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Structural and functional characterization of the maltose transporter of *Saccharomyces*

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STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE MALTOSE TRANSPORTER OF *SACCHAROMYCES*

by

Qi Cheng

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ABSTRACT

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION
OF THE MALTOSE TRANSPORTER OF *SACCHAROMYCES*

by

Qi Cheng

Advisor: Professor Corinne A. Michels

Maltose fermentation in yeast *Saccharomyces* strains requires the presence of one or more of five unlinked, structural and functional homologous *MAL* loci: *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6*. Each locus is a complex locus consisting of three essential maltose fermentation genes: GENEs 1, 2, and 3. GENE 3 encodes the *MAL*-activator which is required for the inducible transcription of two structural genes: GENE 1 and GENE 2. While GENE 2 was identified to encode maltase, GENE 1 was found to be required for the synthesis of maltose permease, the maltose transporter.

To determine the structure and function of the GENE 1 product, we have sequenced the *MAL61* gene, GENE 1 in *MAL6* locus. The *MAL61* gene contains an open reading frame of 1842 basepairs which encodes the 614 residue putative MAL61 protein. Hydropathy analysis suggests that the secondary structure of the MAL61 protein contains two blocks of six transmembrane domains separated by an approximately 71 residue intracellular region. Significant sequence and structural homology is seen between the MAL61 protein and a family of

known sugar transporters including the human glucose transporters.

The cellular localization of MAL61 was determined by analyzing a functional *MAL61-lacZ* gene fusion product. Our results demonstrated that *MAL61* encodes an integral membrane protein.

We elucidated the biochemical and physiological nature of MAL61 by carrying out kinetic studies of maltose uptake in genetically defined *Saccharomyces* strains. *MAL11* and *MAL61* were shown to encode an inducible, high-affinity maltose transporter. A low-affinity maltose transporter, which is also detected in maltose-fermenting strains containing *MAL1* or *MAL6* locus, was found to be expressed constitutively and not to be related to *MAL11* or *MAL61*.

In addition to structural and functional studies, we investigated the glucose-induced inactivation of the maltose transport system in a genetically defined strain carrying the *MAL6* locus. We have demonstrated that the *MAL61*-encoded maltose transporter is subject to glucose-induced inactivation and this inactivation process is distinguishable from glucose repression of the maltose fermentative enzymes including both maltose permease and maltase. We have shown that glucose specifically targets the inactivation of the MAL61 maltose transporter and that a protein synthesis inhibitor, cycloheximide, gives rise to the same affect. The mechanism of the inactivation of maltose transport system is discussed.

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INTRODUCTION

Sugar transport in *Saccharomyces*

The plasma membrane is a semi-permeable barrier separating the cytoplasm from the environment in all living cells. Other than certain small lipid soluble molecules, traffic across the cell membrane is mediated by membrane proteins, such as amino acid transporters, sugar transporters, ion transporters and channel forming proteins. A great deal of research has been devoted to the field of membrane transport relating to the isolation, purification and molecular and functional characterization of various carrier proteins, yet elucidation of their molecular mechanism of action remains an unsolved problem.

Sugar transport has became a vital area of research in human biology and pathophysiology. A family of facilitated glucose transporters including GLUT1, GLUT2, GLUT3, GLUT4 and GLUT5 has been identified (reviewed in Silverman 1991). The erythrocyte-type glucose transporter, GLUT1, was the first human glucose transporter to be studied and its deduced amino acid sequence was reported from the sequence of a cDNA clone containing the GLUT1 gene (Mueckler et al. 1985). Analysis of its primary structure suggests the presence of twelve transmembrane domains which are organized into two groups of six each, with a highly hydrophilic amino terminus, carboxyl terminus, and central domain predicted to lie on the

cytoplasmic face of the membrane (Mueckler et al. 1985). Studies using peptide-specific antibodies to various hydrophilic regions of the protein have confirmed many of the topological features of this model (Davies et al. 1987). Over the past a few years, GLUT2-5 have been isolated and characterized in succession. Despite the fact that they exhibit considerable homology to GLUT1 in their primary sequences and structure, studies analyzing their protein and mRNA distribution indicate that they display a marked tissue-specific pattern of expression (reviewed in Gould and Bell 1990). Most interestingly, GLUT4 was found to be expressed in insulin-responsive tissues and shown to play a central role in mediating insulin-stimulated glucose transport. It was found that, in the presence of glucose, insulin controls glucose transport by promoting translocation of the GLUT4 transporter molecules residing in the cytosol to the plasma membrane (reviewed in Gould and Bell 1990). While the insulin receptor appears not to be involved in the abnormal cellular response in diabetes mellitus, a human disease in which insulin is incapable of appropriately increasing the transport of glucose into target tissues, the identification of GLUT4 has provided new insights into understanding the molecular and cellular basis of insulin resistance in diabetes. In addition, the molecular and functional analysis of different forms of human glucose transporters represents an opportunity to determine how nature has subtly modified a family of proteins with the

same basic function in order to best regulate the utilization of glucose.

Recently, two retroviruses: ecotropic murine leukaemia virus (MuLV-E) and gibbon ape leukaemia virus (GALV) have been shown to gain entry into their target cells via the cell-membrane permease proteins (reviewed in Vile and Weiss 1991). The receptor for MuLV-E (ecoR) was demonstrated as a functional permease capable of transporting basic amino acids (Kim et al. 1991; Wang et al. 1991), and the receptor for GALV was identified as a membrane protein with a great similarity to a phosphate transporter protein (reviewed in Vile and Weiss 1991). These two findings provide evidence for a novel family of human viral receptors, the permeases. The molecular and functional characterization of these membrane permeases opens up a new chapter for studying the viral pathogenesis in leukaemia.

The utilization of sugars is a major aspect of the metabolism of *Saccharomyces* yeast. It has been extensively analyzed over past 40 years because of its practical value for the brewing and baking industries. Early biochemical and physiological studies revealed that sugar transport is the initial rate-limiting step in the utilization of most sugars in yeast and yeast is able to selectively internalize sugars from the environment by using different sugar transporters or permeases. While the mechanisms of sugar uptake and metabolism in most eukaryotic systems remain unknown, an

elucidation of the molecular basis controlling sugar transport in yeast would provide an insight into the regulatory mechanisms of sugar utilization in humans.

Saccharomyces cerevisiae is an excellent model eukaryote for examining sugar transport systems. Classical yeast genetic analysis has been used to localize and identify many genes involved in sugar utilization including sugar transport. Biochemical approaches were employed to determine the functional characteristics of many of these gene products. In addition, the advanced molecular biology techniques available in *Saccharomyces* has enabled researchers to carry out the gene isolation and detailed structural and functional examination of these gene products.

The glucose transport system of *Saccharomyces* has been studied most extensively. Attempts to isolate glucose transport deficient mutants among the mutant strains selected for the inability to utilize glucose suggested either the existence of multiple glucose transport systems with overlapping physiological functions or that the loss of glucose transport is a lethal event (Clifton, Weinstock and Fraenkel 1978). The results of kinetic studies indicated that wild type *Saccharomyces* strains contain multiple forms of glucose transport, including a high-affinity and a low-affinity glucose transporter (Bisson et al. 1987). The presence of both high- and low-affinity uptake processes is characteristic of most sugar transport processes in yeasts

(Bisson and Fraenkel 1983b; Bisson and Fraenkel 1984; Busturia and Lagunas 1985; De Bruijne et al. 1988; Lang and Cirillo 1987; Ramos, Szkutnicka and Cirillo 1989; Serrano and De la Fuente 1974; Spencer-Martins and van Uden 1985; van Steveninck 1972; van Steveninck and Dawson 1968; Verma, Spencer-Martins and van Uden 1987), but the relationship between them is not known. There are some indications that the reported low-affinity species is an artifact resulting from incorrect calculations at high substrate concentrations (particularly for facilitated diffusion transporters) or as a result of non-specific extracellular binding. Since sucrose is cleaved by the extracellular enzyme invertase yielding glucose and fructose, it was thought that a deficiency in sucrose utilization could result from a defect in glucose and fructose uptake or metabolism (Goldstein and Lampen 1975). The first glucose uptake mutant was isolated from a series of mutants deficient in utilization of sucrose (sucrose nonfermenting, *snf*) from the study of Neigeborn and Carlson in 1984. To identify potential genes involved in glucose transport, kinetic studies of glucose uptake in both wild type and *snf* mutant strains were carried out (Bisson et al. 1987). Kinetic studies in *snf3* mutants of the strain S288C revealed a defect in high-affinity glucose transport indicating that *SNF3* is required for the synthesis of a high-affinity glucose transporter in *Saccharomyces* (Bisson et al. 1987). The *SNF3* gene has been isolated and sequenced (Celenza, Marshall-

Carlson and Carlson 1988). Its predicted amino acid sequence shows that it contains 886 amino acid residues and encodes a 97 kilodalton protein that is homologous to mammalian glucose transporters. Just like the human glucose transporter, it consists of twelve putative membrane-spanning regions organized into two groups of six. A functional *SNF3-lacZ* gene fusion cofractionates with membrane proteins and is localized to the cell surface (Celenza, Marshall-Carlson and Carlson 1988). These facts suggest that SNF3 acts directly at the glucose transport step and encodes the high-affinity glucose transporter. Based on the study of multicopy suppressors of *snf3* mutation, which function to complement the defect in *snf3* glucose transport, the *HXT1* and *HXT2* genes were isolated and their products characterized (Kruckeberg and Bisson 1990; Lewis and Bisson 1991). Like the SNF3 protein, the HXT1 and HXT2 proteins contain twelve putative membrane-spanning domains with a central hydrophilic domain and hydrophilic N- and C-terminal domains and exhibit significant homology to the human glucose transporters (Kruckeberg and Bisson 1990; Lewis and Bisson 1991). Disruption of the *HXT1* or *HXT2* genes resulted in loss of a portion of the high-affinity glucose transport, and wild-type levels of glucose transport required the presence of the *SNF3*, *HXT1* and *HXT2* genes, suggesting that they all belong to the family of high-affinity glucose transporters in *Saccharomyces* (Kruckeberg and Bisson 1990; Lewis and Bisson 1991). The role of each gene product in

glucose transport in *Saccharomyces* should be elucidated by further gene expression analysis under different physiological and genetic conditions as well as protein localization studies under different growth conditions.

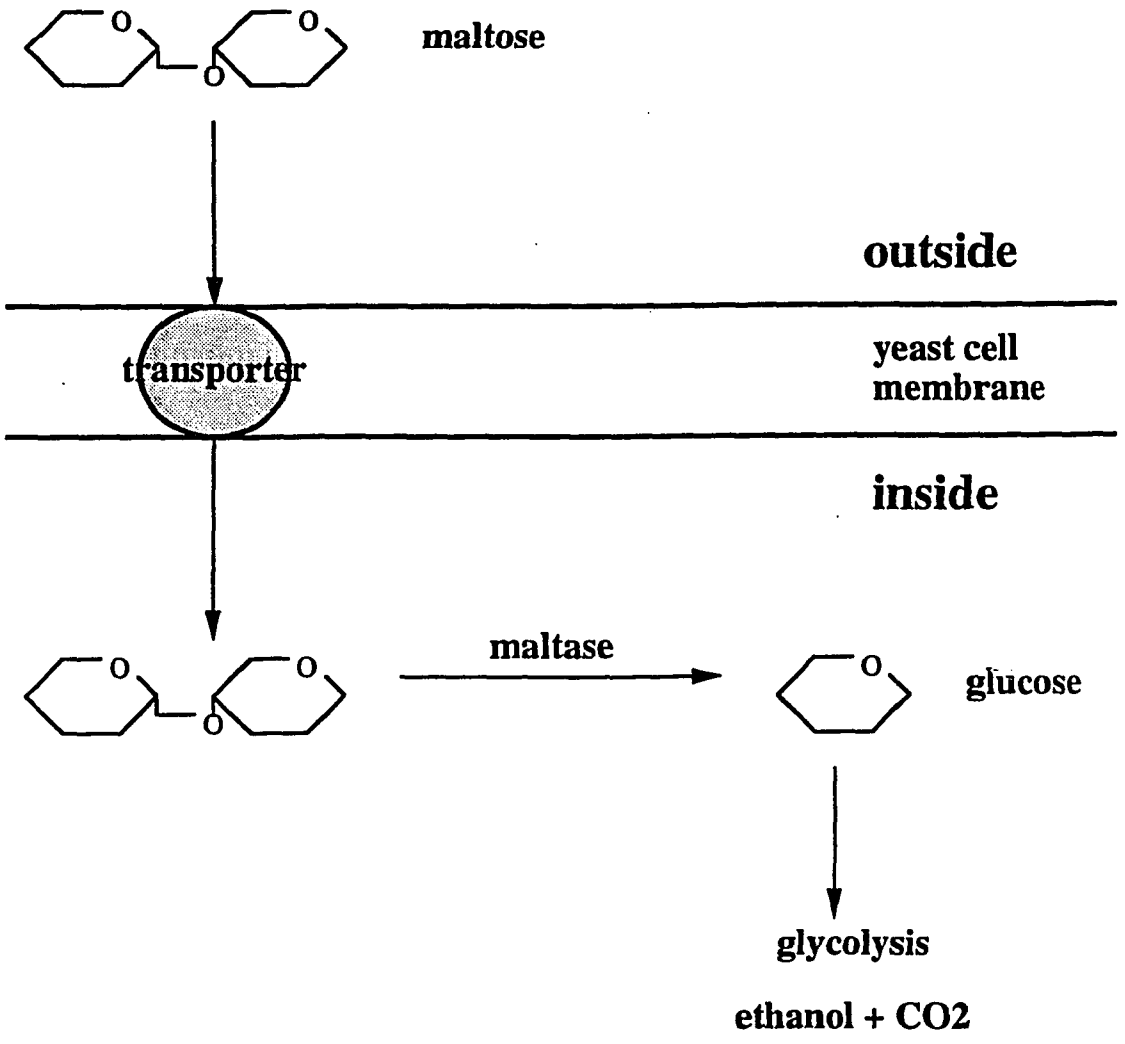
Genetic, biochemical and molecular biology approaches have also been used to characterize the galactose transport system in *Saccharomyces cerevisiae*. *GAL2* was first proposed to encode a protein required for the transport of galactose based on an analysis of the galactose uptake deficient phenotype of *gal2* mutant (Douglas and Condie 1954). By characterizing the kinetics of galactose uptake in the wild type *GAL2* strain and in the mutant *gal2* strain, it has been reported that galactose induces two transport processes, a galactokinase-independent, low-affinity process and a galactokinase-dependent, high-affinity process, and both processes depend on the presence of the *GAL2* gene (Ramos, Szkutnicka and Cirillo 1989). In uninduced cultures, or induced *gal2* mutants, galactose enters the cell only as a poor analog of glucose and it is transported by a constitutive, low-affinity facilitated diffusion process (Cirillo 1968; van Steveninck 1972; van Steveninck and Dawson 1968). Further evidence supporting the *GAL2* gene as a permease gene came from the sequencing analysis (Nehlin, Carlberg and Ronne 1989; Szkutnicka et al. 1989). The nucleotide sequence of *GAL2* predicts a protein with 574 amino acids and a molecular weight of 63,789 dalton (Szkutnicka et al. 1989). This predicted

GAL2 protein contains twelve transmembrane domains and shows both sequence and structural homology with the superfamily of sugar transporters which contains members from several species including the human glucose transporter GLUT1 (Szkutnicka et al. 1989). The protein product of *GAL2* has been purified and reconstituted in liposomes, and this experiment fully demonstrated that the GAL2 protein is a functional galactose transporter (Ramos, Szkutnicka and Cirillo 1989).

A major effort of my research has been a structural and functional characterization of the maltose transport system of *Saccharomyces*. The transport of maltose is the initial step involved in maltose fermentation in *Saccharomyces* (De La Fuente and Sols 1961) (Figure 1). Maltose is a disaccharide consisting of two molecules of glucose (Barnett 1976). It is transported into the yeast cell via a protein carrier, referred to as maltose transporter or maltose permease, and the transported maltose then is split into two molecules of glucose by the cytoplasmic cleavage enzyme, maltase (De La Fuente and Sols 1961; Harris and Thompson 1961). Fermentation of maltose is completed via the glycolysis pathway to generate ethanol and CO_2.

Maltose fermentation is an inducible process. In the absence of maltose, the level of maltose uptake and maltase activity is very low (Robertson and Halvorson 1956). The synthesis of both maltose permease and maltase is coordinately induced in the presence of maltose and is repressed in the

Figure 1. The physiological pathway of maltose fermentation in *Saccharomyces*.


maltose
outside
transporter
yeast cell
membrane
inside
maltase
glucose
glycolysis
ethanol + CO2

presence of glucose (de Kroon and Koningsberger 1970; Robertson and Halvorson 1956; van Rijn and van Wijk 1972).

The transport of maltose is a highly specific process (Harris and Thompson 1961). Of a number of sugars and sugar derivatives, including glucose, 2-deoxyglucose, sucrose, fructose, lactose, methyl-α-glucoside, maltotriose, raffinose, cellobiose and turanose, only methyl-α-glucoside and turanose at comparatively high concentrations inhibit the uptake of maltose, and maltose is far more effective as an inducer of maltose permease expression than any of the other sugars (De la Fuente and Sols 1961; Harris and Thompson 1961).

Maltose transport is an active transport system. The uptake of maltose by induced yeast cells is inhibited by various energy uncouplers, indicating that maltose transport requires an energy supply (Harris and Thompson 1961; Serrano 1977). The energy source of maltose transport has been found to be independent of the intracellular ATP level and is provided by the electrochemical gradient of protons in the cell membrane. It appears that the maltose uptake system is a maltose/proton symport system (Seaston, Idkson and Eddy 1973; Serrano 1977).

Like the multiple forms of the glucose and galactose transport systems in yeast, both high-affinity and low-affinity forms of maltose transport system also were revealed in genetically-undefined baker's *Saccharomyces* strains (Busturia and Lagunas 1985). While the biochemical process of

maltose transport has been extensively studied, the molecular and genetic basis of these two transporters is poorly understood.

