 7 compares these regions of homology of MAL11-2 to those of the ten other members of the sugar transporter superfamily. The residues of MAL11-2 which are identical to ones found in 5 of the other transporters are shown in boldface. A consensus sequence is shown at the bottom. Those residues where MAL11-2 and MAL61 are distinctly different in character from the consensus are indicated by an asterisk. The significance of these conserved regions and the variations seen in the maltose transporters is not yet understood.

Structural comparison of MAL11-2 and MAL61 trans-porters. Figure 8 presents a sequence comparison of MAL11-2 and MAL61, both maltose transporters. MAL11-2 protein shares 75% similarity and 57% identity with MAL61 protein. Similarity and/or identity is not localized to any one region. Regions

Figure 7. Sugar transporter superfamily homology. The degree of homology over four regions of 11 different transporters is shown. The proposed topological location of each region is indicated. MAL11-2 and MAL61 are the high-affinity maltose transporters of *S. cerevisiae*; GAL2 is the high-affinity galactose transporter of *S. cerevisiae*; HXT1, HXT2 and SNF3 are the high-affinity glucose transporters of *S. cerevisiae*; GLUT1 is the human facilitative glucose transporter of human; Ratglu is the facilitative glucose transporter of rat; XylE is the xylose transporter of *E. coli*; LAC12 is the lactose transporter of *K. lactis*; PHO84 is the inorganic phosphate transporter of *S. cerevisiae*. Alignment was performed using the GCG program PILEUP. Numbers indicate the actual residue number of the regions starting from the first residue of each protein.

The single letter representation for amino acid residues was used. Letters in bold face for each column indicate agreement of the MAL proteins with five or more other transporters. On the consensus line agreement of five or more transporters with each other is shown by the lower case letter, seven or more in agreement is indicated by the upper case letter. In addition, on the consensus line L (or l) represents the hydrophobic amino acids L, I, V; X (or x) represents the polar amino acids E, D, R, K, N, Q. Distinctive differences between the maltose transporters MAL11-2 and MAL61 and other transporters are indicated by an asterisk above the appropriate columns.

I. Region between TMD 4 and TMD 5

```
                      *      *         *      *      *              *
MAL11-2       212   VGQIL SAIPWGCFQS LAVTYASEVC PLALRYYMTS YSNICWLFGQ
MAL61         206   VGQAL CGMPWGCFQC LTVSYASEIC PLALRYYLTT YSNLCWTFGQ
GAL2          178   IGRII SGLGVGGIAV LCPMLISEIA PKHLRGTLVS CYQLMITAGI
HXT1          171   IGRII SGLGVGGITV LSPMLISEVA PSEMRGTLVS CYQVMITLGI
HXT2          163   IGRII SGMGVGGIAV LSPTLISETA PKHIRGTCVS FYQLMITLGI
SNF3          200   VGRVI SGIGIGAISA VVPLYQAEAT HKSLRGAIIS TYQWAITWGL
GLUT1         124   LGRFI IGVYCGLTTG FVPMYVGEVS PTAFRGALGT LHQLGIVVGI
Ratglu        124   LGRFI IGVYCGLTTG FVPMYVGEVS PTALRGALGT LHQLGIVVGI
XylE          131   IYRII GGIGVGLASM LSPMYIAELA PAHIRGKLVS FNQFAIIFGQ
LAC12         171   GGRWF VAFFATIANA AAPTYCAEVA PAHLRGKVAG LYNTLWSVGS
PHO84         175   FYRIV MGIGIGGDYP LSSIITSEFA TTKWRGAIMG AVFANQAWGQ
Consensus           LGRiI sGLgLG.... L.PmYLsEva P..LRG.Lls .yQL.I.lGL
```

II. Region extending from TMD 6 into 70 residue cytoplasmic loop

```
                     *             *   *                  *     *                 *
MAL11-2       289   APLMIGIFFA PESPWWLVRK DRVAEARKSL SRILSGKGAE KDIQVDLTLK QIELTIEKER
MAL61         283   LPLAVGIFLA PESPWWLVKK GRIDQARRSL ERILSGKGPE KELLVSMELD KIKTTIEKEQ
GAL2          253   LFMIGALTLV PESPRYLCEV NKVEDAKRSI AK..SNKVSP EDPAVQAELD LIMAGIEAEK
HXT1          246   LFMIGGMMFV PESPRYLVEA GRIDEARASL AK..VNKCPP DHPYIQYELS TIEASVEEMR
HXT2          238   IFMIAGMLMV PESPRFLVEK GRYEDAKRSL AK..SNKVTI EDPSIVAEMD TIMANVETER
SNF3          275   SFLAIGMFFL PESPRYYVLK DKLDEAAKSL SF..LRGVPV HDSGLLEELV EIKATYDYEA
GLUT1         198   LLQCIVLPFC PESPRFLLIN RNEENRAKSV LK..KLRGTA DVTHDLQEMK EESRQMMREK
Ratglu        198   LLQCILLPFC PESPRFLLIN RNEENRAKSV LK..KLRGTA DVTRDLQEMK EEGRQMMREK
XylE          211   LLFLMLLYTV PESPRWLM.S RGKQEQAEGI LR..KIMGNT LATQAVQEIK HS...LDHGR
LAC12         248   GLVCIFGWLI PESPRWLVGV GREEEAREFI IK.YHLNGDR THPLLDMEMA EIIESFHGTD
PHO84         270   LACLYFRLTI PESPRYQLDV NAKLELAAAA QEQDGEKKIM DTSDEDMAIN GLERASTAVE
Consensus           Ll.llgl.fL PESPR.LvxX XK.DeAKKSL LK...xkg.. Dd..L..ELX xI...lexEK
```

