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Yeast Sugar Transporters

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ABSTRACT: Transport of sugars is a fundamental property of all eukaryotic cells. Of particular importance is the uptake of glucose, a preferred carbon and energy source. The rate of glucose utilization in yeast is often dictated by the activity and concentration of glucose transporters in the plasma membrane. Given the importance of transport as a site of control of glycolytic flux, the regulation of glucose transporters is necessarily complex. The molecular analysis of these transporters in *Saccharomyces* has revealed the existence of a multigene family of sugar carriers. Recent data have raised the question of the actual role of all of these proteins in sugar catabolism, as some appear to be lowly expressed, and point mutations of these genes may confer pleiotropic phenotypes, inconsistent with a simple role as catabolic transporters. The transporters themselves appear to be intimately involved in the process of sensing glucose, a model for which there is growing support.

KEY WORDS: yeast, sugar, transport, *Saccharomyces*, glucose sensing, glucose transporter family, sugar transport.

I. INTRODUCTION

The transport of sugar into a cell is the first, and perhaps most important, step of sugar metabolism. Regulation of sugar entry is a common biological strategy for modulation of and response to glycolytic flux and cellular activities. The complexities of sugar transport and its control are only recently becoming apparent and appreciated.

Mammalian systems, as well as the yeast *Saccharomyces*, are now known to possess families of highly homologous sugar transporter genes. Members of these multigene families display a similar predicted secondary structure of twelve putative membrane-spanning regions with a characteristic and conserved spacing and have several amino acid motifs in common.[117,183] In mammalian systems, tissue specificity of expression has been invoked to explain the need for and presence of a multigene family.[86,93,94,107,139,140,171,238,239,273] However, such an explanation for the existence of a multigene glucose-transporter family obviously would not apply to a unicellular organism such as *Saccharomyces*. Prokaryotic systems also display families of transporters,[9,112,117–119,166] but in this case, they are differentiable by substrate specificity, which, again, is not the situation in *Saccharomyces*. Whereas there are sugar transporters with clearly differing substrate specificities in this yeast, four putative transporters have been identified in *Saccharomyces* that affect glucose transport, and other related genes remain to be characterized. Mutations of some, but not all, of these putative transporters also simultaneously affect fructose and mannose utilization. All three sugars are believed to be transported by the same transport systems in *Saccharomyces*. Recent data to be described later, however, suggest that this might

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not be the case. The physiological functions of and needs for this multitude of putative transporters in yeast are not yet clear. This review will focus on the biochemical, physiological, and genetic analysis of sugar transporters in *Saccharomyces* and other yeasts and discuss putative roles of these proteins in cellular metabolism and physiology.

II. MULTIPLE ROLES OF GLUCOSE IN EUKARYOTIC CELL BIOLOGY AND METABOLISM

The catabolism of sugars plays a fundamental metabolic role in biological systems as a provider of carbon for new cell material and of energy to drive cellular processes. From a physiological perspective, it is important to distinguish roles as a carbon source, necessary only during periods of net growth, from that as an energy source, energy being necessary not only during growth but during quiescent phases as well. The provision of glucose to "resting" cells can thus have a profound impact on cellular activities, irrespective of growth, simply because metabolic energy is now available. Sugars can also be taken up for incorporation into storage carbohydrate rather than immediate metabolism. In all cases, the sugar must first be transported into the cell, although the total amount needed and the rate at which sugar must be taken up to feed metabolism will differ depending on the physiological and environmental circumstances. Regulatory mechanisms dictating sugar uptake and catabolism will almost certainly reflect the cellular needs for carbon or carbon and energy vs. energy alone.