The genetics of maltose fermentation in *Saccharomyces*

Classical genetic analysis identified five *MAL* loci (*MAL1*, *MAL2*, *MAL3*, *MAL4* and *MAL6*) involved in maltose fermentation in *Saccharomyces* (reviewed in Barnett 1976; Federoff et al. 1982; Needleman et al. 1984; Needleman and Michels 1983; Winge and Roberts 1950). These *MAL* loci appeared to be functionally equivalent; each alone was capable of enabling *Saccharomyces* strains to ferment. The five *MAL* loci have been genetically mapped, and all are located at or near a telomere (Figure 2): *MAL1*, at the right arm of chromosome VII (Celenza and Carlson 1985); *MAL2*, at the right arm of chromosome III; *MAL3*, at the right arm of chromosome II; *MAL4*, at the right arm of chromosome XI (Mortimer and Schild 1980); *MAL6*, at the chromosome VIII (Charron et al. 1989). Each *MAL* locus is a complex consisting of three genes (referred to as GENE 1, GENE 2 and GENE 3), all essential for maltose fermentation (Charron, Dubin and Michels 1986; Cohen et al. 1984; Cohen et al. 1985; Michels and Needleman 1984; Needleman et al. 1984) (Figure 2). Restriction enzyme mapping and Southern analysis as well as functional analysis by complemetation studies revealed that each of the five *MAL* loci exhibited extensive sequence and functional homology to each

Figure 2. The genetic organization and nomenclature of the *MAL* loci of *Saccharomyces*.

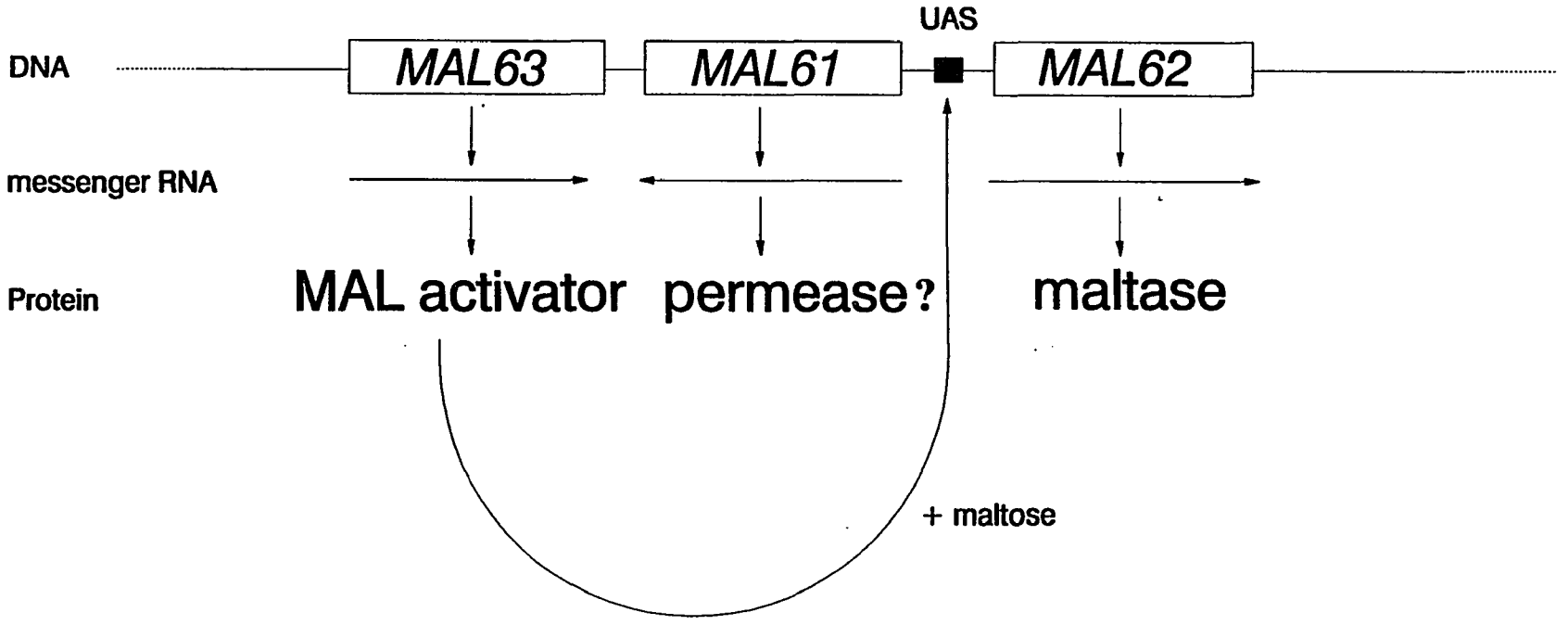
Locus	Chromosome	GENE3	GENE1	GENE2
MAL 1	VII	*MAL13*	*MAL11*	*MAL12*
MAL2	III	*MAL23*	*MAL21*	*MAL22*
MAL3	II	*MAL33*	*MAL31*	*MAL32*
MAL4	XI	*MAL43*	*MAL41*	*MAL42*
MAL6	VIII	*MAL63*	*MAL61*	*MAL62*

other with only a few restriction site polymorphism (Charron, Dubin and Michels 1986; Charron et al. 1989; Needleman et al. 1984). A nomenclature has been established to indicate both locus position and GENE function (Figure 2). For example, *MAL61* represents GENE 1 at the MAL6 locus and *MAL11* represents GENE 1 at the *MAL1* locus (Needleman et al. 1984; Charron and Michels 1986).

For the past twelve years, research work in the laboratory of Dr. Corinne Michels has focused on the molecular and functional analysis of these three maltose fermentation gene products as well as characterizing the mechanisms of their regulation and interaction in response to maltose and glucose. The *MAL6*- and *MAL1*-encoded maltose fermentation genes have been studied in greatest detail. Figure 3 represents an overview of the functional and transcriptional regulation of the maltose fermentation genes at the *MAL6* locus.

MAL63 (GENE 3 homolog) encodes a transcription activator, the MAL-activator, which functions as a positive regulator of maltose fermentation and is required for the inducible expression of *MAL61* and *MAL62* (Chang et al. 1988; Needleman et al. 1984). The *MAL63* gene has been sequenced and its deduced protein sequence indicates that the MAL63 protein is a DNA-binding protein based on the presence of a cysteine and basic amino acid rich sequence similar to the DNA-binding domains of several yeast transcription activators (Kim and Michels 1988;

Figure 3. An overview of the functional and transcriptional regulation of the maltose fermentation genes at the *MAL6* locus.


UAS
DNA
MAL63
MAL61
MAL62
messenger RNA
Protein
MAL activator
permease?
maltase
+ maltose

Sollitti and Marmur 1988). Recent studies has confirmed that the cysteine-rich region of the MAL63 protein is responsible for binding to the UAS_{MAL} (J. Kim, unpublished results), the bidirectional promotor element localized within the intergenic region of *MAL61* and *MAL62* (Levine, Tanouye and Michels, submitted). The MAL-activator functions along with the UAS_{MAL} to render the maltose inducible expression of the *MAL61* and the *MAL62* genes. In the presence of maltose, the *MAL*-activator is proposed to bind in an active form to the UAS_{MAL} region to initiate a divergent, coordinate transcription of the *MAL61* and *MAL62* genes (Figure 3). This maltose inducible process is repressed in the presence of glucose by an yet undefined mechanism.

MAL62 (GENE 2 homolog) has been sequenced (Hong and Marmur 1986). It encodes the cleavage enzyme maltase. This conclusion is based on genetic studies in which a temperature sensitive maltase variant was identified and mapped to the *MAL12* gene of the *MAL1* locus, a homolog of *MAL62*. Additionally, a deletion/disruption of GENE 2 leads to the abolition of maltase expression and function (Cohen et al. 1984; Dubin et al. 1985; Charron, Dubin and Michels 1986). Biochemical evidence revealed that in each of the *MAL* genotypes, only a single species of maltase was observed (Halvorson, Winderman and Gorman 1963). The maltase produced in *Saccharomyces* in response to the 5 non-allelic *MAL* loci were purified and found to be indistinguishable with regard to

heat inactivation, electrophoretic mobility, chromatography on CM-cellulose or DEAE-cellulose columns, neutralization with specific antiserum, and substrate specificity (Halvorson, Winderman and Gorman 1963; Needleman et al. 1978).

The first evidence that the gene encoding maltose permease mapped to each of the *MAL* loci came from the identification of a *MAL1*-linked temperature-sensitive mutation affecting maltose transport (Goldenthal, Cohen and Marmur 1983). Genetic analysis of mutations in *MAL61*, a GENE 1 homolog located at the *MAL6* locus, strongly suggested that *MAL61* encodes maltose permease or a regulator that is required for the synthesis of maltose permease (Chang et al. 1989). Using a carbon dioxide electrode to determine CO_2 evolution, maltose transport was measured indirectly in a genetically-defined *MAL6* wild type and several *mal6* mutant strains. The results showed that a deletion/disruption of the *MAL61* gene completely abolished maltose transport activity. Transformation of this *mal61Δ* mutant strain with high copy plasmids carrying the *MAL61* gene led to up to a tenfold increase in maltose permease activity as compared to the single-copy parental strain. Integration of a DNA fragment carrying the yeast *URA3* gene into the coding region of *MAL61* near the N-terminal end resulted in a low level constitutive transcription of *MAL61* and in a low level constitutive synthesis of maltose permease (Chang et al. 1989).

To determine whether *MAL61* encodes maltose permease

itself or a regulator required for the synthesis or function of maltose permease, it was essential to analyze the sequence structure of the *MAL61* gene to see if the *MAL61*-encoded protein exhibits characteristics that would be expected of an integral membrane transport protein. As an initial step in pursuing this study, we sequenced the *MAL61* gene and analyzed the structural features of the predicted MAL61 protein. The predicted protein sequence of the *MAL61* gene was found to be highly homologous to the sequences of a group of known sugar transporters including the human glucose transporter GLUT1. These results were used to support the hypothesis that MAL61 protein could be a sugar transporter protein. This part of work is described in the Chapter 1 entitled, "The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters".

To further elucidate the nature of MAL61, we have identified its subcellular localization by constructing an in-frame gene fusion of *MAL61* to the *E. coli lacZ* gene encoding ß-galactosidase. Our results revealed that the *MAL61-lacZ* fusion product localized predominantly to the yeast membrane fraction, thus demonstrating that MAL61 is a membrane protein. This part of work is described in the Chapter 2 entitled, "*MAL61* of *Saccharomyces* encodes an integral membrane protein".

To determine the function of the MAL61 protein as a maltose transporter, a kinetic study of maltose uptake was

performed in a genetically well-defined strain. Early kinetic study of maltose uptake in genetically undefined maltose fermenting strains of *Saccharomyces cerevisiae* indicated the apparent presence of at least two forms of the maltose transporter: a low-affinity transporter with a Km of 70 mM, and a high-affinity transporter with a Km of 4 mM (Busturia and Lagunas 1985). Given the polygenic nature of the *MAL* loci, we wished to explore the possibility that the two kinetically distinct maltose transporters could be the products of different *MAL* genes or at least one of them could be the product of GENE 1. Therefore, the kinetics of maltose uptake was studied in a genetically well-characterized strain carrying a single copy of either the *MAL11* or *MAL61* gene to further elucidate the biochemical nature of the MAL11 and MAL61 proteins. This part of work is described in Chapter 3 entitled, "*MAL11* and *MAL61* encode the inducible high-affinity maltose transporter of *Saccharomyces cerevisiae*".

Glucose-induced inactivation in *Saccharomyces*

Sugar utilization in yeast is governed not only by the substrate specificity of the transport proteins encoded in the strains' genome but also by the characteristics of the regulatory mechanisms controlling expression of these transporters. The synthesis of GENE 1 and GENE 2 of the *MAL* loci is regulated by both maltose and glucose. Another part of my thesis work is a study on the glucose regulation of

maltose transport system, specifically, a demonstration of glucose-induced inactivation of *MAL61*-encoded maltose transport system.

Glucose repression (or catabolite repression) and glucose-induced inactivation (or catabolite inactivation) are the two reported mechanisms controlling the sugar fermentation enzymes as well as gluconeogenic enzymes at both the transcriptional and post-translational levels (reviewed in Carlson 1987; reviewed in Holzer 1976; Sedivy and Fraenkel 1985). Because of these two regulatory processes, in the presence of glucose, yeast cells are able to eliminate the further synthesis of glucose as well as the utilization of other sugars such as galactose, sucrose and maltose, and thereby to preferentially utilize glucose which enters the glycolytic pathway directly.

Glucose repression is a global regulatory system governing the response of cells to the availability of glucose. It has been found to affect the expression of the sucrose fermentation genes, the galactose fermentation genes, the maltose fermentation genes as well as the genes encoding the gluconeogenic enzymes (reviewed in Carlson 1987). Glucose repression occurs at the mRNA synthesis level. In the presence of glucose, none of these sugar fermentation enzymes or gluconeogenic enzymes are further synthesized. Genetic and biochemical studies have shown that glucose repression is a complex regulatory system involving more than a dozen putative

regulatory genes (Entian 1986; Gancedo and Gancedo 1986; Carlson 1987). The yeast gene *SNF1*, which is known to play a central role in glucose repression and control the expression of a series of glucose repressible genes in the yeast *Saccharomyces cerevisiae*, has been found to encode a protein kinase, indicating that glucose repression may involve protein phosphorylation (Celenza and Carlson 1986). The detailed molecular mechanism of glucose repression is not known at present.

In addition to glucose repression, yeast cells are able to rapidly inactivate the pre-existing sugar transport activity and gluconeogenic enzyme activity by an as yet undefined, post-translational control mechanism, referred to as glucose-induced inactivation. Glucose-induced inactivation has been reported to affect the *SNF3*-encoded high-affinity glucose transporter (Bisson and Fraenkel 1983b; Ramos, Szkutnicka and Cirillo 1988), the *GAL2*-encoded inducible high- and low-affinity galactose transporters (Matern and Holzer 1977; Ramos, Szkutnicka and Cirillo 1989), the maltose transport activity (Busturia and Lagunas 1985; Gorts 1969), the activity of the gluconeogenic enzymes including fructose-1,6-bisphosphatase, cytoplasmic malate dehydrogenase and phosphoenolpyruvate carboxykinase (Ferguson, Jr and Holzer 1967; Gancedo 1971; Haarasilta and Oura 1975; Witt, Kronau and Holzer 1966), aminopeptidase I and uridine nucleosidase (Frey and Rohm 1979; Magni et al. 1977). The addition of glucose

leads to a complete loss of the enzymatic activity of these proteins within one to two hours and such a rapid inactivation could not simply be explained by growth dilution of the pre-existing proteins after cessation of their synthesis under glucose repression (reviewed in Holzer, 1976).

The mechanism of glucose-induced inactivation of these proteins has been studied by various research groups and the inactivation of each of these proteins has been described as an irreversible process which might involve proteolysis since the recovery from glucose-induced inactivation requires *de novo* protein synthesis (reviewed in Holzer 1976). Studies on aminopeptidase I and the gluconeogenic enzymes from *Saccharomyces* indicate that glucose-induced inactivation of these enzyme activities is paralleled by a decrease in the amount of cross-reacting material, strongly suggesting that inactivation results from their selective proteolysis (Frey and Rohm 1979; Funayama, Gancedo and Gancedo 1980; Muller, Muller and Holzer 1981; Neeff et al. 1978; Tortora et al. 1981). A detailed study of fructose-1,6-bisphosphatase, one of the key enzymes in gluconeogenesis of *Saccharomyces*, has shown that the addition of glucose to derepressed yeast cells leads to its biphasic inactivation (Lenz and Holzer 1980). Within the first 3 minutes after glucose addition, the enzyme is reversibly phosphorylated resulting in the reversible inactivation of 60% of the enzyme activity (Muller and Holzer 1981; Maron, Gancedo and Gancedo 1980). During the second

irreversible phase, proteolysis leads to an almost complete disappearance of the enzyme activity and cross-reacting material within one hour (Funayama, Ganceko and Gancedo 1980; Tortora et al. 1981). The reversible phase of glucose inactivation was not detected for cytoplasmic malate dehydrogenase and phosphoenolpyruvate carboxykinase and no phosphorylation of phosphoenolpyruvate carboxykinase was evidenced (Tortora et al. 1984), therefore the role of phosphorylation in glucose-induced inactivation remains unclear. A recent study has revealed that glucose-induced inactivation and proteolytic degradation of fructose 1,6-bisphosphatase requires an intact *PEP4* gene, which encodes a protease that regulates the other yeast vacuolar proteases (Chiang and Schekman 1991). In response to glucose, yeast cells redistribute the cytosolic fructose 1,6-bisphosphatase to the yeast vacuole and enzyme degradation occurs in the vacuole following this relocalization (Chiang and Schekman 1991).

Kinetic studies in mutant *Saccharomyces* strains affecting cyclic-AMP dependent protein kinase (cAPK) have shown that the glucose-induced inactivation of the high-affinity glucose transporter as well as the induced high- and low-affinity galactose transporter is mediated by cAMP-dependent protein kinase (Ramos and Cirillo 1989). In *tpk1* mutants which lack cAPK, these sugar transporters are not inactivated in the presence of glucose. In *bcy1* mutants, which do not contain

the inhibitory subunit of cAPK and thus express unregulated cAPK, the activities of these sugar transporters are greatly reduced indicating that constitutively expressed cAPK leads to constitutive, glucose-induced inactivation of these sugar transporters (Ramos and Cirillo 1989). It has not been determined whether the cAPK-dependent phosphorylation during glucose-induced inactivation affects these sugar transporters themselves or affects some other protein(s) involved in the inactivation process.

Maltose transport is a highly regulated process. While maltose transporter is coregulated with maltase by glucose repression, studies in genetically undefined *Saccharomyces* strains indicated that maltose transporter is additionally subject to glucose-induced inactivation (Busturia and Lagunas 1985; Gorts 1969; van Rijn and van Wijk 1972). To explore the complexity of glucose regulation of maltose transport for the *MAL61*-encoded maltose transporter, a careful and detailed functional analysis was performed in genetically-well defined *Saccharomyces* strains. This part of work is described in Chapter 3 entitled, "A demonstration of glucose-induced inactivation of *MAL61*-encoded maltose transporter of *Saccharomyces*".

CHAPTER 1

The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters

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ABSTRACT

The *MAL61* gene of *Saccharomyces cerevisiae* encodes maltose permease a protein required for the transport of maltose across the plasma membrane. Here we report the nucleotide sequence of the cloned *MAL61* gene. A single 1842 base pair open reading frame is present within this region encoding the 614 residue putative MAL61 protein. Hydropathy analysis suggests that the secondary structure consists of two blocks of six transmembrane domains separated by an approximately 71 residue intracellular region. The N-terminal and C-terminal domains of 100 and 67 residues in length, respectively, also appear to be intracellular. Significant sequence and structural homology is seen between the MAL61 protein and the Saccharomyces high-affinity glucose transporter encoded by the *SNF3* gene, the *Kluyveromyces lactis* lactose permease encoded by the *LAC12* gene, the human HepG2 glucose transporter and the *Escherichia coli* xylose and arabinose transporters encoded by the *xylE* and *araE* genes indicating that all are members of a family of sugar transporters and are related either functionally or evolutionarily. A mechanism for glucose-induced inactivation of maltose transport activity is discussed.