III. Region from TMD 8 to TMD 9

```
                     *           *                *
MAL11-2       415   LGLAGTLCSW VISGRVGRWT ILTYGLAFQM VCLFIIGGMG
MAL61         408   LGIAATFVSW WASKYCGRFD LYAFGLAFQA IMFFIIGGLG
GAL2          375   VNFASTFFSL WTVENLGRRK CLLLGAATMM ACMVIYASVG
HXT1          368   VNFFSTCCSL YTVDRFGRRN CLMWGAVGMV CCYVVYASVG
HXT2          360   VNFASTFVAL YTVDKFGRRK CLLGGSASMA ICFVIFSTVG
SNF3          400   VNVVFNVPGL FFVEFFGRRK VLVVGGVIMT IANFIVAIVG
GLUT1         316   VNTAFTVVSL FVVERAGRRT LHLIGLAGMA GCAILM.TIA
Ratglu        316   VNTAFTVVSL FVVERAGRRT LHLIGLAGMA GCAVLM.TIA
XylE          324   INLTFTVLAI MTVDKFGRKP LQIIGALGMA IGMF...SLG
LAC12         376   VTWISSICGA FFIDKIGRRE GFLGSISGAA LALTGLSICT
PHO84         410   GSLPGYWVSV FTVDIIGRKP IQLAGFIILT A.LF..CVIG
Consensus           VNlA.TlvSL ftVDKlGRRX LlL.GLagma Lc.fi...LG
```

IV. Region from the end of TMD 12

```
                       *    *                 **
MAL11-2       549   VIIDLPETTG RTFSEINELF NQGVPARKFA
MAL61         542   AVVDLPETAG RTFIEINELF RLGVPARKFK
GAL2          515   VFFFVPETKG LSLEEIQELW EEGVLPWK..
HXT1          508   VFFFVPETKG LSLEEVNDMY AEGVLPWK..
HXT2          500   VFFFVCETKG LTLEEVNEMY VEGVKPWK..
SNF3          536   VYLTVYETKG LTLEEIDELY IKSSTGVV..
GLUT1         448   TYFKVPETKG RTFDEIASGF RQGGASQS..
Ratglu        448   TYFKVPETKG RTFDEIASGF RQGGASQS..
XylE          459   MWKFVPETKG KTLEEL.EAL WEPETKKT..
LAC12         510   IYFFFVETKG RSLEEL.EVV FEAPNPRKA.
PHO84         547   TTLLIPETKR KTLEEINELY HDEIDPATLN
CONSENSUS           v.ffVPETKG KTLEEInEL. XXGv.p.K..
```

Figure 8. Sequence comparison of the MAL11-2 and MAL61 transport proteins. The single letter code for amino acid residues was used. Identity is indicated by a solid line between residues, and similarity is indicated by double dots (:) or single dots (.) depending on the degree of evolutionary similarity of the residues (Devereux, Haeberli and Smithes, 1984). Potential transmembrane domains are underlined. Charged residues of transmembrane domains are circled.

```
MAL11-2    1  MKNIISLVSKKKAASKNEDKNISESSRDIVNQQEVFNTEDFEEGKKDSAFELDHLEFTTN  60
              ||.: ||:.:||. ....  .:|.::     ||. | ||. ::||. |.|.|:|.|||:...
MAL61      1  MKGLSSLINRKKDRNDSHLDEIENG....VNATE.FNSIEMEEQGKKSDFDLSHLEYGPG  55

MAL11-2   61  SAQLGDSDEDNENVINEMNATDDANEANSEEKSMTLKQALLKYPKAALWSILVSTTLVME 120
              |    .|.:|: .::::|  | :||.||:..|::|.|. || .||||| ||:||||||: |
MAL61     56  SLIPNDNNEEVPDLLDE..AMQDAKEADESERGMPLMTALKTYPKAAAWSLLVSTTLIQE 113

MAL11-2  121  GYDTALLSALYALPVFQRKFGTLNGE.GSYEITSQWQIGLNMCVLCGEMIGLQITTYMVE 179
              |||||:|:|:|||||||:|:|.||:: |.|||.   ||||| :| :.||::|||:|.  |:
MAL61    114  GYDTAILGAFYALPVFQKKYGSLNSNTGDYEISVSWQIGLCLCYMAGEIVGLQVTGPSVD 173

MAL11-2  180  FMGNRYTMITALGLLTAYIFILYYCKSLAMIAVGQILSAIPWGCFQSLAVTYASEVCPLA 239
              :||||||:| || :|.|:|||||:||||:||||||  |:::||||||:|.|.||||:||||
MAL61    174  YMGNRYTLIMALFFLAAFIFILYFCKSLGMIAVGQALCGMPWGCFQCLTVSYASEICPLA 233

MAL11-2  240  LRYYMTSYSNICWLFGQIFASGIMKNSQENLGNSDLGYKLPFALQWIWPAPLMIGIFFAP 299
              ||||:|.|||:|| |||:||.|||||||:..:||:||||||||||||| || :|||:||
MAL61    234  LRYYLTTYSNLCWTFGQLFAAGIMKNSQNKYANSELGYKLPFALQWIWPLPLAVGIFLAP 293

MAL11-2  300  ESPWWLVRKDRVAEARKSLSRILSGKGAEKDIQVDLTLKQIELTIEKERLLASKSGSFFN 359
              |||||||:|:|:.:||:||.|||||||:||::  |.:.|..|. |||||. : |..|.:::
MAL61    294  ESPWWLVKKGRIDQARRSLERILSGKGPEKELLVSMELDKIKTTIEKEQKM.SDEGTYWD 352

MAL11-2  360  CFK.GVNGRRTRLACLTWVAQNSSGAVLLGYSTYFFEKKQVMATDKAFTFSLIQYCLGLA 418
              |.| |:| ||||:|||.|::| |:|| |:|||||:|| .| .||.|||||:||||||:|
MAL61    353  CVKDGINRRRTRIACLCWIGQCSCGASLIGYSTYFYEKAGV.STDTAFTFSIIQYCLGIA 411

MAL11-2  419  GTLCSWVISGRVGRWTILTYGLAFQMVCLFIIGGMGFGSGSSASNGAGGLLLALSFFYNA 478
              :|:.||  |  .||:.:..:||||| : :|||||:| :.. :|. |.|:||:.:.||||
MAL61    412  ATFVSWWASKYCGRFDLYAFGLAFQAIMFFIIGGLGCSDTHGAKMGSGALLMVVAFFYNL 471

MAL11-2  479  GIGAVVYCIVAEIPSAELRTKTIVLARICYNLMAVINAILTPYMLNVSDWNWGAKTGLYW 538
              ||::||:|:|.|:||. |||||:||| .||:.|: .:|. | || ..||||||.|::|
MAL61    472  GIAPVVFCLVSEMPSSRLRTKTIILARNAYNVIQVVVTVLIMYQLNSEKWNWGAKSGFFW 531

MAL11-2  539  GGFTAVTLAWVIIDLPETTGRTFSEINELFNQGVPARKFASTVVDPFGKGKTQ....... 591
              |||. .||||.::|||||.|||| ||||||. ||||||| || ||||: :|..
MAL61    532  GGFCLATLAWAVVDLPETAGRTFIEINELFRLGVPARKFKSTKVDPFAAAKAAAAEINVK 591

MAL11-2  592  ......HDSLADESISQSSSIKQRELNAADKC............................ 617
                    ..|:.||: | .| :..
MAL61    592  DPKEDLETSVVDEGRSTPSVVNK*.................................... 615
```