Glucose uptake in eukaryotic cells has been shown to be modulated by growth rate, growth phase, nutrient supply, external glucose concentration, and rate of glycolytic flux and responds quickly to changes in growth status and to the glucose concentration in the medium. The complexity of regulation of sugar transport may explain the existence of multigene families of glucose transporters. A single protein simply might not be capable of responding to the plethora of physiological and environmental signals that must impact glucose uptake activity. Instead, a class of proteins responding in subtly, and perhaps not-so-subtly, different fashions to regulatory events may be the simplest mechanism of control of glucose transport and subsequent coordination with metabolism. Glucose transport in yeast is more closely analogous to a water faucet than to a simple on/off switch. The rate of uptake is adjusted to match the rate of flux through glycolysis. Unraveling the intricacies of control of glucose uptake should provide new insight into the mechanisms of regulation of substrate transport and utilization in general.

Glucose, the preferred carbon and energy source of eukaryotes and many prokaryotes, plays yet another role in many cells — that of a molecular signal directing cellular processes such as initiation of a new cell cycle and regulation of gene expression at the transcriptional level. In many respects, glucose can be thought of as a hormone. In multicellular organisms such as humans, there are basically two types of cells: those that adapt their metabolic activities in direct response to changing glucose concentrations in their environment to maintain the energy levels required for function and those that adjust glucose uptake in response to hormonal signals such as insulin, glucagon, and growth hormones. Neural cells are glucose responsive, whereas adipose and many types of muscle are hormone responsive. In this way, cells with a higher overall priority for maintenance of function, such as those of the brain, do not have to compete with other relatively less vital tissues for an essential energy source. This prioritization of cells for glucose consumption is particularly important under conditions of limiting substrate.

The yeast *Saccharomyces* behaves in an analogous metabolic fashion to the glucose-responsive mammalian cells. Addition of glucose to glucose-starved nongrowing cells results in the synthesis of several second messengers and can signal the initiation of growth.[245–247] Glucose controls the RAS-adenylate cyclase pathway in *Saccharomyces;* addition of glucose to resting cells rapidly results in the synthesis of a burst of cAMP.[14,46,176,185] In addition, glucose activates the yeast plasma membrane proton-pumping ATPase,[211,213] resulting in the uptake of calcium and potassium ions.[73,255] There is some evidence

that provision of glucose also results in the synthesis of the inositol phosphate family of second messengers[89,136] and induces cAMP-independent changes in stationary phase cells.[110]

Glucose is responsible for three other global regulatory phenomena: glucose inactivation, glucose induction, and glucose repression. Several proteins not required for the fermentation of glucose are inactivated on exposure of the cells to glucose, a phenomenon called "glucose inactivation."[124] This includes transporters such as the maltose and galactose carriers and enzymes of gluconeogenesis such as fructose bisphosphatase.[72,75,82,95,99,106,155,210,214] The high-affinity glucose transport system also displays catabolite inactivation.[27,36] The exact mechanism of glucose inactivation is not known. Phosphorylation of the target protein appears to be an initial step but is not absolutely required for inactivation.[150,175,184] Inactivation ultimately involves loss of immunoreactive protein, presumably as a result of proteolytic degradation.[191]

Glucose also regulates gene expression at the transcriptional level. Expression of pyruvate decarboxylase, an enzyme required for ethanol production as an end product of fermentation, is inducible at the transcriptional level by glucose.[121,226] Loss of a gene known as glycolysis regulation *(GCR1)* makes the genes encoding the reversible steps of glycolysis also glucose inducible for expression.[58] In *gcr1* mutants in the absence of glucose, the enzymes of glycolysis are expressed to about 5% of normal, increasing to 20 to 50% of normal in the presence of glucose.[58] The *GCR1* protein is a transcription factor, binding to DNA in the presence of other transcriptional factors, and seems to act specifically on glycolytic genes.[7,8,61,122,127] The mechanism by which glucose mediates this induction is unknown, but it is clear that enzymes involved in glucose catabolism are differentially expressed at the transcriptional level, according to glucose availability.

Enzymes involved in respiration and metabolism of nonglucose substrates are also controlled by glucose at the transcriptional level and are repressed in the presence of fermentable sugars such as glucose.[15,79,250] Several genes have been identified that affect glucose repression,[98,250] and some appear to encode transcription factors, whereas others have as yet unidentified functions.