INTRODUCTION

Maltose fermentation in the Saccharomyces yeasts is initiated by the transport of the disaccharide across the plasma membrane. This transport is carried out by maltose permease and the process is the rate limiting step in fermentation. An understanding of the mechanisms controlling maltose transport is therefore fundamental to an understanding of the factors regulating maltose fermentation. The Saccharomyces maltose uptake system is an inducible active transport system (Harris and Thompson 1961; Okada and Halvorson 1964; De Kroon and Koningsberger 1970; Serrano 1977). Serrano (1977) reports that this transport is independent of intracellular ATP levels but is coupled to the electrochemical gradient of protons. That is, maltose transport occurs via a proton symport system. As has been seen in the glucose and galactose transport systems of Saccharomyces, the maltose transport system exists in both a high and a low affinity form (Bisson and Fraenkel 1983a; Bisson and Fraenkel 1983b; Bisson and Fraenkel 1984; Ramos, Szkutnicka and Cirillo 1989; Busturia and Lagunas 1985). The basis of the difference between the two forms of these sugar transporters is not understood.

Saccharomyces strains able to ferment maltose carry any one of five *MAL* loci: *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6* (reviewed by Barnett 1976). The first indication that the

gene encoding maltose permease mapped to any of the *MAL* loci came from the identification of a *MAL1*-linked temperature-sensitive maltose transport mutation (Goldenthal, Cohen and Marmur 1983). All of the *MAL* loci have been cloned and structurally and functionally compared (Federoff et al. 1982; Needleman and Michels 1983; Charron, Dubin and Michels 1986; Charron and Michels 1987; Charron et al. 1989). The *MAL* loci are all highly sequence homologous exhibiting only a few restriction site polymorphisms. Each locus is a complex locus containing three genes required for maltose fermentation: GENEs 1, 2, and 3 (Needleman et al. 1984). We have established a two digit numbering system in order to distinguish the GENE 1, 2 or 3 functions mapping to the different *MAL* loci. The first digit indicates the locus position and the second the GENE function (Needleman et al. 1984; Charron and Michels 1987; Charron and Michels 1988). Thus, the *MAL61* gene is the GENE 1 function mapping to the *MAL6* locus.

Transcription of GENEs 1 and 2 is induced by maltose and repressed by glucose (Needleman et al. 1984). That GENE 2 encodes maltase is inferred from the identification of an allele of the *MAL12* gene (that is, GENE 2 of the *MAL1* locus) that encodes a temperature-sensitive maltase (Dubin et al. 1985). GENE 1 encodes maltose permease. This conclusion is based on several lines of evidence reported by Y. S. Chang, R. A. Dubin, E. Perkens, C. A. Michels and R. B. Needleman (Chang

et al. 1989). Point mutations in the *MAL61* gene as well as a deletion/disruption of the *MAL61* gene completely abolish maltose transport activity. Transformation of these mutant strains with high copy plasmids carrying the *MAL61* gene leads to up to a 10-fold increase in maltose permease activity as compared to the single-copy parental strain. Most significantly, the integration of a fragment carrying the yeast *URA3* gene into the coding region of *MAL61* near the N-terminal end results in a low level constitutive transcription of *MAL61* and in a low level constitutive synthesis of maltose permease. GENE 3 encodes the MAL activator and the product of this gene is a cysteine-zinc finger protein (Chang et al. 1988; Kim and Michels 1988; Solliti and Marmur 1988).

This report presents the sequence of the *MAL61* gene. Analysis of the deduced amino acid sequence of the proposed MAL61 protein indicates that it is an integral membrane protein. Additionally, MAL61 protein shows significant homology to several other sugar transport proteins from yeast and other species. This homology is seen both on the level of the primary sequence and on the level of secondary structure.

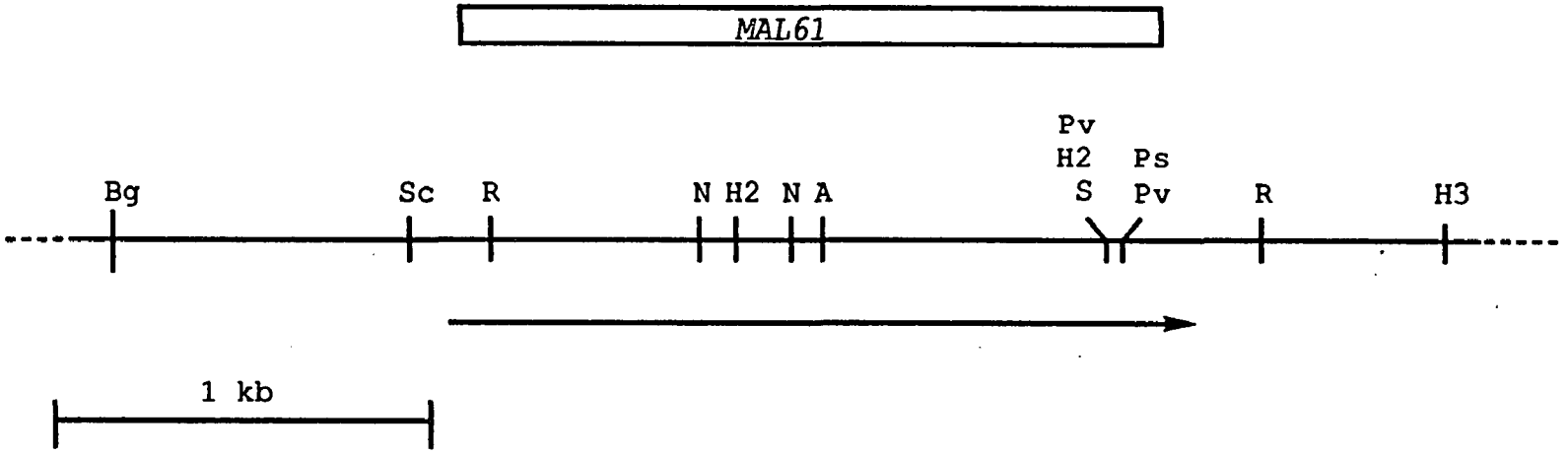
MATERIALS AND METHODS

Sequencing: Figure 1 shows a restriction endonuclease map of the *MAL61* gene. Sequencing was done according to the method of Sanger, Nicklen and Coulson (1977). The region was divided into three fragments: the *Pst*I-*Eco*RI fragment containing the *MAL61* upstream sequences and the 5'-end of the gene; the 1.7 Kb *Eco*RI-*Sal*I fragment containing sequences internal to the *MAL61* gene; and the *Sal*I-*Hind*III fragment containing the 3'-end of the gene. Each of these was then sequenced by a combination of methods. Nested deletions within the *MAL61*-insert fragments were constructed with the fragment cloned into the M13 sequencing vector mp18 using exonuclease III and these were sequenced using the universal primer (Messing 1983; Henikoff 1984). Gaps were filled by using oligonucleotide primers identical to known sequences. Nested deletions were also constructed using Bal31 to degrade the *MAL61*-insert fragment cloned in the plasmid vector pBR325. For sequencing, these deletions were subcloned into the M13 sequencing vectors. Sequencing of the second strand was carried out using the 3.6 kb *Bgl*II - *Hind*III fragment containing the entire *MAL61* gene cloned into the M13 vector mp19. This was sequenced with a series of oligonucleotide primers complementary to known *MAL61* sequence.

Computer analysis: Sequence data was analyzed using the programs of IntelliGenetics, Inc. of Palo Alto, California.

Figure 1. Restriction endonuclease map of the *MAL61* gene of *S. cerevisiae*.

The restriction endonuclease map of the 3.6 kb DNA fragment containing the *MAL61* gene and its flanking sequence is shown. The abbreviations used are: A, AvaI; B, BglII; H2, HindII; H3, HindIII; N, NcoI; Ps, PstI; Pv, PvuII; R, EcoRI; Sc, ScaI.


MAL61
Pv
H2
S
Ps
Pv
Bg
Sc
R
N
H2
N
A
R
H3
1 kb

Alignment of the MAL61 protein sequence with several other transport protein sequences was carried out using the GENALIGN program. GENALIGN is a copyrighted software product of IntelliGenetics, Inc.; the program was developed by Dr. Hugo Martinez of the University of California at San Francisco. The hydropathy plots shown in Figure 4 comparing MAL61 and SNF3 proteins are the gift of John Celenza, Linda Marshall-Carlson and Marian Carlson of the Department of Genetics and Development, Columbia University College of Physicians and Surgeons, New York; the profiles were made using the algorithm developed by Kyte and Doolittle (1982) and utilized the values of Eisenberg (1984) with a 21-residue window.

RESULTS

Sequence of the *MAL61* gene and the proposed secondary structure of the deduced protein: Figure 2 presents the sequence of the DNA fragment containing the *MAL61* gene starting at the *Sca*I site shown in Figure 1 and extending to the right for 2000 base pairs. A single large open reading frame is observed with the AUG codon of the N-terminal methionine located 105 basepairs from the *Sca*I site. No other large open reading frames are observed in any of the five other reading frames. The orientation of this single 1842 base pair open reading frame is consistent with the size of the maltose inducible transcript of the *MAL61* gene (2.0 kilobasepairs) and with the direction of transcription of the *MAL61* gene as reported in Needleman et al (1984). Construction of a *MAL61-lacz* fusion at the *Eco*RI site near the N-terminal end of the coding region supports the conclusion that the AUG codon indicated as the translation initiation can function as such in Saccharomyces (Levine, Tanouye and Michels, submitted). A consensus "TATA" sequence is located at position -89 to -94. The sequence of the open reading frame predicts a 67,174 dalton protein of 614 amino acid residues.

Figure 2 also depicts the positions of twelve postulated hydrophobic transmembrane domains. Each of the twelve postulated 21-residue transmembrane domains has an average

Figure 2. Nucleotide sequence of the *MAL61* gene and predicted amino acid sequence of the gene product.

The nucleotide sequence of the *MAL61* gene is given starting at the upstream ScaI 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. Asterisks indicate the termination codons. Putative 21 residue membrane-spanning regions are boxed and shaded. The location of these is based on the algorithm of Kyte and Doolittle (1982) using the hydropathy parameters of Eisenberg (1984).

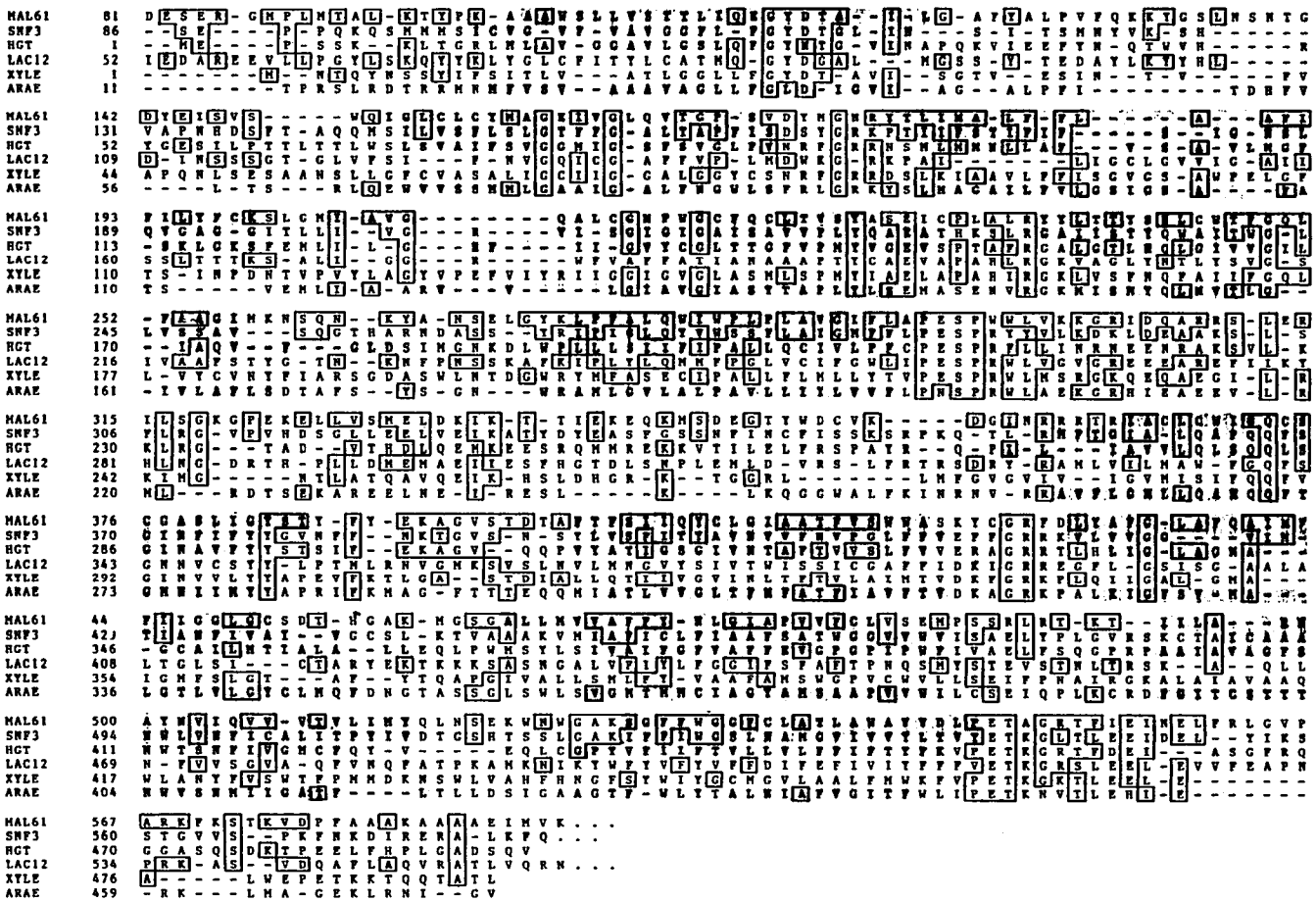
```
-105                AGTACTCAGCATATAAAGAGACACAATATACTCCATACTTGTTGTGAGTGGTTTTAGCGTATTCAGTATAACAATAAGAATTACATCCAAGACTATTAATTAACT

    Met Lys Gly Leu Ser Ser Leu Ile Asn Arg Lys Lys Asp Arg Asn Asp Ser His Leu Asp Glu Ile Glu Asn Gly Val Asn Ala Thr Glu   30
1   ATG AAG GGA TTA TCC TCA TTA ATA AAC AGA AAA AAA GAC AGG AAC GAC TCA CAC TTA GAT GAG ATC GAG AAT GGC GTG AAC GCT ACC GAA

    Phe Asn Ser Ile Glu Met Glu Glu Gln Gly Lys Lys Ser Asp Phe Asp leu Ser His Leu Glu Tyr Gly Pro Gly Ser Leu Ile Pro Asn   60
91  TTC AAC TCG ATA GAG ATG GAG GAG CAA GGT AAG AAA AGT GAT TTT GAT CTT TCC CAT CTT GAG TAC GGT CCA GGT TCA CTA ATA CCA AAC

    Asp Asn Asn Glu Glu Val Pro Asp Leu Leu Asp Glu Ala Met Gln Asp Ala Lys Glu Ala Asp Glu Ser Glu Arg Gly Met Pro Leu Met   90
181 GAT AAT AAT GAA GAA GTC CCC GAC CTT CTC GAT GAA GCT ATG CAG GAC GCC AAA GAG GCA GAT GAA AGT GAG AGG GGA ATG CCA CTC ATG

    Thr Ala Leu Lys Thr Tyr Pro Lys Ala Ala Ala Trp Ser Leu Leu Val Ser Thr Thr Leu Ile Gln Glu Gly Tyr Asp Thr Ala Ile Leu  120
271 ACA GCT TTG AAG ACA TAT CCA AAA GCT GCT GCT TGG TCA CTA TTA GTT TCC ACA ACA TTG ATT CAA GAG GGT TAT GAC ACA GCC ATT CTA

    Gly Ala Phe Tyr Ala Leu Pro Val Phe Gln Lys Lys Tyr Gly Ser Leu Asn Ser Asn Thr Gly Asp Tyr Glu Ile Ser Val Ser Trp Gln  150
361 GGA GCT TTC TAT GCC CTG CCT GTT TTT CAA AAA AAA TAT GGT TCT TTG AAT AGC AAT ACA GGA GAT TAT GAA ATT TCA GTT TCC TGG CAA

    Ile Gly Leu Cys Leu Cys Tyr Met Ala Gly Glu Ile Val Gly Leu Gln Val Thr Gly Pro Ser Val Asp Tyr Met Gly Asn Arg Tyr Thr  180
451 ATC GGT CTA TGT CTA TGC TAC ATG GCA GGT GAG ATT GTC GGT TTG CAA GTG ACT GGG CCT TCT GTA GAT TAC ATG GGC AAC CGT TAC ACT

    Leu Ile Met Ala Leu Phe Phe Leu Ala Ala Phe Ile Phe Ile Leu Tyr Phe Cys Lys Ser Leu Gly Met Ile Ala Val Gly Gln Ala Leu  210
541 CTG ATC ATG GCG TTG TTC TTT TTA GCG GCT TTC ATT TTC ATT CTG TAT TTT TGC AAG AGT TTG GGT ATG ATT GCC GTG GGA CAG GCA TTG

    Cys Gly Met Pro Trp Gly Cys Phe Gln Cys Leu Thr Val Ser Tyr Ala Ser Glu Ile Cys Pro Leu Ala Leu Arg Tyr Tyr Leu Thr Thr  240
631 TGT GGT ATG CCA TGG GGT TGT TTC CAA TGT TTG ACC GTT TCT TAT GCT TCT GAA ATT TGT CCT TTG GCC CTA AGA TAC TAT TTG ACG ACT

    Tyr Ser Asn Leu Cys Trp Thr Phe Gly Gln Leu Phe Ala Ala Gly Ile Met Lys Asn Ser Gln Asn Lys Tyr Ala Asn Ser Glu Leu Gly  270
721 TAT TCT AAT TTA TGT TGG ACG TTC GGT CAA CTT TTC GCT GCT GGT ATT ATG AAA AAT TCC CAG AAC AAA TAT GCC AAC TCA GAA CTA GGA

    Tyr Lys Leu Pro Phe Ala Leu Gln Trp Ile Trp Pro Leu Pro Leu Ala Val Gly Ile Phe Leu Ala Pro Glu Ser Pro Trp Trp Leu Val  300
811 TAT AAG CTA CCT TTT GCT TTG CAG TGG ATC TGG CCC CTT CCT TTG GCG GTA GGT ATT TTT TTG GCA CCA GAG TCT CCA TGG TGG CTG GTT

    Lys Lys Gly Arg Ile Asp Gln Ala Arg Arg Ser Leu Glu Arg Ile Leu Ser Gly Lys Gly Pro Glu Lys Glu Leu Leu Val Ser Met Glu  330
901 AAA AAA GGA AGG ATT GAT CAG GCG AGG AGA TCA CTT GAA AGA ATA TTA AGT GGT AAA GGA CCC GAG AAA GAA TTA CTA GTG AGT ATG GAA

    Leu Asp Lys Ile Lys Thr Thr Ile Glu Lys Glu Gln Lys Met Ser Asp Glu Gly Thr Tyr Trp Asp Cys Val Lys Asp Gly Ile Asn Arg  360
991 CTC GAT AAA ATC AAA ACT ACT ATA GAA AAG GAG CAG AAA ATG TCT GAT GAA GGA ACT TAC TGG GAT TGT GTG AAA GAT GGT ATT AAC AGG

    Arg Arg Thr Arg Ile Ala Cys Leu Cys Trp Ile Gly Gln Cys Ser Cys Gly Ala Ser Leu Ile Gly Tyr Ser Thr Tyr Phe Tyr Glu Lys  390
1081 AGA AGA ACG AGA ATA GCT TGT TTA TGT TGG ATC GGT CAA TGC TCC TGT GGT GCA TCA TTA ATT GGT TAT TCA ACT TAC TTT TAT GAA AAA

    Ala Gly Val Ser Thr Asp Thr Ala Phe Thr Phe Ser Ile Ile Gln Tyr Cys Leu Gly Ile Ala Ala Thr Phe Val Ser Trp Trp Ala Ser  420
1171 GCT GGT GTT AGC ACT GAT ACG GCT TTT ACT TTC AGT ATT ATC CAA TAT TGT CTT GGT ATT GCT GCA ACG TTT GTA TCC TGG TGG GCT TCA

    Lys Tyr Cys Gly Arg Phe Asp Leu Tyr Ala Phe Gly Leu Ala Phe Gln Ala Ile Met Phe Phe Ile Ile Gly Gly Leu Gly Cys Ser Asp  450
1261 AAA TAT TGT GGC AGA TTT GAC CTT TAT GCT TTT GGG CTG GCT TTT CAG GCT ATT ATG TTC TTC ATT ATC GGT GGT TTA GGA TGT TCA GAC

    Thr His Gly Ala Lys Met Gly Ser Gly Ala Leu Leu Met Val Val Ala Phe Phe Tyr Asn Leu Gly Ile Ala Pro Val Val Phe Cys Leu  480
1351 ACT CAT GGC GCT AAA ATG GGT AGT GGT GCT CTT CTA ATG GTT GTC GCG TTC TTT TAC AAC CTC GGT ATT GCA CCT GTT GTT TTT TGC TTA

    Val Ser Glu Met Pro Ser Ser Arg Leu Arg Thr Lys Thr Ile Ile Leu Ala Arg Asn Ala Tyr Asn Val Ile Gln Val Val Val Thr Val  510
1441 GTG TCT GAA ATG CCG TCT TCA AGG CTA AGA ACC AAA ACA ATT ATT TTG GCT CGT AAT GCT TAC AAT GTG ATC CAA GTT GTA GTT ACA GTT

    Leu Ile Met Tyr Gln Leu Asn Ser Glu Lys Trp Asn Trp Gly Ala Lys Ser Gly Phe Phe Trp Gly Gly Phe Cys Leu Ala Thr Leu Ala  540
1531 TTG ATC ATG TAC CAA TTG AAC TCA GAG AAA TGG AAT TGG GGT GCT AAA TCA GGC TTT TTC TGG GGA GGA TTT TGT CTG GCC ACT TTA GCT

    Trp Ala Val Val Asp Leu Pro Glu Thr Ala Gly Arg Thr Phe Ile Glu Ile Asn Glu Leu Phe Arg Leu Gly Val Pro Ala Arg Lys Phe  570
1621 TGG GCT GTT GTC GAT TTA CCA GAA ACC GCT GGC AGG ACT TTT ATT GAG ATA AAT GAA TTG TTT AGA CTT GGT GTT CCA GCA AGA AAG TTC

    Lys Ser Thr Lys Val Asp Pro Phe Ala Ala Ala Lys Ala Ala Ala Ala Glu Ile Asn Val Lys Asp Pro Lys Glu Asp Leu Glu Thr Ser  600
1711 AAG TCG ACT AAA GTC GAC CCT TTT GCA GCT GCC AAA GCA GCA GCT GCA GAA ATT AAT GTT AAA GAT CCG AAG GAA GAT TTG GAA ACT TCT

    Val Val Asp Glu Gly Arg Ser Thr Pro Ser Val Val Asn Lys ***       ***                                                  614
1801 GTG GTA GAT GAA GGG CGA AGC ACC CCA TCT GTT GTG AAC AAA TGATTTTTTTAGCCAGTAGGTAGATCGGCGTTATTTAATTTTATTTTATATAA
```