with the least identity are found in the 109 residue cytoplasmic N-terminus (43% identity), the last 32 residues of the C-terminus (37% identity) and transmembrane domains 2, 9 and 11 (45%, 52% and 43% identity respectively). The greatest degree of identity lies in transmembrane domains 1 and 6 (76% and 81% respectively) and in the two proposed cytoplasmic regions following TMD 6 and TMD 12 (with 63% and 80% identity over 32 residues respectively). Regions which show the greatest degree of divergence probably lack functional significance, while regions with a high degree of identity are likely to be responsible for functions the two proteins have in common, maltose binding, proton symport and regulation (discussed below). In an effort to reveal additional structural features common to both maltose transporters we carried out a more detailed analysis of the proposed transmembrane domains of MAL11-2 and MAL61. Models of membrane protein structure propose that the hydrophobic transmembrane domains (TMD) form an α-helical configuration (Mueckler et al., 1985). Several proposed TMD of both proteins contain polar and charged residues which might be of functional significance. When hydrophilic residues are localized to one face of the helical TMD, they are considered amphipathic. By comparing hydrophobicity plots of each membrane protein using a 19 residue window to a plot for the same protein using a 7 residue window (using the GCG program PEPPLOT) the amphipathic nature of each transmembrane domain

can be revealed (Fasman and Gilbert, 1990). According to this prediction transmembrane domains 1, 4, 7, 8 and 11 are potentially amphipathic in nature in both the MAL11-2 and in MAL61 proteins. More detailed information on the amphipathic nature of MAL11-2 protein was obtained using the GCG program HELICALWHEELS. HELICALWHEELS computes and plots the location of each residue on the face of a presumed α-helix. Since a protein helix would form one turn every seven residues and requires 18 residues to span the phospholipid membrane, HELICALWHEELS plots the residues on the face of an eighteen position wheel. In this representation adjacent residues are located on the same face of the α-helix. The use of HELICALWHEELS confirmed TMDs 1, 4, 7, 8 and 11 are amphipathic in both proteins. In addition, TMD 2 of MAL11-2 was found to be amphipathic by this method.

Transmembrane domains 1 and 8 are the most highly conserved between the two proteins. Detailed comparison of the helical wheels produced for MAL11-2 and MAL61 proteins revealed that most hydrophilic residues of the transmembrane helices are conserved both in number and in position relative to each other. In Figure 8 the charged residues of each transmembrane domain are circled.

Recent evidence indicates that the presence of leucine and/or isoleucine residues at intervals of seven along a protein helix is a motif, the leucine zipper, for protein-protein interaction (White and Weber, 1989). Lewis

and Bisson (1991) report the region preceding the second transmembrane domains of HXT1, HXT2, GAL2 and the mammalian glucose transporters contains a leucine zipper motif. The leucine zipper may form the basis for oligomerization of the transporters. Indeed, GLUT1 may exist as a homotetramer as determined by radiation inactivation studies of intact red cell membranes (Silverman, 1991). A leucine zipper is also found near TMD 2 (and TMD 6 and 11) of MAL11-2. Interestingly, whereas the MAL11-2 transporter contains three helices with leucine-zippers no leucine-zippers are found in MAL61. Therefore, the leucine zipper motif is not essential to the structure or function of the maltose transporters.

Potential post-translational modification of MAL11-2. The MAL11-2 protein may be post-translationally modified. Several potential sites for post-translational modification of the MAL11-2 protein were identified using the GCG program MOTIFS. One post-translational modification seen in some members of the sugar transporter superfamily is glycosylation (Gould and Bell, 1990; Silverman, 1991). MAL11-2 exhibits two potential glycosylation sites. One is located within TMD 7, the second is found in the carboxy terminus, neither site is likely to be glycosylated in the native protein based on its location.