Little is understood in any system regarding the very early metabolic events involved in receipt and transmission of the glucose signal. Glucose receptors or sensors analogous to hormone receptors have not been identified. Although definitive data are lacking, a current popular model suggests that the transporters themselves are the glucose sensors, either alone or in combination with the first enzymatic step of glycolysis, sugar phosphorylation, or as part of a larger sensing complex.[188,194,256] Glucose serves many important functions in eukaryotic cells: as carbon and energy source and as regulator of cellular activities and metabolism. The glucose transporters appear to be involved in all of these roles.

Because of its biochemical and genetic tractability, *Saccharomyces* is an excellent experimental system in which to dissect the processes of glucose transport, sensing, and signal transduction. This yeast has been the focus of intensive analysis of glucose transport. Other yeasts (*Kluyveromyces, Pichia, Candida,* and the fungus, *Neurospora*) have also been investigated, largely for comparative purposes.[12,69,70,101,178,227,234,235,259,263] These studies have revealed that the transport of sugars in fungi is highly controlled and is a far more complex process than previously suspected.

III. GLUCOSE METABOLISM IN *SACCHAROMYCES*

The natural habitat of *Saccharomyces* is grape juice, rich in an equimolar mixture of glucose and fructose. Grape juice is approximately 20 to 24% (w/v) (1 to 1.5 *M*) in total sugar. *Saccharomyces* rapidly ferments this sugar to ethanol via glycolysis, pyruvate decarboxylase, and alcohol dehydrogenase. Thus, sugar transport in *Saccharomyces* must function over very broad ranges of sugar concentration, osmolarity, and specific gravity. The plasma membrane of *Saccharomyces* undergoes dramatic compositional changes to maintain ethanol tolerance, so transporters must function in a changing microenvironment as well. The typi-

cal fermentation is anaerobic, but *Saccharomyces* is facultative and can respire on low sugar concentrations or while using respiratory substrates. *Saccharomyces* has a relatively narrow range of sugars that serve as good growth substrates: glucose, fructose, mannose, galactose, the disaccharides sucrose and maltose, and the trisaccharide raffinose. This yeast can also utilize the respiratory substrates, ethanol, acetic acid, pyruvate, lactate, and some strains can use glycerol poorly as a substrate. Glycerol does stimulate growth on low concentrations of glucose such as those typically found in media made using yeast extract and on lactate. Maltose and galactose have their own specific sugar transporters that are inducible by substrate, repressed by glucose, and inactivated by the fermentable sugars glucose and fructose. Glucose, fructose, and mannose were thought to share the same transporters, but data to be described later indicate that these sugars may have specific transporters. Other non-*Saccharomyces* yeasts are able to use a much broader range of sugar and nonsugar substrates. Sugar metabolism in *Saccharomyces* has been reviewed recently.[24,281]

In yeast, as in many other organisms, cytoplasmic glucose concentrations are normally low to barely detectable. This observation led to the conclusion that sugar uptake is the rate-limiting step of glycolysis.[16] However, low internal substrate concentration can be a consequence of stringent coordination of sugar uptake and sugar consumption and does not necessarily indicate that transport is the rate-limiting step of glucose metabolism. Instead, the rate of transport may be tightly regulated so that only the amount of glucose that can be used is brought in to the cell at a rate equivalent to that at which it can be consumed. Uncontrolled glucose uptake leads to the depletion of ATP if sugar kinase activity is unchecked. The activity of the sugar kinases in *Saccharomyces* is not regulated by the concentration of the phosphorylated end products. If there is a block anywhere downstream of sugar kinase activity in the glycolytic pathway, this uncoupling of upper and lower glycolysis and subsequent loss of ATP could have disastrous consequences for the cell. Indeed, misregulation of maltose transport and metabolism can lead to glucose poisoning and cell death.[77] To prevent ATP depletion, the most appropriate site of regulation of glycolysis is glucose uptake.[16]