hydrophobicity value of greater than 0.42. As in the SNF3 protein, no signal sequence is seen at the N-terminal end of the MAL61 protein and the first predicted transmembrane domain begins at residue 100 suggesting that the N-terminal 100 amino acid residues lie on the cytoplasmic face of the plasma membrane. The overall secondary structure of the MAL61 protein thus appears to consist of two blocks of six transmembrane domains separated by an approximately 71 residue intracellular region. Both the 100 N-terminal residues and the 67 C-terminal residues are predicted to lie on the cytoplasmic face of the plasma membrane. Although two potential N-linked glycosylation sites are found at Asn-15 and Asn-27, these may not be modified since they lie within the proposed cytoplasmic N-terminal region. This remains to be determined since it has been shown that tunicamycin inhibits the synthesis of the maltose transport system in Saccharomyces (Lagunas, Dejuan and Benito 1986). Work is now in progress in our laboratory to verify the predicted membrane topology of the maltose permease.

Homology of MAL61 protein to other sugar transporters: Comparison of the deduced sequence of the MAL61 protein to that of the SNF3 protein reveals an approximate 24% sequence homology (Figure 3). The *SNF3* gene encodes the high-affinity glucose transporter of Saccharomyces or a component of this transport system (Celenza, Marshall-Carlson and Carlson 1988). More impressive than the sequence homology is the structural

Figure 3. Sequence and structural homology among MAL61 protein and other sugar transporters.

Amino acid sequences of the MAL61 protein, the high-affinity glucose transporter of *Saccharomyces* encoded by *SNF3* gene (Celenza, Marshall-Carlson and Carlson 1988), the lactose permease of *Kluyveromyces lactis* encoded by the *LAC12* gene (Chang and Dickson 1988), Human HepG2 glucose transporter (Mueckler et al. 1985) and the *Escherichia coli* xylose and arabinose transporter (Maiden et al. 1987) are shown using standard single-letter amino acid symbols. The proteins are aligned so as to maximize identity to the MAL61 protein sequence. Gaps (indicated by dashes) are introduced to optimize the alignment. Identities with the MAL61 protein are boxed. Shaded regions indicate the putative transmembrane regions in the MAL61, SNF3 (Celenza, Marshall-Carlson and Carlson 1988), human HepG2 (Mueckle et al. 1985), and AraE (Maiden et al. 1987) protein sequences. Amino acids are numbered on the left.

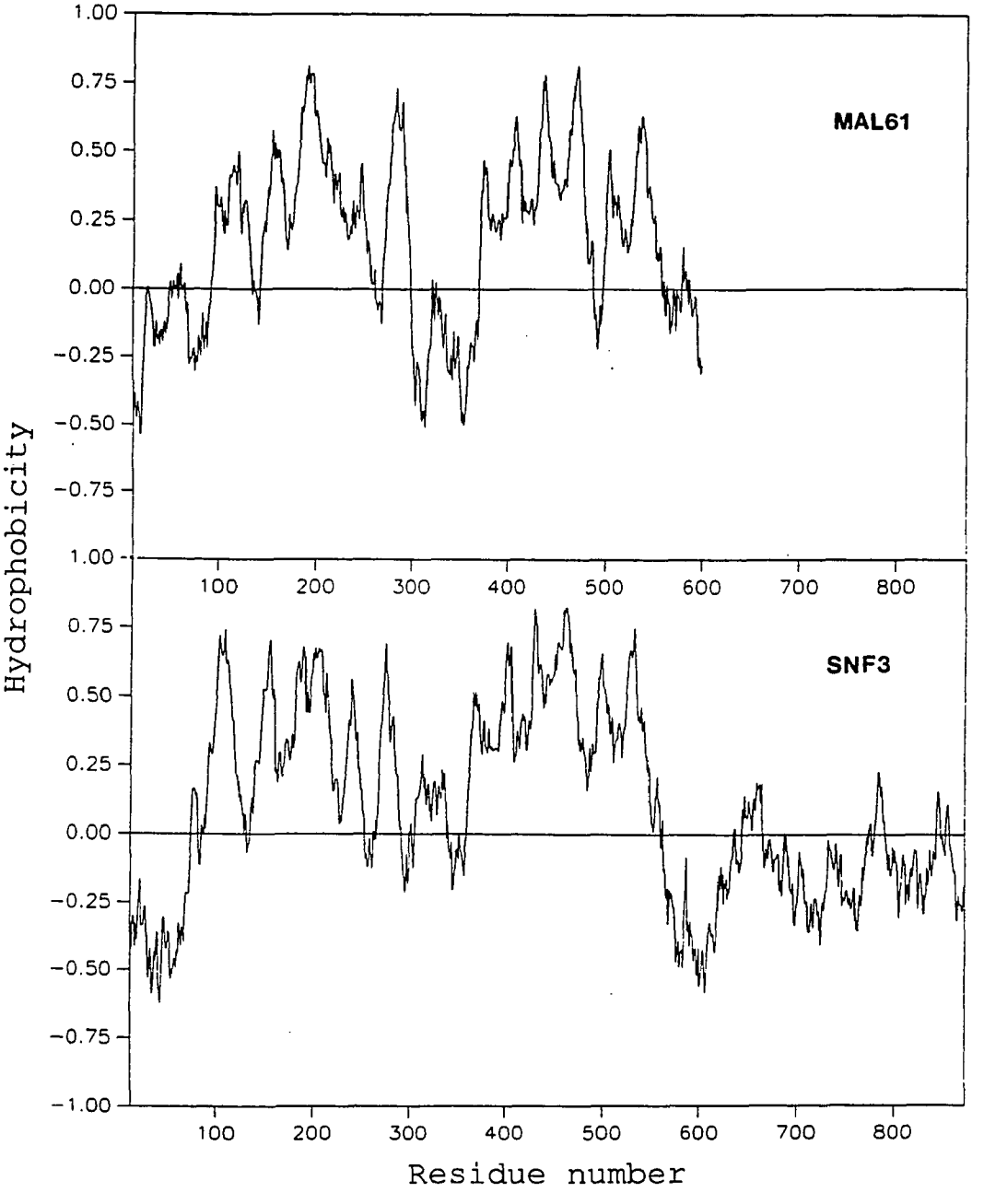


homology between these two proteins. Figure 4 depicts the hydropathy plots of both MAL61 and SNF3 proteins. One clearly sees the twelve proposed transmembrane domains organized into two blocks of six each. The spatial distribution of these domains is so similar that the plots are nearly perfectly superimposable. Both proteins contain an hydrophilic N-terminal domain of similar size. While the MAL61 protein, like the SNF3 protein, contains an hydrophilic C-terminal domain, this domain is significantly smaller in the MAL61 protein.

Equivalent sequence and structural homology is seen to other sugar transport proteins from other species. Figure 3 aligns the amino acid sequence of the MAL61 protein with those of the SNF3 protein, the human HepG2 glucose transporter, the *Kluyveromyces lactis* lactose permease encoded by the *LAC12* gene, and the *Escherichia coli* xylose and arabinose transporters (Mueckler et al. 1985; Maiden et al. 1987; Celenza, Marshall-Carlson and Carlson 1988; Chang and Dickson 1988). Homologous sequences are seen in both hydrophobic membrane spanning domains as well as in hydrophilic regions. The proposed secondary structure of these sugar transporters is also remarkably similar and this is illustrated in Figure 3. Comparison of the MAL61 protein to the *E. coli* lactose permease and to the yeast plasma membrane ATPase (encoded by the *PMA1* gene) reveals little, if any, sequence homology even though all are proton transporters (Serrano 1977; Kaback 1983;

Figure 4. Hydrophobicity profile of the predicted MAL61 protein and SNF3 proteins.

The profiles were determined as described in Materials and Methods and in Figure 2.


MAL61
SNF3
Hydrophobicity
Residue number
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
100
200
300
400
500
600
700
800

Serrano, Kielland-Brandt and Fink 1986).

DISCUSSION

The results presented here offer additional strong evidence that the *MAL61* gene encodes the maltose permease. Transcription of the *MAL61* gene is maltose induced and glucose repressed (Needleman et al. 1984). Genetic evidence indicates that *MAL61* is required for maltose transport in MAL6 strains (Chang et al. 1989). Our results clearly demonstrate that the MAL61 protein is homologous both in sequence and in secondary structure to other sugar transporters, particularly the Saccharomyces high-affinity glucose transporter encoded by the *SNF3* gene. While the results presented here do not demonstrate that the MAL61 protein is a plasma membrane protein they support this conclusion. The primary sequence of the MAL61 protein exhibits twelve highly hydrophobic regions approximately 21-residues in length and such a structure is consistent with that of an integral membrane protein (Kyte and Doolittle 1982; Eisenberg 1984). In another study (to be reported elsewhere), analysis of a series of *MAL61-phoA* fusions selected in *E. coli* by transposition of a Tn5-derivative carrying a truncated copy of the *E. coli phoA* gene into a plasmid carrying the *MAL61* gene also supports the localization of the MAL61 protein to the plasma membrane (Manoil and Beckwith 1988; C. A. Michels and L. Seeccoomer, unpublished results). *E. coli* strains carrying these fusion plasmids express alkaline phosphatase activity in whole cells

and preliminary mapping localizes the fusion junction sites to the region of the second group of six transmembrane domains.

The homology demonstrated here among the Saccharomyces maltose permease, the Saccharomyces high-affinity glucose transporter, the human HepG2 glucose transporter, the *Kluyveromyces lactis* lactose permease and the *E. coli* xylose and arabinose transporters is remarkable. These proteins appear to be members of a family of related sugar transporters even though their mechanisms of transport differ. Some are active transporters utilizing proton symport and others function by facilitated diffusion. The Saccharomyces galactose transporter, which transports by facilitated diffusion, also is reported to be a member of this family of sugar transporters (Szkutnicka et al. 1989). An evolutionary relationship is strongly implied among all of these proteins however, homology resulting from a common ancestry is difficult to distinguish from convergent evolution. Sequence convergence could result from the fact that all are sugar transporters with similar functional constraints placed upon their structures.

Transmembrane domains 1, 2, 4, 5, 7, 8 and 11 of MAL61 protein contain several polar and negatively charged residues (serine, threonine, asparagine, glutamine, aspartate and glutamate). Particularly noteworthy is domain 1 which contains seven polar residues (4 threonine, 2 serine, 1 glutamine) and two charged residues (1 aspartate, 1 glutamate)

and has an average hydrophobicity value that just exceeds the 0.42 minimum proposed by Eisenberg (1984). Graphic analysis of the membrane-spanning domains indicates that the polar and charged residues contained in these domains would largely be localized to the same face of the proposed alpha-helical structure in the case of domains 2, 4, 5, 8 and 11. Mueckler et al. (1985) suggest that the hydroxyl and amide side chains in an amphipathic alpha-helix could line a transmembrane channel and function in the transport of the sugar. A similar structural organization may exist in the maltose permease. Additionally, since the transport of maltose in Saccharomyces is a proton symport system, it is possible that the charged residues located in the transmembrane domains of the MAL61 protein function in proton transport in a fashion similar to that seen in the lactose permease of *E. coli* (Herzlinger, Carrasco and Kaback 1985; Carrasco et al. 1986; Serrano 1977).

The addition of glucose to maltose-induced fermenting cultures not only leads to the cessation of synthesis of maltose permease but also leads to the loss of any existing maltose permease activity by an as yet undefined process referred to as glucose-induced inactivation (Gorts 1969; Busturia and Lagunas 1985). Glucose-induced inactivation also affects the activity of several other enzymes in Saccharomyces (reviewed in Holzer 1984; Jones 1984; Achstetter and Wolf 1985). These include enzymes of the gluconeogenic pathway (fructose-1,6-bisphosphatase, cytoplasmic malate dehydrogenase

and phosphoenolpyruvate carboxykinase), aminopeptidase I, uridine nucleosidase, the high-affinity glucose transporter (SNF3 protein) and galactose permease (Witt, Kronau and Holyer 1966; Ferguson, Boll and Holyer 1967; Gancedo 1971; Haarasilta and Oura 1975; Matern and Holzer 1977; Magni et al. 1977; Frey and Rohm 1979; Bisson and Fraenkel 1984; Ramos, Szkutnicka and Cirillo 1988). Maltose uptake is almost completely inactivated within 90 minutes following the addition of glucose to the culture medium (Gorts 1969; Busturia and Lagunas 1985). The most recent results indicate that this deadaptation inactivates both the high and low affinity uptake systems but earlier studies report that only the high affinity transport is affected. Glucose specifically initiates the inactivation since the transfer of a maltose fermenting culture to a non-inducing medium containing ethanol does not lead to the rapid loss of maltose transport activity. Recovery from glucose inhibition requires that the cells be returned to inducing medium and recovery does not occur if *de novo* protein synthesis is inhibited. These results imply that glucose-induced inactivation irreversibly destroys the maltose transport protein.