Phosphorylation of serine, threonine and/or tyrosine residues is commonly found in proteins and frequently plays a regulatory role (Darnell, Lodish and Baltimore, 1986). Three classes of potential phosphorylation recognition sites were

found in MAL11-2: protein kinase C, c-AMP dependent protein kinase and casein kinase II. Eight potential protein kinase C phosphorylation sites are predicted for the MAL11-2 transporter. Of these only one is found to be similar to a site in the MAL61 transporter. This site is located within the 70 residue cytoplasmic loop, at residue 323 and ten of the thirteen residues adjacent to this site are identical in MAL11-2 and MAL61. MAL11-2 was found to have a single potential cytoplasmic c-AMP phosphorylation site at its amino terminus (KKDS, residue 50). The MAL61 protein was also found to have one potential site, however its location is at the beginning of the seventh TMD (RRRT). A secondary c-AMP dependent kinase phosphorylation site is located at the end of TMD 6 (RKS in MAL11-2, RRS in MAL61). As indicated previously, this region is highly conserved. Several potential casein kinase II phosphorylation sites were located in both proteins. Only the residues around sites 560 and 590 are conserved (11 out of 13 residues in 560 site, 8 of 13 residues in the 590 site). Tyrosine kinase phosphorylation sites were not found in MAL11-2 protein but were identified for MAL61.

In addition to glycosylation and phosphorylation, MOTIFS recognized amidation sites for MAL11-2 protein. The significance of any of these sites is not known.

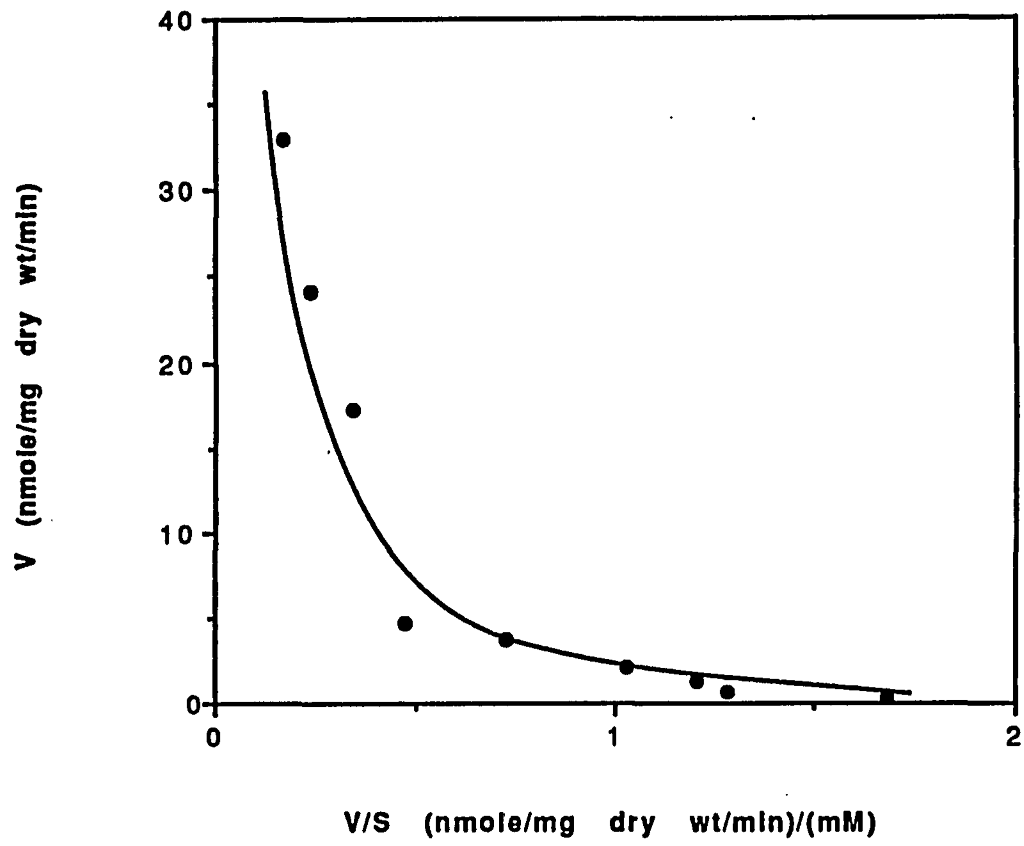
Functional characteristics of the MAL11-2 transporter. Previous work on MAL11-1 and MAL61 transporters showed both to

encode high-affinity, maltose/proton symporters (Cheng and Michels, 1991). We carried out similar analysis of the MAL11-2 maltose permease. Strain 100-1A is a maltose nonfermenting strain containing the *MAL1* locus with a deletion disruption of *MAL11-1*. Eadie-Hofstee analysis of strain 100-1A grown either induced (2% maltose plus 2% galactose) or uninduced (2% galactose) demonstrated the presence of only a constitutively expressed low-affinity (Km 70 - 80 Mm) maltose permease (Cheng and Michels, 1991). Transformation of 100-1A with the plasmid pMAL11-2 restores the ability to ferment maltose. Figure 9 shows an Eadie-Hofstee analysis of transformant 100-1A [pMAL11-2] following growth under induced (2% maltose) conditions. Two kinetically distinct maltose permeases, a high-affinity transporter (Km 4-5 mM) and the low affinity transporter (Km 70-80 mM) are seen. Analysis of maltose transport by 100-1A[pMAL11-2] following growth in uninduced (2% galactose) conditions reveals only the low-affinity transporter. Thus MAL11-2 encodes a maltose-inducible high-affinity maltose permease.

The MAL61 transporter is a proton symporter (Cheng and Michels 1991; Serrano, 1977) To determine if the MAL11-2 transporter is also a proton symporter the uptake of ^{14}C-maltose was determined at pH 4.2 and at pH 7.0. At neutral pH high affinity maltose transport was absent. This result confirms that MAL11-2 is a proton symporter.

Since *MAL11-2* is a maltose inducible gene its upstream

Figure 9. Eadie-Hofstee plot for the MAL11-2 transporter. Strain 100-1A[pMAL11-2] was grown in YP medium supplemented with 2% (wt/vol) maltose. Uptake of ^{14}C-maltose was performed as described in Materials and Methods


V (nmole/mg dry wt/min)
40
30
20
10
0
0
1
2
V/S (nmole/mg dry wt/min)/(mM)

sequence is expected to exhibit sequence similarity to the UAS_{MAL} of the *MAL* structural genes. The upstream sequence of the *MAL6* structural genes, *MAL61-MAL62*, has been studied in detail and, based on deletion analysis and footprint analysis has been localized to an 81 base pair sequence (Hong and Marmur, 1987; Levine, Tanouye and Michels, 1991; Ni and Needleman, 1990). The upstream sequence of *MAL11-2* should also contain a similar sequence since it too is a maltose inducible gene whose expression is dependent on the MAL activator (Charron and Michels, 1988). The intergenic sequence of *MAL1g* from the start codon of *MAL11-2* to the start codon of *MAL12* is 785 base pairs whereas the *MAL61-MAL62* intergenic region of the *MAL6* locus is 875 bp. Figure 10 compares these two sequences. The 315 bp immediately upstream of *MAL11-2* show no significant homology to the comparable region upstream of MAL61. But, starting at base pair -316 and extending for 469 bp to the start of *MAL12*, the sequences are almost 100% identical. This region contains the UAS_{MAL} (indicated by a solid bar in Figure 10) of the *MAL61-MAL62* intergenic region thereby providing the UAS_{MAL} for the maltose inducibility of *MAL11-2*.

The substrate specificity of the MAL11-1 and MAL11-2 maltose transporters. Determination of substrate specificity of the known maltose transporters separate from their inducibility was undertaken by transformation of strains carrying the MAL11-1 and MAL11-2 transporters with a

Figure 10. Comparison of the nucleotide sequence of *MAL1g* (*MAL11-2*) and *MAL61-MAL62* intergenic regions. Bases are numbered starting just upstream of the open reading frame of *MAL11-2*. *MAL61/62* starts at -88 from the *MAL61* open reading frame. The sequence extends to the base just before the start of the open reading frame for either *MAL12* or *MAL62* gene. double bars between bases indicates identity. Underlined is the region containing the UAS_{MAL} of MAL61/MAL62.

```
MAL11-2     -1  ATTATACTATTTTTTTAGTTGTTTGATGTTCTTCTATGTAGCATCAGAAAGAAACACCAACCCGAAAATTCTTCAAACAA  -80
MAL61/62   -88  .CTTTATATGCTGAGTACTTATGCAATATGCGCTCACTCAGGATGAAATGTACACAGCCGAAAGTATATTGAAAGCTGCC  -166

MAL11-2    -81  TCAATACCAAACCGCTTTATATAAAAAATTAAGATGTCGACATTCCTTATTTTTTACTGAGTTCGTTAAAGTTGGGTACA  -160
MAL61/62  -167  TCTGTGGAAACTTCTATCTAATGTTGTCTCCAGATGTAGACTATGAGGCCTGAAGAAGTCTTTAGGCACCTGTTGGAGAG  -246

MAL11-2   -161  CTCTTGATTACTGTAATTGTCTCTGTATGTCCCTCAAGCCCGGTACGTTGTCATTTTCTAGTACGCATCAACGGAGTGTT  -240
MAL61/62  -247  TATAAGGAGACTGCTACAACAACGTCTTCCCCACAAAAATTATGTGGAGGCCGGTATGATACCTGCACAAACGTTAAGTT  -326

MAL11-21  -241  ACATGATAGATAGACCGAGTAGAATCTATGGCTATGGGGTAATTAAAACCTTAAAGCTCCTTTCGCTGC...CATAGTAA  -317
MAL61/62  -327  ACACATGAAAAAGAAACTGACATAACTTTGATCTCTGAAAATATGTTTTCCCCTGAGTAGCTTCACTGCTTGGATACCAA  -406

MAL11-2   -318  TACGAATAGACCTTGGCTATAGTAAGTTGCATCTGTACCGTAGAGATTCTTGCAACTCGCTTAAACTCTCGCTTTTAGAT  -397
MAL61/62  -407  TACGAATAGACCTTGGCTATAGTAAGTTGCATCTGTACCGTAGAGATTCTTGCAACTCGCTTAAACTCTCGCTTTTAGAT  -486

MAL11-2   -398  AATATTTCTCCTTATTGCGCGCTTCGTTGAAAATTTCGCTAAACACGGGGTTTAAGTTAAAGTTTACAGGATTTATCCGG  -477
MAL61/62  -487  AATATTTCTCCTTATTGCGCGCTTCGTTGAAAATTTCGCTAAACACGGGGTTTAAGTTAAAGTTTACAGGATTTATCCGG  -566

MAL11-2   -478  AAATTTTCGCGGACCCCACACAATTAAGAATTGGCTCGAAGAGTGATAACGCATACTTTTCTTTTCTTTTTTTAGTTCCT  -557
MAL61/62  -567  AAATTTTCGCGGACCCCACACAATTAAGAATTGGCTCGAAGAGTGATAACGCATACTTTTCTTTTCTTTTTTTAGTTCCT  -646

MAL11-2   -558  AGCGTACCTAACGTAGGTAACATGATTTGGATCGTGGGATGATACAAACAACGTAAGATGAATAGTTCCTTCCTCAATTC  -637
MAL61/62  -647  AGCGTACCTAACGTAGGTAACATGATTTGGATCGTGGGATGATACAAACAACGTAAGATGAGTAGTTCCTTCCTCAATTC  -726

MAL11-2   -638  TTCTTGCAGCATCATTTTCTTGAGGCGCTCTGGGCAAGGTATAAAAAGTTCCATTAATACGTCTCTAAAAAATTAAATCA  -717
MAL61/62  -727  TTCTTGCAGCATCATTTTCTTGAGGCGCTCTGGGCAAGGTATAAAAAGTTCCATTAATACGTCTCTAAAAAATTAAATCA  -806

MAL11-2   -718  TCCATCTCTTAAGCAGTTTTTTTGATAATCTCAAATGTACATCAGTCAAGCGTAACTAAATTACATAA.............  -785
MAL61/62  -807  TCCATCTCTTAAGCAGTTTTTTTGATAATCTCAAATGTACATCAGTCAAGCGTAACTAAATTACATAA.............  -874
```