The mechanism of this irreversible inactivation is unknown. Studies on aminopeptidase I and the gluconeogenic enzymes fructose-1,6-bisphosphatase, cytoplasmic malate dehydrogenase and phosphoenolpyruvate carboxykinase from Saccharomyces indicate that the glucose-induced inactivation

of these enzyme activities is paralleled by a decrease in the amount of cross-reacting material suggesting that inactivation results from their selective proteolysis (Neeff et al. 1978; Frey and Rohm 1979; Funayama, Gancedo and Gancedo 1980; Muller, Muller and Holzer 1981; Tortora et al. 1981). While maltose permease is an integral membrane protein and these enzymes are cytosolic proteins, it is never-the-less tempting to propose that the irreversible inactivation of maltose permease is the result of proteolytic degradation. In a survey of several proteins from different eukaryotic organisms, Rechsteiner and coworkers have found a correlation between short half-life and the presence of sequences rich in proline, glutamate, serine and threonine (so-called PEST-regions) which they propose target proteins for proteolysis (Rogers, Well and Rechsteiner 1986; Rechsteiner 1987; Rechsteiner, Rogers and Rote 1987). A search of the MAL61 and SNF3 proteins reveals potential PEST sequences located in the N-terminal cytoplasmic regions. These are found at residues 49-78 of MAL61 protein (score of 0.64) and at residues 1-13 (score of 7.85) and 63-91 (score of 1.66) of SNF3 protein. Rogers, Well and Rechsteiner (1987) also suggest that a protein containing a region with a low positive PEST score could be subject to degradation only under certain physiological conditions, such as when that region were phosphorylated thereby increasing the negative charge of the region. In such cases, the degradation of a protein could be

regulated as is seen in glucose-induced inactivation of the gluconeogenic enzymes. It is worth noting that the N-terminal peptide of Saccharomyces fructose-1,6-bisphosphatase contains a PEST-region (score of 1.7). Whether or not PEST regions are relevant in the Saccharomyces system remains to be determined but the possibly that a common mechanism exists for the glucose-induced inactivation of all of these proteins even though they are located in different cellular compartments and lack any obvious sequence similarities is an interesting one to pursue.

CHAPTER 2

MAL61 of *Saccharomyces* encodes an integral membrane protein

ABSTRACT

Two *MAL61-lacZ* in-frame gene fusions were constructed and their expression in different compartments of yeast cells was studied. A functional *MAL61-lacZ* gene product containing codons 1-586 of *MAL61* is found predominantly in the membrane fraction of the cell. The control fusion product containing codons 1-30 of *MAL61* cofractionates with the soluble cytoplasmic fraction. Our results demonstrate that *MAL61* encodes an integral membrane protein.

INTRODUCTION

Computer analysis of the sequence of *MAL61* indicated the predicted MAL61 protein is a highly hydrophobic protein and is strongly homologous to known sugar transporters (Cheng and Michels 1989). Like the other members of human glucose transporter superfamily (HGT superfamily), the MAL61 protein contains 12 potential transmembrane domains organized into two blocks of 6 domains each separated by a hydrophilic central region. The highly hydrophilic amino terminals (about 100 amino acid residues), carboxyl terminus (about 67 amino acid residues) and central domain (about 71 amino acid residues) are predicted to lie on the cytoplasmic face of the membrane based on the topological results for human glucose transporter GLUT1 (Cheng and Michels 1989; Davies et al. 1987).

To confirm that *MAL61* encodes a membrane protein, we have constructed two gene fusions between *MAL61* and the *E. coli lacZ* gene encoding ß-galactosidase. ß-galactosidase is a soluble protein but can be targeted to the membrane if it is fused to a membrane protein (Hagen et al. 1986). It has been used as a reporter to demonstrate the membrane location of various proteins such as *Saccharomyces* glucose transporter SNF3 and *E. coli* maltose transporter malF (Celenza, Marshall-Carlson and Carlson 1988; Silhavy et al. 1976). The analysis of *MAL61-lacZ* fusion products in this report shows that *MAL61* encodes an integral membrane protein.

MATERIALS AND METHODS

Construction of *MAL61-lacZ* fusions. A *MAL61-lacZ* fusion plasmid pMAL61$_{(586)}$-lacZ was constructed by inserting a *Bam*HI-*Pst*I DNA fragment containing the entire 5'-noncoding upstream sequence and codons 1-586 of *MAL61* into vector YEp366. The plasmid pMAL61$_{(30)}$-lacZ (referred to as pIGR61,62 in Levine, Tanouye and Michels, submitted) contains the entire 5'-noncoding upstream sequence and codons 1-30 of *MAL61* in vector YEp356R. Plasmids YEp366 and YEp356R are from a series of yeast shuttle vectors with multiple cloning sites suitable for construction of *lacZ* fusions (Myers et al. 1986). Both are yeast/*E. coli* shuttle plasmids containing the 2 micron circle replication origin and are therefore present in 20-50 copies per cell. The *LEU2* gene of YEp366 and the *URA3* gene of YEp356R are used for selection in yeast.

Yeast transformation. pMAL61$_{(586)}$-lacZ and pMAL61$_{(30)}$-lacZ were transformed or cotransformed into a maltose-fermenting *Saccharomyces* strain 600-1B carrying the *MAL1* locus (Charron, Dubin and Michels 1986). Transformation was done using the lithium chloride method (Ito et al. 1983). Transformants were isolated as leucine and/or uracil prototrophs on SD medium (yeast nitrogen base w/o amino acids plus 2% (wt/vol) dextrose and appropriate amino acids; Difco Laboratories).

Protein fractionation and Western Blot Analysis. Transformed yeast strains 600-1B[pMAL61$_{(586)}$-lacZ], 600-

1B[pMAL61$_{(30)}$-lacZ] and 600-1B[pMAL61$_{(586)}$-lacZ, pMAL61$_{(30)}$-lacZ] were grown in leucine or uracil selective SD media with 2% (wt/vol) maltose as carbon source. Mid-log phase cultures containing 4 x 10^{10} cells from each maltose-induced strain were harvested, washed and resuspended into 1 ml of solution (pH7.5) containing 10mM Tris/HCL, 1mM EDTA, 2mM phenylmethyl-sulfonyl fluoride (PMSF). The crude yeast proteins were extracted using the method described in Ausubel et al (1989). Yeast cells were disrupted by vortexing with glass beads for 15x10 seconds on the ice. The extract supernatants were collected using trimmed pipettes and then centrifuged at 8000rpm for 10 minutes at 4^0C. A 0.3 ml aliquot of the supernatant, which represents the protein extract from the soluble cytoplasmic fraction, was removed from each strain sample and saved at -70^0C for up to one week. The remaining supernatant was further centrifuged at 14,000rpm for 30 minutes at 4^0C. The supernatants were then discarded and the cell pellets were dissolved in 0.3 ml of 1% deoxycholate, 10mM Tris/HCL, 2mM PMSF (pH7.5) and incubated on ice for one hour. Every 15 minutes, the samples were vortexed thoroughly for 20 seconds. Extracts were then centrifuged at 14,000rpm for 30 minutes at 4^0C. The supernatant, which represent the membrane protein fraction, was removed and saved at -70^0C for up to one week. The protein concentration in both soluble cytoplasmic fraction and membrane fraction was determined using a BCA protein assay kit from PIERCE. Samples containing equal

amounts of protein were loaded into a 6% SDS-polyacrylamide gel and Western Blot analysis was carried out using a standard method (Ausubel et al. 1989) with anti-ß-galactosidase as the primary antibody. A horseradish peroxidase conjugation detection system ECL (Amersham) was used to detect the fusion proteins.

RESULTS AND DISCUSSION

The fusion product of pMAL61$_{(586)}$-lacZ is functional and equivalent to the MAL61 protein. It restores the maltose fermenting activity of a mutant strain 100-1A, which carries a deletion/disruption of *MAL11*, a GENE 1 homologue of *MAL61* (Charron and Michels 1988). Transforming strain 100-1A with either pMAL61$_{(30)}$-lacZ or either vector lacking the *MAL61* insert does not restore the maltose fermenting activity (data not shown). In plasmids pMAL61$_{(586)}$-lacZ and pMAL61$_{(30)}$-lacZ, *MAL61* was fused in frame at codons 586 and 30, respectively, to codon 8 of the *E. coli* ß-galactosidase gene *lacZ*. Because both fusion plasmids contain the full 874 basepairs of *MAL61* upstream sequence, they express a maltose-regulated ß-galactosidase activity (data not shown).

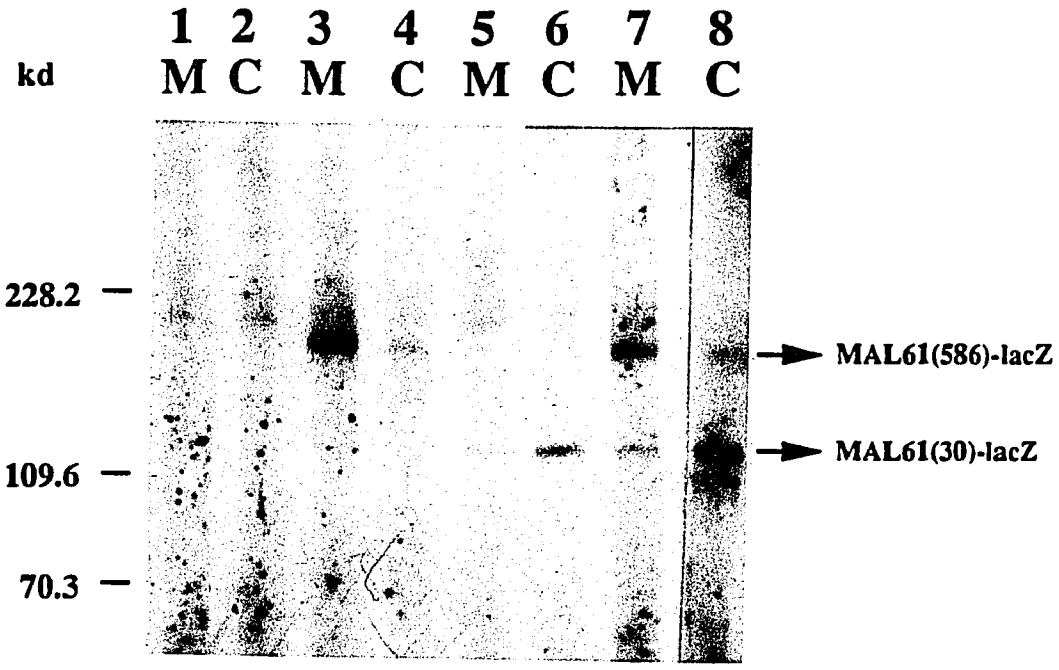
The amino acid residue position 586 is located downstream from the 12 potential transmembrane domains (Cheng and Michels 1989). If MAL61 is an integral membrane protein as predicted from computer analysis, one would expect to detect the majority of the *MAL61*$_{(586)}$-*lacZ* fusion product in the protein extracted from the membrane fraction. In contrast, the product of the *MAL61*$_{(30)}$-*lacZ* fusion would be expected to be present mostly in the soluble cytoplasmic fraction, since the amino acid residue position 30 is in the predicted hydrophilic N-terminus of MAL61 and N-terminal to the 12 potential transmembrane domains.

The subcellular location of the *MAL61-lacZ* fusion products was determined by Western Blot analysis (Figure 1). In the pMAL61$_{(586)}$-lacZ transformed strain, a 180 kd protein, which is at predicted size of the fusion product, is present predominantly in the membrane fraction and is almost absent in the soluble cytoplasmic fraction. In the pMAL61$_{(30)}$-lacZ transformed strain, the predicted fusion product with a molecular weight of about 115 kd is expressed predominantly in the soluble cytoplasmic fraction as expected. Most significantly, in a simultaneously expressed, cotransformed strain, the *MAL61*$_{(586)}$*-lacZ* gene product is found predominantly in the membrane fraction while the majority of the *MAL61*$_{(30)}$*-lacZ* gene product is in soluble cytoplasmic fraction of the cells. Transforming the same strain with the YEp vector alone does not give rise to any detectable ß-galactosidase protein.

Our results show that ß-galactosidase is converted to a membrane-bound state by a gene fusion containing the twelve potential transmembrane domains of the MAL61 protein, while a fusion product containing a short N-terminal hydrophilic portion of MAL61 remains in the cytoplasm. These results fully demonstrate that *MAL61* encodes an integral membrane protein and provide supporting evidence for the proposed topological model of the predicted MAL61 protein.

Figure 1. Western analysis of *MAL61-lacZ* fusion products.

Transformed yeast strain 600-1B[YEp366] (lanes 1 and 2), 600-1B[pMAL61$_{(586)}$-lacZ] (lanes 3 and 4), 600-1B[pMAL61$_{(30)}$-lacZ] (lanes 5 and 6) and 600-1B[pMAL61$_{(586)}$-lacZ, pMAL61$_{(30)}$-lacZ] (lanes 7 and 8) were grown in maltose-containing media to mid-log phase. Equal amount of proteins from the membrane fraction (M) and the soluble cytoplasmic fraction (C) from the same culture were loaded in each lane. The size of the fusion proteins was estimated by comparison to standard protein size markers whose position in the gel is indicated.


1 2 3 4 5 6 7 8
kd
M C M C M C M C
228.2
109.6
70.3
MAL61(586)-lacZ
MAL61(30)-lacZ

CHAPTER 3

MAL11 and *MAL61* encode the inducible high-affinity maltose transporter of *Saccharomyces cerevisiae*

ABSTRACT

We have investigated the transport of maltose in a genetically defined maltose fermenting strain of *Saccharomyces cerevisiae* carrying the *MAL1* locus. Two kinetically different systems were identified: a high-affinity transporter with a Km of 4 mM and a low-affinity transporter with a Km of 70-80 mM. The high-affinity maltose transporter is maltose inducible and is encoded by the *MAL11* (and/or *MAL61*) gene of the *MAL1* (and/or *MAL6*) locus. The low-affinity maltose transporter is expressed constitutively and is not related to *MAL11* and/or *MAL61*. Both maltose transporters seem to be subject to the glucose-induced inactivation.

INTRODUCTION

In *Saccharomyces* strains, the *MAL11* and *MAL61* genes, located at the *MAL1* and *MAL6* loci, respectively, are reported to encode maltose permease (Charron, Dubin and Michels 1986; Needleman et al. 1984). Genetic complementation studies, restriction endonuclease mapping, and Southern and Northern hybridization analysis have demonstrated that *MAL11* and *MAL61* are sequence and functional homologues.

Genetic analysis of mutations in *MAL61* strongly suggests that *MAL61* encodes maltose permease (Chang et al. 1989). This suggestion is supported by sequence analysis of the *MAL61* gene (Cheng and Michels 1989). Significant sequence and structural homology is seen between the MAL61 protein and the *Saccharomyces* high-affinity glucose transporter encoded by the *SNF3* gene, the *Saccharomyces* galactose permease encoded by the *GAL2* gene, the *Kluyveromyces lactis* lactose permease encoded by the *LAC12* gene, the human glucose transporter *GLUT1* and the *Escherichia coli* xylose and arabinose transporters encoded by the *XylE* and *AraE* genes. Hydropathy analysis suggests that the secondary structure of all of these sugar transporters consists of two blocks of six transmembrane domains separated by a relatively long intracellular region. It seems that all of them are members of a family of sugar transporters and are related either functionally and/or evolutionarily.

We have further elucidated the nature of the MAL11 and

MAL61 protein biochemically. Here we describe a kinetic study of maltose uptake in a genetically well characterized strain carrying a single copy of either the *MAL11* or *MAL61* gene. Our results clearly demonstrate that *MAL11* and *MAL61* encode the maltose inducible, high-affinity maltose transporter. A second maltose permease is present in this strain, a constitutive, low-affinity transporter. This low-affinity transporter is not encoded by *MAL11* or *MAL61*. Both forms of the maltose permease are sensitive to glucose-induced inactivation.

MATERIALS AND METHODS

Yeast strains and growth conditions. Strains 600-1B (*MATa MAL1 SUC1 ura3-52 leu2-3,112*) and 100-1A (*MATa SUC1 mal11Δ::URA3 leu2-3,112*) have been described previously (Charron, Dubin and Michels 1986; Charron and Michels 1988). All strains were grown on YP medium (1% yeast extract, 2% peptone; Difco Laboratories) supplemented with the appropriate sugar as indicated.

Construction of plasmid pMAL61 and yeast transformation. The *Hind*III-*Bgl*II fragment containing the entire *MAL61* gene and its upstream region was isolated from plasmid pY6 and cloned into the CEN plasmid pRS315 which carries the *LEU2* gene as its yeast selective marker (Needleman et al. 1984; Sikorski and Hieter 1989). Transformation of strain 100-1A was done using the lithium chloride method (Ito et al. 1983). Transformants were isolated as leucine prototrophs on SD (yeast nitrogen base w/o amino acids plus 2% dextrose and appropriate amino acids; Difco Laboratories) medium and maltose fermenting transformants were selected. Maltose fermentation was determined by the production of acid and gas 1 to 3 days after inoculation in 5 ml of YEP plus 2% (wt/vol) maltose medium in Durham tubes. Plasmid stability in this transformed strain, 100-1A[pMAL61], during its growth on the YP medium supplemented with 2% (wt/vol) maltose was tested by calculating the rate of loss of the LEU+ phenotype.

Approximately 60% of the cells retained the plasmid at the time of harvesting for the transport assay. Loss of the plasmid *LEU2* gene correlated with the loss of the ability to ferment.

Measurement of maltose transport by the uptake of [U-^{14}C] maltose. The method used to measure the uptake of radiolabelled maltose was as described in Kruckeberg and Bisson (Kruckeberg and Bisson 1990) with slight modifications according to Serrano (Serrano 1977). Yeast strains were grown on a rotary shaker at 250 rpm at 30^{0}C. The mid to late log phase cells (O.D.$_{600}$ = 1 to 1.2) were harvested, rapidly filtered, washed with and resuspended in 0.1 M tartaric acid/Tris pH 4.2 buffer to a final concentration of 90-110 mg wet weight per ml. Cells and [U-^{14}C] maltose were incubated at 25^{0}C for 10 seconds. Incorporation was stopped by the addition of 10 ml ice-cold water. Cells were immediately filtered, washed and counted in a Beckman liquid scintillation counter. Under the experimental conditions used here, uptake appears to be linear for at least 15 seconds (data not shown). We determined the initial velocity of maltose uptake over a range of substrate concentrations from 0.2 mM to 200 mM and plotted the data as velocity (V) vs. velocity/substrate concentrations (V/S) according to the Eadie-Hofstee transformation (Eadie 1952; Hofstee 1952).