constitutive activator for their expression. To determine the substrate specificity of the two permeases independent of the inducibility of *MAL11-1* and *MAL11-2* expression by the various α-glucosides, we used the *MAL64-R10* allele encoding a constitutive MAL-activator (Wojciechowicz et al. 1991) by allowing both the maltase gene and transporter genes to be expressed in all growth conditions. Thus, the ability of a transformed strain carrying a constitutive activator to ferment a particular α-glucoside sugar is a measure of the ability of the sugar to be transported by the constitutively present transporter. Maltase has been shown to cleave all of the α-glucosides used in this study (Barnett, 1976; Perkins and Needleman, 1988). Strain 600-1B represents an inducible strain expressing *MAL11-1*. Strain 100-1A [pMAL11-2] is isogenic to 600-1B, but the *MAL11-1* gene has been deleted and replaced with the *MAL11-2* gene carried on a stable episomal plasmid. Both of these strains were transformed with plasmid pMAL64-R10, carrying the constitutive *MAL64-R10* allele, by targeted integration at the chromosomal *ura3-52* gene as described in Materials and Methods. These transformants, 600-1B:pMAL64-R10 and 100-1A:pMAL64-R10[pMAL11-2] represent strains constitutively expressing the resident maltose transporter gene, *MAL11-1* and *MAL11-2* respectively. The ability of these strains to ferment various α-glucosides is shown in Table 1. The wild type *MAL1* strain 600-1B is able to ferment maltose and turanose. Strain 600-1B:pMAL64-R10, does

Table 1. Comparison of the substrate specificity of the MAL11-2 and MAL11-1 maltose transporters. 600-1B is a maltose fermenting *MAL1* strain expressing the MAL11-1 transporter. 100-1A is isogenic to 600-1B with a deletion of the *MAL11-1* gene. 100-1A[pMAL11-2] is 100-1A transformed with plasmid pMAL11-2. Plasmid pMAL11-2 is an episomal plasmid containing the *MAL11-2* gene as described in Materials and Methods. *MAL64-R10* encodes the constitutive MAL activator (Dubin et al., 1989) introduced on an integration plasmid pMAL64-R10 which carries the *MAL64-R10* gene in a derivative of the *E. coli*/yeast shuttle vector YIp5 (Wojciechowicz, Gibson and Michels, 1991). Fermentation (+) was determined as gas production in 3-5 days of incubation at 30^0 C (as described in Materials and Methods.

Yeast strain	600-1B		100-1A		100-1A[pMAL11-2]	
MAL related genotype	*MAL11-1 MAL12 MAL13*		*mal11-1Δ MAL12 MAL13*		*mal11-1Δ MAL12 MAL13[MAL11-2]*	
Constitutive activator	none	*MAL64-R10*	none	*MAL64-R10*	none	*MAL64-R10*
maltose	+	+	-	-	+	+
turanose	+	+	-	-	+	+
palatinose	-	-	-	-	+	+
maltotriose	-	-	-	-	+	+
isomaltose	-	-	-	-	-	+
α-methylglucoside	-	-	-	-	-	+
melezitose	-	-	-	-	-	-
trehalose	-	-	-	-	-	-

not acquire the ability to ferment any other α-glucoside clearly indicating that the constitutively produced MAL11-1 transporter is able to transport only maltose and turanose. Deletion of the native transporter, *MAL11-1*, leads to loss of the ability to ferment maltose and turanose in both untransformed and pMAL64-R10 transformed cells, allowing us to conclude that MAL11-1 is the only α-glucoside transporter in this strain responsive to activation by the MAL64-R10 constitutive activator. Transformation of strain 100-1A with pMAL11-2 permits the fermentation of maltose, turanose, palatinose and maltotriose, indicating that MAL11-2 transports all of these sugars. Furthermore, this result clearly shows the *MAL13* encoded MAL-activator is capable of responding to the presence of α-glucosides other than maltose and turanose. Isomaltose and α-methylglucoside failed to be fermented but transformation of 100-1A[pMAL11-2] with the constitutive activator gene *MAL64-R10*, permitted fermentation of isomaltose and α-methylglucoside in addition to the other α-glucosides. This result indicates that MAL11-2 is able to transport isomaltose and α-methylglucoside but that these sugars do not function as inducers for the *MAL13* encoded MAL-activator. The only α-glucosides tested which were not fermented, and therefore not transported were trehalose and melezitose. The transport of sucrose could not be tested as 600-1B carries the *SUC1* locus.

Strain 340-FCB, a strain carrying the *MAL1g* allele and

lacking a gene encoding the MAL-activator, failed to ferment any of the α-glucosides tested. However, the pMAL64-R10 transformed 340-FCB fermented maltose, turanose, palatinose, maltotriose, isomaltose and α-methylglucoside and with acid production in melezitose. A second *MAL1g* strain, 340-2A, when transformed with a plasmid containing the *MAL4*-encoded constitutive MAL-activator on a CEN plasmid, YCpMAL43-C, is also able to ferment all seven α-glucosides (data not shown). Since the results reported in Table 1 demonstrate that the MAL11-2 transporter is unable to transport melezitose, another transporter for this α-glucoside may be encoded by these strains. Alternatively, the activator protein synthesized in strain 100-1A[pMAL11-2]:pMAL64-R10 may not be responding to melezitose as an inducer. This strain should contain a mixed population of MAL activator: MAL13/MAL13 and MAL64-R10/MAL64-R10 homodimers and MAL13/MAL64-R10 heterodimer. If only the MAL64-R10/MAL64-R10 constitutive homodimer is capable of activating transcription because the others do not respond to melezitose, it is possible that adequate levels are not present to activate synthesis of the transporter enough to ferment melezitose. In the 340-2A and 340-FCB strains where only the constitutive activator is present, MAL11-2 is synthesized constitutively and fermentation is possible. Strain 340-2A apparently also contains a gene encoding melezitase since fermentation of 2% melezitose by the pMAL64-R10 transformed strain was very rapid

with production of a volume of gas equal to that in 2% maltose indicating the constitutive MAL-activator is most probably responsible for the expression of melezitase as well.

Discussion

In this work we present a structural and functional characterization of the maltose permease encoded by the *MAL1g* allele of the *MAL1* locus of *Saccharomyces cerevisiae*, referred to as *MAL11-2*. Plasmid pMAL11-2, carrying the *MAL11-2* gene, restores maltose fermentation to a strain deleted of its sole maltose transporter. Sequencing this fragment revealed that *MAL11-2* encodes a highly hydrophobic proposed integral membrane protein with significant sequence and structural homology to MAL61, a *Saccharomyces* high-affinity maltose transporter. The deduced MAL11-2 and MAL61 sequences were 57% identical and 75% similar. Our results show that MAL11-2 is a high-affinity maltose/proton symporter with a Km of 4-5 mM. MAL11-2 expression is induced by maltose and the upstream region is nearly 100% identical over 368 base pairs to the upstream region containing the UAS_{MAL} of *MAL61*. Substrate specificities of MAL11-1 and MAL11-2 were found to be overlapping but distinct. MAL11-1 is capable of transporting only maltose and turanose. MAL11-2 is able to transport maltose, turanose, maltotriose, palatinose, isomaltose and α-methylglucoside. Neither transports melezitose.

MAL11-2 is a member of the sugar transporter superfamily.

Sequence and secondary structural characteristics of MAL11-2 indicate that it is a member of the sugar transporter superfamily. More than fifteen proteins have been identified

and classified as being encoded by this superfamily of genes including: *MAL11-2*, *MAL61* (Cheng and Michels, 1989), *SNF3* (Celenza et al. 1988), *HXT1* (Lewis and Bisson, 1991), *HXT2* (Kruckeberg and Bisson, 1990) and *GAL2* (Nehlin, Carlberg and Ronne, 1989; Szkutnicka et al., 1989) of *S. cerevisiae*; *LAC12* (Chang and Dickson, 1988) of *K. lactis*; *AraE*, *XylE* and *Cit* (Maiden et al., 1987) of *E. coli*; *GLUT1* through *GLUT5* (Gould and Bell, 1990) of human. The secondary structural features of the superfamily of sugar transporters are best revealed in hydropathy plots and are shown to contain three characteristic elements: twelve hydrophobic domains; a long (65-75 residue) highly charged proposed cytoplasmic loop between hydrophobic domains six and seven; a long (20-30 residues) extracellular domain between hydrophobic domains one and two (Silverman, 1991; Gould and Bell, 1990; Walmsley, 1988). Each of the hydrophobic domains is thought to be oriented transverse to the membrane. This is based on computer and graphical analysis of the predicted protein sequence of GLUT1 and on the results of chemical and proteolytic digestion experiments performed on the native, membrane- bound protein (Mueckler et al., 1985).

Four consensus sequences have been identified as characteristic of this family of sugar transporters. Figure 7 compares these four conserved regions for eleven members of this family of transporters. The functional and structural significance of these consensus regions and of the variations

of some sequences from the consensus is not known. The secondary structure of these proteins and the PESP sequences at the end of TMD 6 and 12 suggest that the N-terminal and C-terminal halves resulted from a genomic duplication event (Maiden et. al, 1987). A Dotplot comparing the two halves of the proteins does not support this hypothesis.

Recently *PHO84*, encoding an inorganic phosphate transporter of *S. cerevisiae*, has been reported as a member of this superfamily (Bun-ya et al., 1991). The presence of a non-sugar transporter in this class of proteins suggests that the secondary structure of these proteins is a basic feature of not only sugar transporters but of transporters of hydrophilic molecules in general. This superfamily also includes proton symporters: the two maltose transporters and AraE and XylE. A group of glucose/Na^+ cotransporters have been sequenced and described, but none of them are related to the GLUT series or to the sugar transporter superfamily (Silverman, 1991).

MAL11-2 contains regions functionally homologous to GLUT1 and LacY transporters.

Information on the functional regions of MAL transporters may be gleaned from a comparison of proposed functional domains of the most completely described transporters. The topology of a human facilitative diffusion glucose transporter GLUT1 has been studied in the greatest detail and may be considered as representative of this structural class of

proteins. Regions of GLUT1 have been identified by various methods as having specific functions. Transmembrane domains (TMD) 3, 5, 7, 8 and 11 of GLUT1 were determined to be amphipathic and perhaps responsible for formation of a channel and/or binding of glucose (Mueckler et al., 1985). Of these TMD 7 and 8 are most likely to form the channel since they contain many serine and glutamine residues (Walmsley, 1988). Fluorescence emission studies indicate TMD 9 and 10 form a hydrophobic cleft that moves during glucose transport (Silverman, 1991; Gorga and Lienhard, 1982; Rampal and Jung, 1987). Therefore, a substrate binding site for glucose appears to be formed by the glucose binding domains 7, 8 and 11 and the hydrophobic cleft of 9 and 10. The same TMD of MAL11-2 and MAL61 have similar characteristics and it is tempting to speculate they are involved in maltose transport. Proton symport is not a feature common to the sugar transporter superfamily. The *S. cerevisiae* maltose transporters are proton symporters and may share structural and functional features with the *E. coli* β-galactoside/ proton symporter. Proton transport activity of the *E. coli* protein has been localized to a region that involves two transmembrane domains (Kaback et al. 1986). Specific residues have been implicated in proton symport and have been shown to regulate the transport of the sugar. Histidine, glutamic acid and arginine residues are involved in a "charge relay" adapted in part to H^+ translocation (Kaback, Bibi and Roepe, 1990).