[U-^{14}C] maltose was purchased from Amersham U.S.A. The specific activity of the labeled [U-^{14}C] maltose is 540

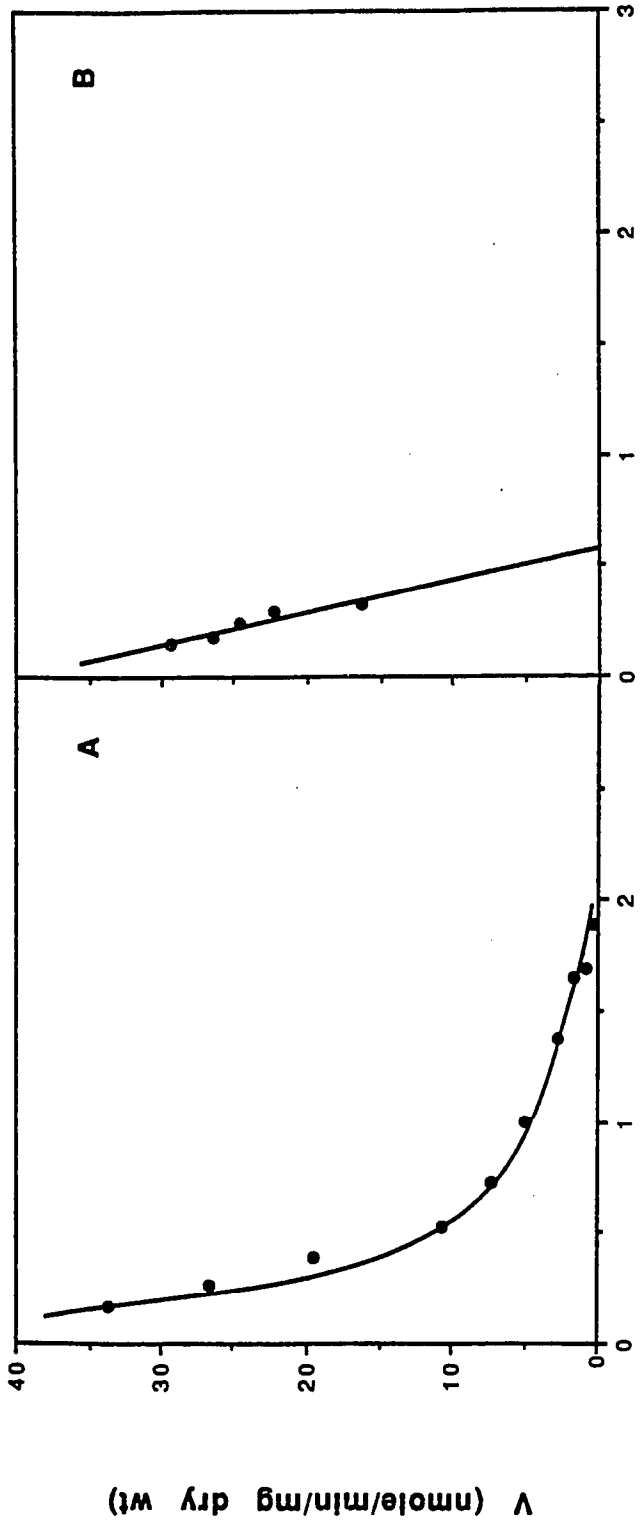
mci/mmol. Maltose was from Sigma Chemical Co. U.S.A.

RESULTS

Identification of two forms of the maltose uptake system in a genetically well characterized strain. The kinetics of maltose uptake were studied in a maltose fermenting *Saccharomyces cerevisiae* strain 600-1B. This strain contains only the *MAL1* locus and no others, and thus contains a single copy of the *MAL11* gene and no other *MAL11*-gene homologues (Charron, Dubin and Michels 1986). Figure 1A shows the Eadie-Hofstee analysis of maltose uptake by strain 600-1B grown under induced growth conditions (in the presence of 2% maltose). Uptake is clearly biphasic implying the presence of at least two forms of maltose transporter in this strain. One is a high-affinity transporter with a Km value of 4 mM, and the other one is a low-affinity transporter with a Km value of 70-80 mM. Following growth of strain 600-1B under non-inducing growth conditions (in the absence of maltose but with 2% galactose as the sugar source), the high-affinity transport activity decreases to an undetectable level and the low-affinity transport activity remains unaffected (Figure 1B), indicating that the high-affinity transporter is maltose inducible, but that the low-affinity transporter is constitutively expressed. The high specific activity of radiolabelled maltose needed to detect this low-affinity system tends to give high background levels and variable results. Never-the-less, this apparent low-affinity transport

Figure 1. Eadie-Hofstee plot of maltose uptake in the *MAL1* strain 600-1B.

Strain 600-1B was grown in (A) YP medium supplemented with 2% (wt/vol) maltose, and (B) YP medium supplemented with 2% (wt/vol) galactose.


B
A
0
1
2
3
0
10
20
30
40
V (nmole/min/mg dry wt)
V/S (nmole/min/mg dry wt)/(mM)

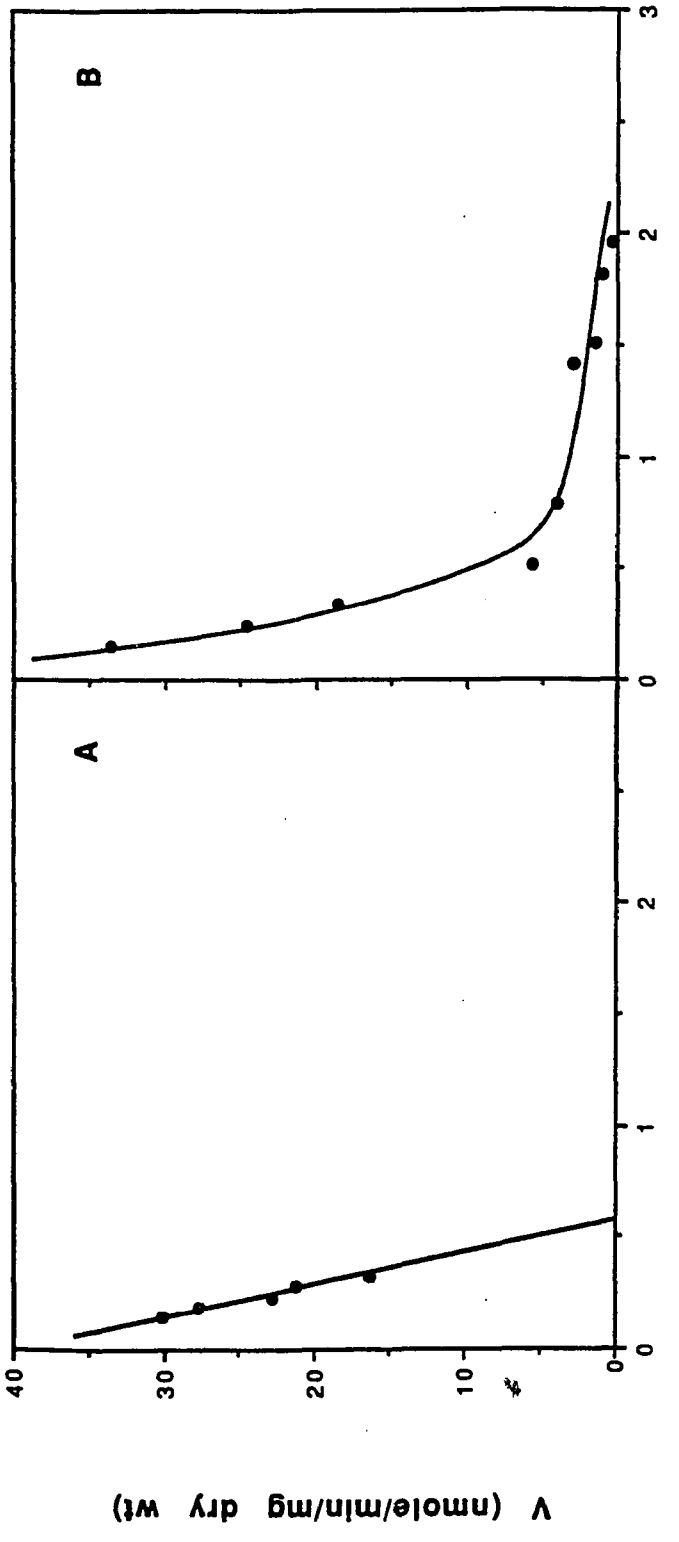
activity is reproducibly present.

The high-affinity maltose transporter is encoded by the *MAL11* and/or its sequence and functional homolog *MAL61*. Strain 100-1A carries a deletion/disruption of the *MAL11* gene at the *MAL1* locus. It does not grow on 2% (wt/vol) maltose YP medium and grows poorly on 10% (wt/vol) maltose YP medium. Eadie-Hofstee analysis of strain 100-1A grown on YP medium supplemented with both 2% maltose and 2% galactose (inducing conditions) is shown in Figure 2A. The high-affinity transport activity is totally absent, but the low-affinity transport activity remains and appears to be unaffected. This clearly demonstrates that the high-affinity transporter alone is encoded by the *MAL11* gene.

Strain 100-1A was transformed with a CEN plasmid carrying the *MAL61* gene, plasmid pMAL61. Both the ability to grow normally on 2% (wt/vol) maltose YP medium and the ability to ferment maltose are restored in this transformant, 100-1A[pMAL61]. Transforming strain 100-1A with the vector plasmid alone does not restore these activities. Figure 2B shows an Eadie-Hofstee analysis of strain 100-1A[pMAL61] grown on YP medium supplemented with 2% maltose. The high-affinity transport activity is fully restored. When 100-1A[pMAL61] cells are grown on YP medium supplemented with both 2% maltose and 2% galactose, the same biphasic Eadie-Hofstee pattern is obtained (data not shown). These results clearly demonstrate that the *MAL11* and/or *MAL61* genes encode the high-affinity

Figure 2. Eadie-Hofstee plot of maltose uptake in the *MAL11* deletion/disruption strain 100-1A and 100-1A transformed with the plasmid borne *MAL61* gene.

(A) Strain 100-1A grown on YP medium supplemented with 2% (wt/vol) galactose plus 2% (wt/vol) maltose. (B) Strain 100-1A[pMAL61] grown on YP medium supplemented with 2% (wt/vol) maltose.


B
A
V/S (nmole/min/mg dry wt)/(mM)
V (nmole/min/mg dry wt)
0
1
2
3
10
20
30
40

maltose transporter.

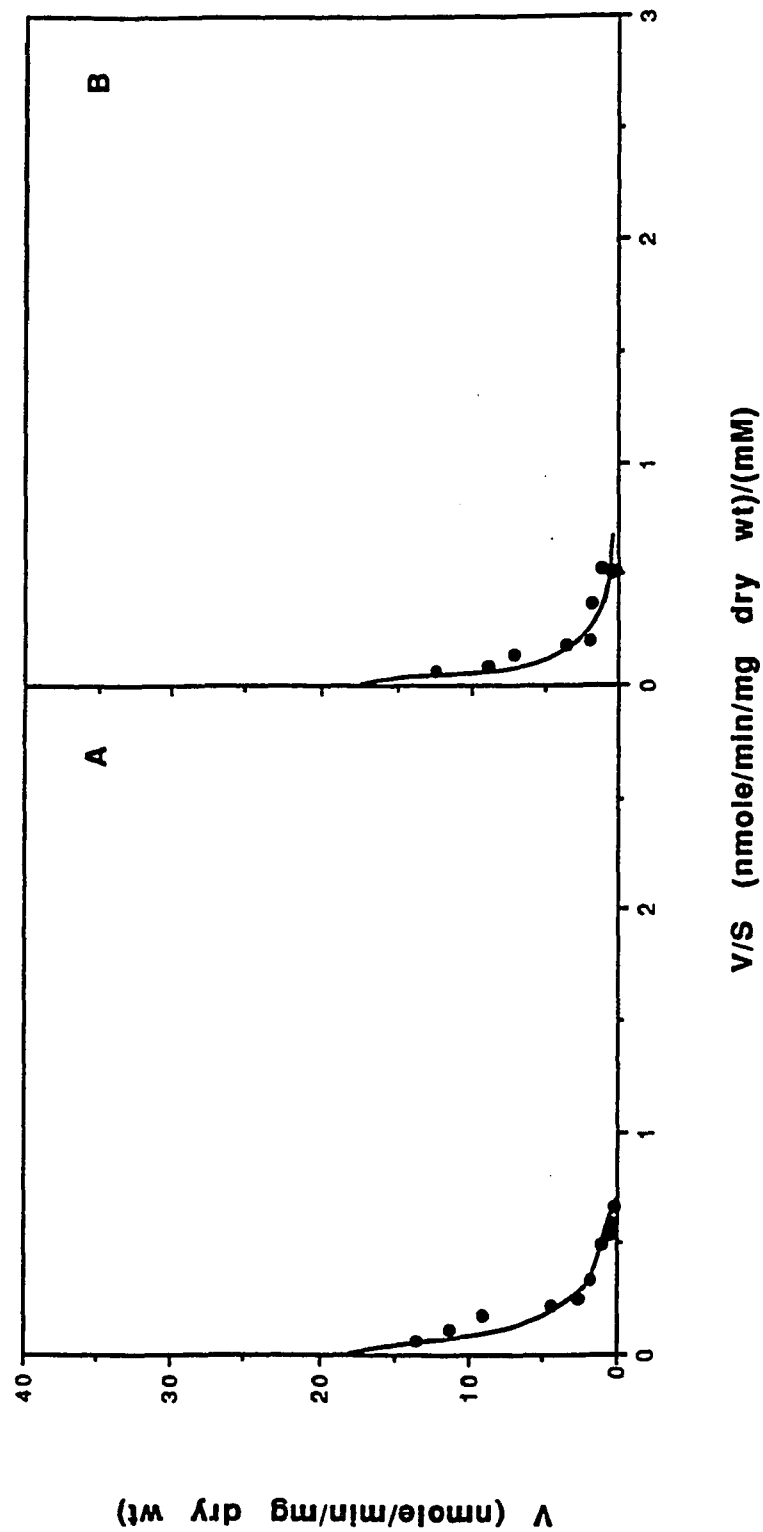
Northern analysis of strains of the *MAL1* and *MAL6 mal1*^{0} genotypes detects two transcripts with homology to *MAL61*-derived probes: a 2.0 kb maltose inducible transcript and a 2.4 kb constitutively expressed transcript (Charron, Dubin and Michels 1986; Needleman et al. 1984). Based on the finding that neither transcript is detectable in strains carrying deletion / disruptions in *MAL11* or *MAL61* and *mal11*, it was concluded that both the inducible and the constitutive transcripts are the product of the *MAL11* and *MAL61* genes (R. A. Dubin, Ph.D. thesis, Queens College and the Graduate School of the City University of New York, New York, 1987). These results, along with those reported here, clearly indicate that the high-affinity maltose transporter is the product of the 2.0 kb inducible transcript alone since, under uninduced condition while the 2.4 kb transcript is still present, no high-affinity transport activity is detected. The constitutive 2.4 kb transcript also does not appear to encode the constitutive, low-affinity transporter because the low-affinity transport activity is present in the *MAL11* deletion/disruption strain while the transcript is undetectable (Charron, Dubin and Michels 1986).

Both the high- and low-affinity maltose transporters are sensitive to glucose-induced inactivation. Strain 600-1B was grown to mid to late log phase in 2% maltose YP medium. The cells were harvested by filtration and transferred to fresh YP

media containing 2% glucose or 2% glucose plus 2% maltose. After 90 minutes the cells were harvested and kinetic studies of maltose uptake were performed. The Eadie-Hofstee analysis results are shown in Figure 3. Both the high- and low-affinity maltose transport activity are found to be subject to glucose-induced inactivation (Figure 3A). Ninety minutes after the addition of glucose, an approximately 30% decrease in the low-affinity and 60-70% decrease in the high-affinity maltose transport activity is observed. The glucose-induced inactivation of maltose transport occurs even in the presence of maltose (Figure 3B).

Figure 3. Pattern of maltose uptake in the *MAL1* strain 600-1B following glucose-induced inactivation.

Strain 600-1B was grown to mid-late log phase on YP medium supplemented with 2% (wt/vol) maltose, harvested by filtration, and resuspended at time zero in (A) YP medium supplemented with 2% (wt/vol) glucose or (B) YP medium supplemented with 2% (wt/vol) maltose plus 2% (wt/vol) glucose. The cells were harvested after 90 minutes at which time an Eadie-Hofstee analysis of maltose transport was carried out.


A
B
0
10
20
30
40
0
1
2
3
V (nmole/min/mg dry wt)
V/S (nmole/min/mg dry wt)/(mM)

DISCUSSION

Early biochemical studies in *Saccharomyces cerevisiae* indicate that the specific transport of maltose is the first, and also rate limiting step in maltose fermentation (De la Fuente and Sols 1962; Harris and Thompson 1961). A protein component, referred to as the maltose permease, is observed to be involved in maltose uptake and accumulation (Harris and Thompson 1961; Robertson and Halvorson 1957). This maltose transport process is specifically inducible by maltose and not by other sugars and the uptake of maltose requires an energy supply (De Kroon and Koningsberger 1970; Harris and Thompson 1961; Serrano 1977). Serrano (1977) reported that maltose transport is not dependent upon intracellular ATP levels and therefore is not associated with phosphorylation. It is coupled to the electrochemical gradient of protons across the plasma membrane and one proton is cotransported with every maltose molecule. In summary, the maltose transport system is an inducible active proton symport system.

Gorts' studies in baker's yeast strains revealed only one maltose transporter with a Km of 4 mM (Gorts 1969). Later studies on genetically undefined strains of *Saccharomyces cerevisiae* indicate the apparent presence of at least two forms of the maltose transporter: a low-affinity transporter with a Km of 70 mM, and a high-affinity transporter with a Km of 4 mM (Busturia and Lagunas 1985). Given the polygenic

nature of the *MAL* loci, we wished to explore the possibility that these two kinetically distinct transport activities could be the products of different *MAL* loci. We report here a kinetic study of maltose uptake in a genetically well characterized *Saccharomyces* strain carrying only a single copy of the *MAL11* gene located at the *MAL1* locus. Even in this strain containing only a single *MAL* locus, our results also reveal at least two forms of the maltose transporter: a constitutively expressed low-affinity transporter with a Km of 70-80 mM, and a maltose inducible high-affinity transporter with a Km of 4 mM. Since deletion / disruption of the *MAL11* gene causes the complete abolishment of high-affinity transport activity, and transforming the *MAL11* deletion / disruption strain with a cloned copy of the *MAL61* gene fully restores the high-affinity transport activity, we conclude that the inducible high-affinity maltose transporter is encoded by the *MAL11* and *MAL61* genes. All five *MAL* loci in *Saccharomyces cerevisiae* exhibit extensive sequence and functional homology (Barnett 1976; Charron, Dubin and Michels 1986; Charron and Michels 1987; Charron et al. 1989; Federoff et al. 1982; Needleman and Michels 1983). Thus we suggest that GENE1 of each *MAL* locus encodes the high-affinity maltose transporter.