Neither MAL transporter protein has histidine in any proposed TMD. Kaback et al. (1990) proposed functional overlap of β-galactoside and proton symport activity. By analogy the maltose binding and proton transport domains should occur in the same functional region. Analysis of the residues in the proposed maltose binding domain indicates the "charge relay" could not occur in TMD 7, 8 or 11. There are no lysine, arginine, glutamic acid or aspartic acid residues in TMD 7, 8 and/or 11. Only TMD 1 and 2 contain the required residues for a "charge relay" system.

***MAL11-2* encodes a distinctly different transporter from *MAL11-1*.**

Our results comparing the intergenic regions of *MAL11-2* and *MAL6* confirm the work of Charron and Michels (1986). Figure 10 presents our comparison of the *MAL6* and *MAL1g* intergenic regions. In the 469 bp region from residues -316 to -785 upstream of *MAL11-2* there is almost 100% identity to the *MAL61-MAL62* intergenic region shown to contain the UAS_{MAL} (Hong and Marmur, 1987; Levine, Tanouye and Michels, 1991). As anticipated from the Southern analysis results of Charron and Michels (1988) the sequence immediately upstream of *MAL11-2* shows no significant homology to the similar sequence from *MAL61*. The *MAL11-2* upstream region to bp -316 is only 41% homologous to the *MAL61* upstream region. Thus base pair -315/-316 represents the junction site of the rearrangement which formed this partially functional allele. Charron and

Michels (1988) suggested a chromosomal inversion or rearrangement occurred to form the *MAL1g* allele. If so, *MAL11-2* sequence should be found in the unrearranged chromosome VII. To test this hypothesis we performed Southern analysis using the *MAL1* strain 600-1B probed with *MAL11-2* sequences. Results showed strain 600-1B contains no sequences homologous to *MAL11-2* derived probes disproving this hypothesis. More likely, the *MAL1g* allele resulted from a telomere translocation event similar to that suggested for the polygenic *MAL* and *SUC* loci (Charron et al., 1989; Carlson, Celenza, and Eng, 1985). Like the *MAL* and *SUC* genes, the *MAL11-2* gene is probably a repeated, telomere associated sequence. Other α-glucoside fermentation genes such as the *MGL* genes have been shown to be repeated, in fact *MAL11-2* may be an *MGL* gene. A broad screening of *Saccharomyces* strains is likely to reveal several containing *MAL11-2* homologous sequences at different chromosomal telomeres. We suggest that two "recombination" events occurred to form *MAL1g*. One translocated *MAL1* sequences to chromosome VII. The second which occurred at a site upstream of *MAL11-2* and GENE 2 sequences juxtaposed the two genes and retained the UAS_{MAL} of the GENE 2. Do such events require sequence homology? Rearrangements such as these have occurred for the *SUC*, X and Y' telomere- associated sequences (Carlson, Celenza and Eng, 1985). It has been suggested that homologous recombination between $C_{1-3}A$ repeats or X sequences is responsible for these

translocations (Carlson, Celenza and Eng, 1985). No $C_{1-3}A$ or X sequences have been identified on the centromere-proximal side of the *MAL* loci and it has been suggested that *MAL* translocation events either occurred by a process not involving homologous recombination or that the homologous sequences were very short, or had been lost by mutation over the course of time since the translocation (Charron et al. 1989). Sequencing of the *MAL11-2* - *MAL12* intergenic region provides the first identification of the exact junction site of a *MAL* translocation event. If homologous recombination is involved in this event the sequences on either side of basepairs -315/-316 should be repeated in the yeast genome. We are now in a position to test this.

Transporter specificity.

The α-glucoside specificity of both the MAL11-1 permease and the MAL11-2 permease was determined using plasmid complementation in a genetically defined strain containing the constitutive MAL-activator *MAL64-R10*. All of the dominant *MAL* loci are highly sequence homologous and therefore the results reported here for the MAL11-1 transporter are likely to hold true for the GENE 1 counterparts of all of the *MAL* loci except *MAL1g*. Our results show one of these dominant MAL transporters, MAL11-1, is capable of transporting only maltose and turanose. This finding supports the results of Perkins and Needleman (1988). The MAL11-2 transporter has a broader substrate specificity and is capable of transporting maltose,

turanose, palatinose, maltotriose, isomaltose and α-methylglucoside. Neither transports melezitose. An interesting off-shoot of these experiments was the finding that only maltose, turanose, palatinose and maltotriose could act as inducers with the *MAL13* encoded MAL-activator. Constitutive expression of MAL11-2, using the MAL64-R10 activator, was needed to demonstrate isomaltose and α-methylglucoside transport by MAL11-2. Therefore, the number of sugars fermented by a strain is a reflection not only of the substrate specificity of the resident transporters but of the inducer responsiveness of the transcription activator as well. Our results also reveal the presence of a melezitose (MZ) transporter in certain strains. Interestingly, this MZ transporter is induced by the constitutive activator MAL64-R10. The MAL64-R10 constitutive MAL activator has also been reported to activate the expression of α-methylglucosidase (Perkins and Needleman, 1988). It appears that the UAS for all of these α-glucoside genes is highly homologous. However this is not necessarily so; the binding of a single activator to dissimilar upstream activation sequences has been demonstrated for HAP1 which activates transcription of the *CYC1* and *CYC7* genes (Pfeifer, Prezant and Guarente, 1987). These results indicate that the ability of a strain to ferment a particular α-glucoside depends on the combination of transporters and activators encoded by the strain. This combination is likely to be very variable based

on the polygenic nature of the genes.

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