The inducible, high-affinity maltose transporter encoded by *MAL11* and *MAL61* is most likely also the active, maltose/proton symporter described by Serrano (1977). The

analysis done by Serrano (1977) was carried out on cells grown under maltose-induced conditions and transport was assayed using low sugar concentrations. Based on the results presented here, almost all of the transport measured by Serrano (1977) must have occurred by means of the high-affinity transporter described here. Consistent with this, we find that at pH 7.0 the maltose transport activity of induced cells is dramatically decreased (data not shown).

We have not investigated the mechanism of transport of the low-affinity transport system nor have we defined the gene(s) encoding this system. In the absence of such studies, any conclusions regarding the low-affinity transport system must be considered tentative. Never-the-less, it is possible that this low-affinity maltose transport is mediated by a common α-glucoside transport system which is less specific for maltose and capable of transporting several different α-glucosides such as melezitose, α-methy-glucoside and turanose in addition to maltose (De la Fuente and Sols 1962; Okada and Halvorson 1964b; Perkins and Needleman 1988). Only identification of the gene(s) encoding the low-affinity transporter can resolve these issues.

In *Saccharomyces*, multiple sugar transport systems with high- and low- affinities have been reported for glucose (Bisson and Fraenkel 1983), fructose (Bisson and Fraenkel 1983), galactose (Ramos, Szkutnicka and Cirillo 1989) and α-methy-glucoside (Okada and Halvorson 1964a), etc. In the

glucose transport systems, the high-affinity glucose transporter seems to be kinase dependent and encoded by the *SNF3* gene (Bisson and Fraenkel 1983; Bisson et al. 1987). Low-affinity uptake appears to be constitutive and to occur by facilitated diffusion (Cirillo 1962; van Steveninck 1969). The exact relationship between the high- and the low-affinity glucose transporters remains unclear. A reciprocal regulatory relationship between these two processes has been observed suggesting that the high- and the low-affinity systems might share a common component (Ramos, Szkutnicka and Cirillo 1988). Recent results also suggest the existence of several high-affinity transporters encoded by different genes (Kruckeberg and Bisson 1990).

There are three forms of galactose transporters in *Saccharomyces*. In non-induced cells, galactose is transported by a constitutive, low-affinity facilitated diffusion process (Cirillo 1968). Growth on galactose induces two additional transporters: a galactokinase-independent, low-affinity transporter with a significantly different Km value from the constitutive low-affinity one, and a galactokinase-dependent high-affinity transporter (Ramos, Szkutnicka and Cirillo 1989). Both galactose-induced transporters are dependent on the presence of the *GAL2* gene, suggesting that the product of the *GAL2* gene could be a common carrier in either of two conformational states, low- or high-affinity, which is dependent upon the absence or presence of a functional

galactokinase, respectively (Ramos, Szkutnicka and Cirillo 1989). While our results clearly show that the *MAL11* and *MAL61* genes encode the high-affinity maltose transporter and that the low-affinity maltose transporter appears not to require the *MAL11* and/or *MAL61* genes, we can not rule out the possibility that there is a maltose induced, low-affinity transporter with a Km which is not distinguishable from the Km of the constitutive low-affinity transporter identified here. If such a low-affinity, maltose inducible transporter were present at low concentration, the loss and recovery of its activity in the *MAL11* deletion / disruption strain 100-1A and the *MAL61* transformed strain 100-1A[pMAL61], respectively, would not be recognized.

Our results (Figure 3) and earlier work (Busturia and Lagunas 1985; Gorts 1969) reveal that both the high- and low-affinity maltose transport systems appear to be sensitive to glucose-induced inactivation. The addition of glucose to maltose-induced fermenting cultures leads to the rapid loss (less than 90 minutes) of about 70% of the high-affinity maltose transport activity and about 30% of the low-affinity maltose transport activity. Work is in progress in our laboratory to fully characterize this inactivation process in genetically defined strains.

CHAPTER 4

A demonstration of glucose-induced inactivation of the *MAL61*-encoded maltose transporter of *Saccharomyces*

ABSTRACT

Glucose-induced inactivation of maltose transport was studied in detail in a genetically well-defined *Saccharomyces* strain. We demonstrated that the *MAL61*-encoded maltose transporter is subject to glucose-induced inactivation and this inactivation process is distinguishable from glucose repression. MAL61 is a functional stable protein under non-inducing carbon source glycerol/lactic acid and galactose but is specifically targeted for inactivation by glucose. Inhibition of protein synthesis by cycloheximide also leads to the inactivation of the maltose transport system. The possible mechanisms resulting in these inactivation processes are discussed.

INTRODUCTION

Glucose-induced inactivation has been reported as a selective post-translational control mechanism affecting maltose transport in genetically undefined *Saccharomyces* strains (Busturia and Lagunas 1985; Gorts 1969; van Rijn and van Wijk 1972). Upon addition of glucose to a maltose-induced fermenting baker's yeast culture, a rapid, time-dependent loss (half-life about 45 minutes) of maltose uptake activity was detected. Studies on membrane protein turnover in growing yeast cells indicated that over a period of three hours, the membrane protein population stays stable (Losson, Jund and Lacroute 1977). It seems that the rapid loss of maltose transport activity in response to glucose can not simply be explained by growth dilution of the pre-existing protein after cessation of its synthesis. Since maltase activity remained rather constant during incubation of maltose-grown cells in a nutrient medium with glucose, glucose-induced inactivation appears to specifically affect maltose permease (Gorts 1969). The presence of maltose in the nutrient medium with glucose did not protect the maltose uptake system against inactivation and incubation with ethanol did not inactivate the uptake system, indicating glucose is a specific factor leading to the inactivation of maltose transport system (Busturia and Lagunas 1985; Gorts 1969).

The studies on glucose-induced inactivation of the

maltose transport system by both Gorts' and Lagunas' groups were carried out in genetically undefined *Saccharomyces* strains. It is likely that these strains carried multiple copies of the *MAL* loci and thus several species of maltose permease might be expressed (Charron et al. 1989; F. E. Cotty, Ph.D. thesis, Queens College and the Graduate School of the City University of New York, New York, 1991). Additionally, since there are seven other genes (designated *DSF*), unlinked to known *MAL* loci, reported to be implicated in maltose fermentation in *Saccharomyces*, and four of the *dsf* mutants exhibited reduced maltose uptake (Zimmermann, Kahn and Eaton 1973), maltose transport or its control may be quite complex. In an obscure genetic background, it would be difficult to further analyze the molecular mechanism of glucose-induced inactivation of maltose transport.

When Gorts proposed the phenomena of glucose-induced inactivation of maltose transport system in 1969, he was using an indirect assay method measuring CO_2 evolution to detect maltose transport activity (Gorts 1969). With much more accurate kinetic methods to directly measure maltose transport activity, Busturia and Lagunas (1985) reported the same phenomena. A time period of over two to six hours was used to follow glucose inactivation in the work of Busturia and Lagunas (1985). This raises the problem of distinguishing between two interacting phenomenon: glucose-induced inactivation and glucose repression. Glucose repression would

result in a loss of maltose transport since growth would dilute the pre-existing maltose transport proteins over such a long monitoring time period.

With the availability of genetically well-defined *Saccharomyces* strains and a newly-established biochemical technique which allows the accurate measuring of maltose transport activity, we have carried out a kinetic study in genetically defined strains and revealed two forms of maltose transport system, the low-affinity maltose transporter and the *MAL*-encoded high-affinity maltose transporter (Cheng and Michels 1991). The addition of glucose to maltose-induced fermenting cultures leads to a loss (less than 90 minutes) of about 70% of the high-affinity maltose transport activity and about 30% of the low-affinity maltose transport activity, suggesting that glucose-induced inactivation preferentially affects the *MAL*-encoded high-affinity maltose transporter (Cheng and Michels 1991).

A time course functional analysis is designed in this report in order to distinguish glucose repression and glucose-induced inactivation as two separate mechanisms affecting maltose transport. By comparing the loss of maltose transport and maltase activity to the rate of growth dilution under different growth conditions in genetically well-defined *Saccharomyces* strains, our results fully demonstrated that the *MAL61*-encoded maltose transporter is subject to glucose-induced inactivation.

MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast *Saccharomyces* strains 332-5A (*MATa MAL64 MAL63 MAL62 MAL61 MAL12 ura3-52 leu2-3,112 trp1 his*) and R10 (*MATa MAL64-R10 MAL63 MAL62 MAL61 MAL12 ura3-52 leu2-3,112 trp1 his*) have been described previously (Dubin et al. 1986). All yeast cells were grown on YP medium (1% yeast extract, 2% peptone; Difco Laboratories) supplemented with an appropriate sugar or other carbon source as indicated.

Maltose transport assays. Mid-log phase yeast cells with an $O.D._{600}$ between 0.6 to 0.8 were harvested, washed and used for assaying maltose uptake. We used a previously described method (Cheng and Michels 1991; Kruckeberg and Bisson 1990; Serrano 1977) to measure the incorporation of U-^{14}C maltose by cell suspensions (90-110 mg wet weight/ml) in 0.1 M tartaric acid/Tris pH 4.2 buffer at 28^{0}C with a incubation time of 10 seconds. The final radiolabelled maltose substrate concentration is 4 mM. All transport activities were an average of two to six independent determinations.

[U-^{14}C] maltose was purchased from Amersham U.S.A. The grade I maltose from Sigma, which contains less than 0.5% of glucose, was used in all growth media as well as substrate solutions.

Measurement of maltase activity. Maltase activity was measured as the rate of release of *p*-nitrophenol from *p*-

nitrophenol-α-D-glupyranoside (PNPG) as described previously (Dubin et al. 1985). All maltase levels are an average of three replicate assays carried on the yeast extracts from the same cell cultures that were used for the maltose transport assays.

Time course studies on comparing the kinetics between the loss of maltose transport activity, maltase activity and the rate of growth dilution. Strain 332-5A was grown in YP + 2% (wt/vol) maltose medium and strain R10 was grown in YP + 3% (wt/vol) glycerol and 2% (wt/vol) lactic acid medium to mid-log phase. Yeast cells were then subjected to further growth for another one and half hours under a specified growth condition, as indicated. Two samples of equal aliquots of cells were removed at time zero, immediately before the exposure to the new condition, and at 0.5 hour, 1 hour and 1.5 hours after growth in the new condition. One cell sample was immediately used to assay for maltose transport activity and the other was saved at -70^0C to be used to measure maltase activity within 24 hours. By standardizing the maltose transport activity and maltase activity at time zero as 100%, the relative maltose transport activity and maltase activity at different time intervals was determined and the data were plotted as activity (%) verses time. The cell density ($O.D._{600}$) at intervals of time x (x=0, 0.5, 1.0, 1.5 hours) were recorded and the rate of growth dilution was defined as the percentage value of Cell $O.D._{600}$ at time 0 divided by the

Cell $O.D._{600}$ at time x.

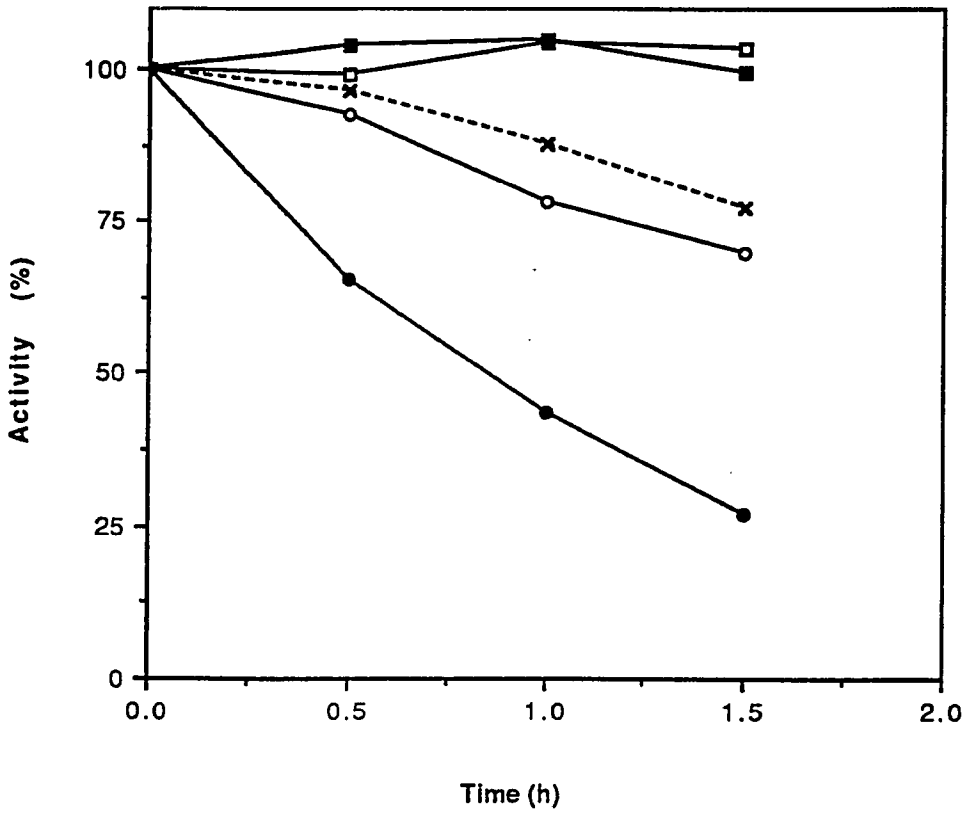
RESULTS

A time course study in strain 332-5A upon addition of glucose. A time course study following addition of glucose to maltose-induced growing culture was carried out in the maltose fermenting *Saccharomyces cerevisiae* strain 332-5A. This strain contains only the *MAL6* and *mal1*^{0} loci and no others, and thus contains a single copy of the *MAL61* gene and no other GENE 1 homologues (Dubin et al. 1986).

The addition of glucose to a mid-log phase, maltose-induced culture of 332-5A led to the loss of about 70-75% of the maltose transport activity over an one and half hour time course (Figure 1). After one and half hours, the maltose transport activity decreased at a much slower rate and stayed relatively constant at 20-25% of the original fully induced level for over two to three hours (data not shown). This remaining maltose transport activity is likely to represent contributions from both the high-affinity and low-affinity activities. Using the Michaels-Menten equation and substituting the substrate concentration as well as the kinetic constants of both high-affinity and low-affinity forms in the Michaels-Menten equation, at 4 mM extracellular substrate concentration only about 85-90% of total maltose transport activity is due to the activity of the *MAL61*-encoded high-affinity maltose transporter. The other 10-15% is from the low-affinity maltose transporter. Therefore, of the 20-

Figure 1. A time course study in *Saccharomyces* strain 332-5A upon addition of glucose.

Saccharomyces strain 332-5A was grown in YP + 2% (wt/vol) maltose to mid-log phase. At time 0, glucose was added to half of the culture to a final concentration of 2% (wt/vol) and cells were incubated further for another one and half hours. At time 0 and at 0.5 hour intervals for 1.5 hours, samples were removed and assayed for maltose transport activity (filled circles) and maltase activity (open circles) as described in Materials and Methods. Results are presented as percent of the activity at time zero. The dashed line indicates the rate of growth dilution. The other half of the culture served as a control (without addition of glucose) and maltose transport activity (filled squares) and maltase activity (open squares) are shown.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Activity (%)
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Time (h)

25% maltose transport activity remaining after the glucose inactivation, much of this could be from the low-affinity maltose transport activity which appears to decrease much slower at one and half hour growth in glucose (Cheng and Michels 1991). The nature of the low-affinity maltose transport is not known. Results from other groups indicated that the low-affinity maltose transport activity could be from non-specific extracellular binding (R. Lagunas, personal communications).

On the other hand, the rate of growth dilution after glucose addition followed much slower kinetics and decreased by approximately 30% (Figure 1). At 1.5 hours, based on the growth rate and the repression of structural gene transcription by glucose, the level of pre-existing maltose transporter protein and maltase should also have decreased by about 30% if no other processes were affecting activity. This appears to be true for maltase activity which decreased with approximately the same kinetics as the growth dilution factor (Figure 1). Maltase has been described as a stable cytoplasmic proteins and the half-life of its mRNA is less than six minutes (Federoff, Eccleshall and Marmur 1983). Without the addition of glucose, both maltose transport and maltase activities remain constant during the one and half hours of growth in maltose medium (Figure 1).

While glucose appears to cause a decrease of both the high-affinity maltose transport activity and maltase activity,

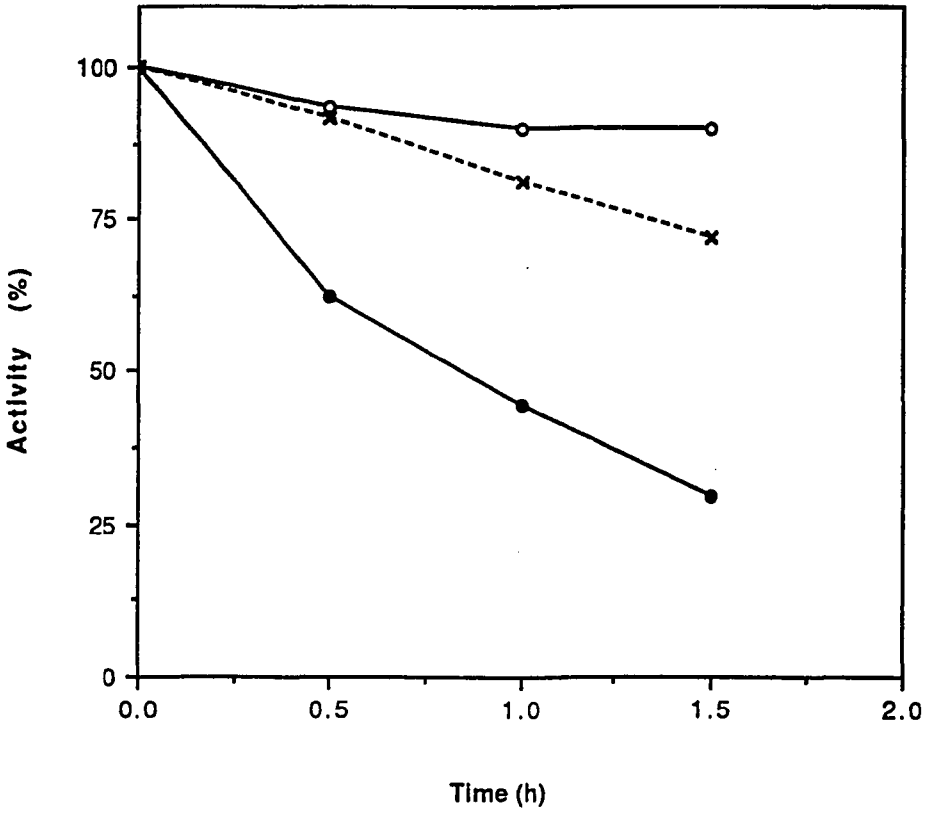
the loss of maltose transport activity is much more rapid than can be explained by growth dilution alone. Reports indicate that most yeast membrane proteins are stable with no detectable or significant turnover during three hours (Lopez and Gancedo 1979; Losson, Jund and Lacroute 1977). Our results therefore suggest that the MAL61 protein is subject to a selective glucose-induced inactivation.

A time course study in strain R10 upon addition of glucose. In order to more fully distinguish the contribution of glucose repression from that of glucose-induced inactivation in the glucose-induced loss of maltose transport activity, we repeated the same inactivation time course study using a partially glucose-repression insensitive, maltose fermenting strain R10 which contains the *MAL64-R10* constitutive MAL-activator allele. This strain was isolated as a maltose-fermenting revertant from an uninducible, non-fermenting strain isogenic to 332-5A but containing a *mal63Δ* deletion/disruption (Dubin et al. 1986). Strain R10 was shown to contain a mutation in the *MAL64* gene, a *MAL63*-linked *MAL63* homolog. In the wild type, *MAL64* is dispensable and plays no apparent role in maltose fermentation. The *MAL64-R10* mutant allele functions as a trans-acting, positive regulator and is required for the constitutive, partially glucose-repression insensitive expression of maltose permease and maltase (Dubin et al. 1986).

Our results (Figure 2) show that upon addition of glucose

Figure 2. A time course study in *Saccharomyces* strain R10 upon addition of glucose.

Saccharomyces strain R10 was grown in YP + 3% (wt/vol) glycerol and 2% (wt/vol) lactic acid medium to mid-log phase. Glucose was added to the growing yeast cells at time 0, and yeast cells were further incubated for another one and half hours. The relative maltose transport activity (filled circles) and relative maltase activity (open circles) as well as the rate of growth dilution (dash lines) were determined according to Material and Methods.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Activity (%)
Time (h)

maltase activity decreased at a slower rate than the growth dilution, as would be expected in a strain in which maltase expresses as a partially glucose repression insensitive enzyme. On the other hand, the loss of maltose transport activity in R10 showed the same kinetics as seen in 332-5A indicating that even with a continued synthesis the *MAL61*-encoded maltose transporter is still targeted for functional inactivation by glucose. Thus glucose-induced inactivation seems to be distinguishable from glucose repression.

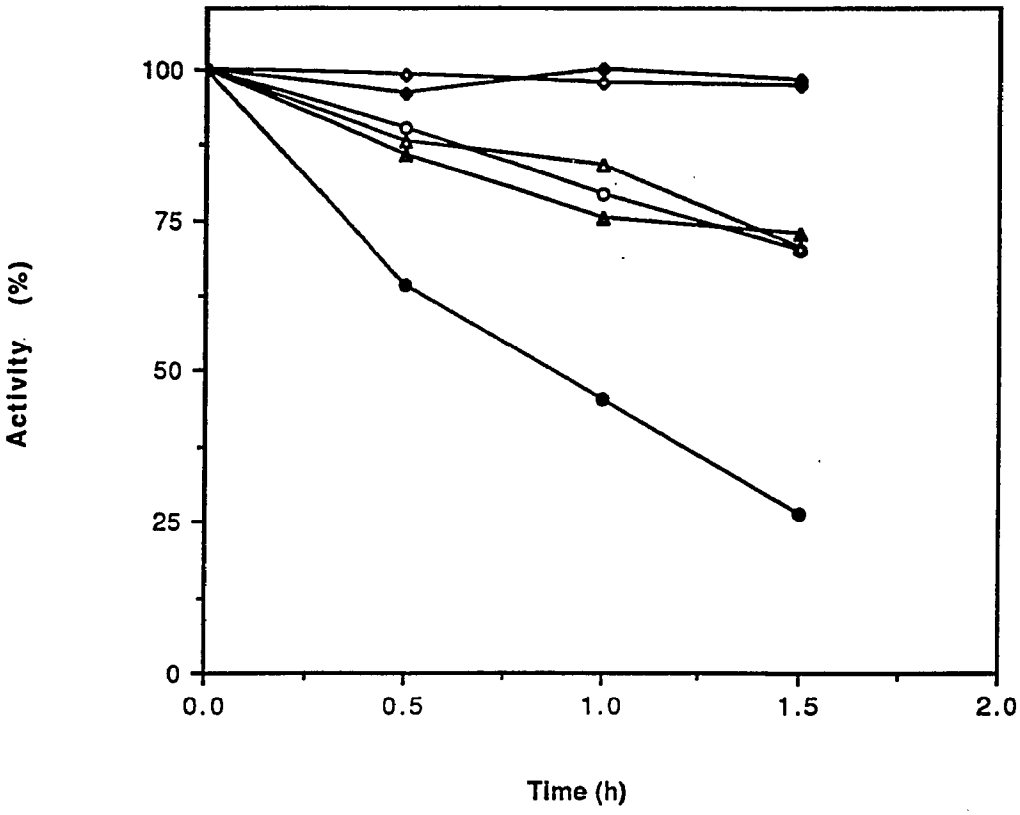
A comparison of the functional stability of the *MAL61*-encoded maltose transporter at different growth conditions. To explore the question of whether or not the inactivation of the MAL61 maltose transport protein is specifically in response to glucose, we decided to repeat the time course study under different carbon source conditions. Strain 332-5A was grown to mid-log phase in maltose-induced condition, harvested by filtration, washed and resuspended in YP + 2% glucose medium, YP + 3% glycerol / 2% lactic acid medium or YP + 2% galactose medium. The levels of maltase activity and maltose transport activity were followed for one and half hours. Glycerol/lactic acid and galactose are non-inducing carbon sources for the maltose fermentative enzymes, therefore, transfer to media containing these energy sources should stop transcription of the structural genes. If the MAL61 maltose transporter, like maltase, is functional stable protein under these conditions, the loss of both maltose

transport activity and maltase activity would follow the same kinetics as the growth dilution rate. As can been seen in Figure 3, the levels of both maltose transport activity and maltase activity stayed relatively constant in glycerol/lactic acid medium reflecting the very slow growth dilution rate on this poor carbon source medium (data not shown). Similarly, resuspending in galactose medium led to a decrease in both maltose transport activity and maltase activity again reflecting the rate of growth on this carbon source. The rate of growth in both YP + 2% glucose and YP + 2% galactose media are approximately the same (data not shown) but as seen in the previous experiment. Resuspending in glucose medium caused a rapid and significant decrease in maltose transport activity while the loss in maltase activity again reflected the growth dilution rate. It appears that the MAL61 maltose transporter is functionally stable in glycerol/lactic acid and in galactose and that glucose specifically targets the MAL61 protein for functional inactivation.

The effects of cycloheximide on maltose transport activity. The stability of the MAL61 maltose transporter in strain 332-5A was further investigated by the addition of a general protein synthesis inhibitor, cycloheximide, into a maltose-induced culture. After the addition of cycloheximide, yeast cells continually grown for about one hour at approximately the same growth rate as before (data not shown). Since the MAL61 maltose transporter is expected to be

Figure 3. A comparison of the functional stability of the *MAL61*-encoded maltose transporter under different growth conditions.

Saccharomyces strain 332-5A was grown in YP + 2% (wt/vol) maltose to mid-log phase. At time 0, yeast cells were harvested by filtration, washed and resuspended in YP + 3% (wt/vol) glycerol and 2% (wt/vol) lactic acid (diamond symbols), YP + 2% (wt/vol) galactose (triangle symbols), and YP + 2% glucose (circle symbols). Aliquots were taken at 0 time and at 0.5 hour, 1.0 hour, 1.5 hour intervals and assayed for maltose transport activity (filled symbols) and maltase activity (open symbols).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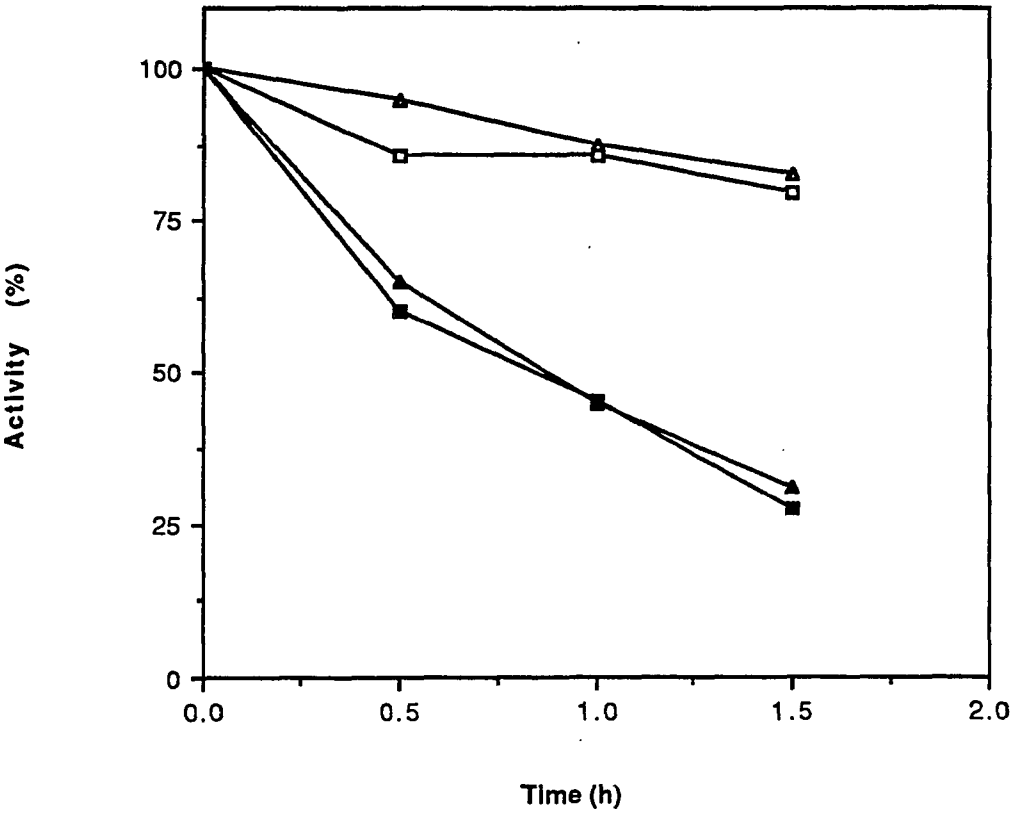
Activity (%)
Time (h)

functionally stable in maltose media, we would expect that in the absence of protein synthesis the loss of maltose transport activity and maltase activity should follow the same kinetics as the growth dilution rate. Surprisingly, both the addition of cycloheximide and the addition of glucose plus cycloheximide resulted in a rapid loss of maltose transport activity, similar to that seen for glucose-induced inactivation (Figure 4). The maltase activity decreased slowly (Figure 4) with kinetics comparable to the growth dilution rate (data not shown).

Figure 4. The effect of cycloheximide on maltose transport activity.

Saccharomyces strain 332-5A was grown in YP + 2% (wt/vol) maltose until mid-log phase. Cycloheximide (10λg/ml) (square symbols) or cycloheximide (10λg/ml) plus glucose (2% final concentration) (triangle symbols) were directly added to the maltose-induced culture at time 0. Aliquots were taken at time 0 and at 0.5 hour, 1.0 hour, 1.5 hours intervals and assayed for maltose transport activities (filled symbols) and maltase activities (open symbols).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Activity (%)
Time (h)

DISCUSSION

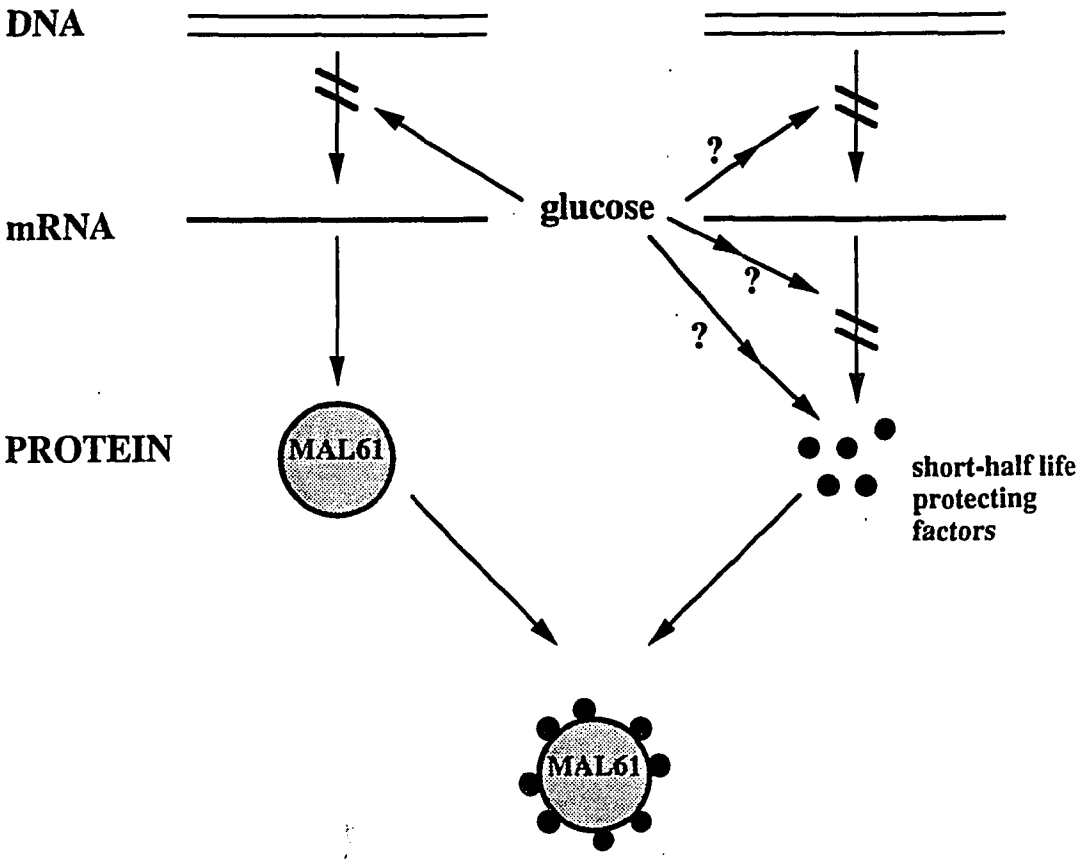
The time course studies reported here reveal that the *MAL61*-encoded maltose transport system is sensitive to glucose-induced inactivation. Additionally, glucose-induced inactivation is a selective mechanism affecting the MAL61 maltose transporter and does not result from the glucose repression of transcription of the genes encoding the maltose fermentation enzymes. The transcriptionally coregulated maltose fermentative enzyme maltase is subject to glucose repression alone, since its loss of activity upon the addition of glucose to the growth medium follows the same kinetics as the growth dilution rate which is 3-5 fold slower than the loss of maltose transport activity. The rapid (half-life of about 45 minutes) inactivation of maltose transport also occurs at a similar rate in a partially glucose-repression insensitive *Saccharomyces* strain (Figure 2), in which the loss rate of maltase activity is decreased compared to the growth dilution.

The mechanism of glucose-induced inactivation of maltose transport has not been determined. The inactivation has been described as an irreversible process which might involve proteolysis since the recovery from glucose inhibition requires that the cells be returned to inducing medium and this recovery is suppressed by cycloheximide (Busturia and Lagunas 1985; Gorts 1969). On the other hand, a model

implying a reversible modification of the maltose-H^+ symport system induced by glucose catabolism has been proposed based on the finding that the effects of glucose depend on the incubation conditions and are partially reversed by carbon starvation even in the presence of cycloheximide (Peinado and Loureiro-Dias 1986).

The time course studies in various growth conditions have shown that the MAL61 maltose transporter is a functionally stable protein except in the presence of glucose (Figure 3) and glucose appears to specifically cause the inactivation of maltose transport system. The functional stability of the maltose transporter is lost in conditions inhibiting *de novo* protein synthesis (Figure 4). This result suggests two possibilities. The first possibility is that in the presence of either glucose or maltose, the maltose permease has a very short half-life but the high rate of induced synthesis in maltose growth conditions maintains a high level of the maltose transport activity. In the absence of continued synthesis, such as would occur as a result of glucose repression or addition of cycloheximide, a rapid loss of maltose transport activity is seen. The second possibility is that the maltose transporter requires protein synthesis for stability. To interpret the latter possibility, we have proposed the Protection Model presented in Figure 5. In this model, unstable protein factors are required to stabilize the active maltose transport system and to protect it from

Figure 5. Protection Model



functional MAL61 maltose transporter

inactivation. These protecting factors can either physically bind to or covalently modify (such as phosphorylation or dephosphorylation) the newly synthesized MAL61 protein to form a functionally stable active maltose transporter. Since these protecting factors have a short half-life, the inhibition of protein synthesis such as by the addition of cycloheximide would be expected to cause the rapid turnover of the protection system which would in turn lead to the rapid functional loss of the MAL61 protein. Glucose could affect the protecting factors at different levels, the transcriptional level (glucose repression), the mRNA level (mRNA stability), the translational level as well as the post-translational protein level, each of which would result in a cessation of further synthesis of the protecting factors and the glucose-induced inactivation of maltose transport.

Several mechanisms of protection via association or modification of the MAL61 protein with protecting factors can be suggested. The protection could increase the physical attachment of the MAL61 protein to the yeast cell membrane thereby maintaining the functional form of the integral membrane MAL61 protein. Alternatively, the protection could prevent MAL61 protein from being exposed to covalent modification, such as phosphorylation or dephosphorylation, which could either directly generate a conformationally changed, inactive form of the MAL61 protein or target the protein for internalization, vacuole relocalization or

proteolysis. Finally, the protection could render the MAL61 protein resistant to degradation by systems such as the yeast vacuolar proteases or the ubiquitin-conjugation systems (Achstetter and Wolf 1985; Ciechanover and Schwartz 1989; Finley and Varshavsky 1985; Hershko 1988; Jentsch et al. 1990). A MAL61 antibody detecting system is essential in order to explore these mechanisms. With an antibody, the protein stability, the phosphorylation state, cellular location and sensitivity to proteolytic degradation could be followed under different growth conditions. This work is currently in progress.

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