A. Mode of Sugar Transport

It is only within the past 10 years that a general agreement has been reached as to the mechanism of sugar transport in *Saccharomyces*. Seemingly conflicting data had been obtained by early workers in the field[55,56,88,132,144] concerning the mechanism of glucose transport in this yeast. Several lines of evidence suggested that glucose transport is a facilitated diffusion process, as is the case in mammalian cells. There was no observable sugar accumulation against a concentration gradient or any demonstrable energy requirement for uptake, and transport was found to be insensitive to uncouplers.[55,56,88] Experiments using a glucose analog, 2-deoxy glucose, revealed that this sugar first appears inside of the cell in the phosphorylated form.[132,261,262] This led Van Steveninck and co-workers[132,261,262] to postulate an active transport mechanism for sugar uptake whereby sugar phosphorylation occurred during sugar entry, as is the case with the phosphotransferase system of *Escherichia coli*.[212] These data, however, are also consistent with a tight coupling between a facilitated diffusion transport system and sugar kinase activity.[228] Several new lines of evidence suggest that the latter is, indeed, the case.

To address the role of sugar phosphorylation in transport, a kinetic analysis of glucose transport in *Saccharomyces* was undertaken in wild-type cells and in cells partially or completely deficient in sugar phosphorylation.[25,152] *Saccharomyces* possesses three enzymes capable of phosphorylating glucose at the six position: hexokinase PI (A), hexokinase PII (B), and glucokinase. Glucokinase also catalyzes the phosphorylation of mannose, but not of fructose, and is thus an aldohexose-specific kinase.[2] The hexokinases phosphorylate mannose as well as fructose, in addition to glucose. Triple kinase mutants lacking all three sugar kinases are not able to use glucose, fructose, or mannose as carbon and energy sources.[60,97,158,159,167,168] Thus, there is no alternate mechanism for the phosphorylation of these sug-

ars in yeast. All three enzymes are soluble in yeast-cell extracts and are not found in the particulate or membrane fractions.[97,186] There is no genetic evidence for direct sugar phosphorylation via a transporter on entry into the yeast cell; however, a tight metabolic association between the transporter and the sugar kinases readily explains the data suggesting vectorial phosphorylation.

In a kinetic analysis of sugar uptake in *Saccharomyces,* Eadie-Hofstee plots of glucose, fructose, and mannose uptake were nonlinear, displaying two putative components of differing affinities. Low-affinity transport (K_m = 10–20 m*M* for glucose, 20–50 m*M* for fructose, and 50–70 m*M* for mannose) appeared to be constitutive, although the activity of this transporter does decrease in stationary phase and under certain other growth conditions.[22,23,25–27] The high affinity system (K_m = 1 m*M* for glucose, 5 m*M* for fructose, and 7 m*M* for mannose) was found to be repressed by high sugar concentration and somehow dependent on the presence of sugar phosphorylation activity.[23] Loss of hexokinase PI and PII *(hxk1 hxk2)* activity resulted in loss of high-affinity uptake of fructose but not glucose. A triple kinase mutant *(hxk1 hxk2 glk),* lacking glucokinase activity, did not display high-affinity uptake of either sugar.[25] These results suggest that the appearance of high-affinity uptake is dependent in some unknown fashion on kinase activity.

Accurate measurement of sugar uptake via facilitated diffusion is inherently difficult in yeast. Metabolism of substrate is rapid and occurs within the seconds time frame of assay. Cell volume is small so that in the absence of metabolism, internal substrate concentration may accumulate to high enough levels as to result in efflux from the cells. Researchers have attempted to address these difficulties in experimental design using various approaches. Nonmetabolizable analogs, such as 6-deoxy glucose, have been used as substrates for uptake to minimize the influence of metabolism on apparent uptake rates. Alternately, mutants deficient in glucose phosphorylation have been analyzed, using both glucose and 6-deoxy glucose. 2-Deoxy-D-glucose, a glucose analog that can be phosphorylated but not metabolized any further has also been examined and, similar to glucose 6-deoxy-D-glucose, displayed both high- and low-affinity transport, with high affinity dependent on the presence of a functioning sugar kinase. However, 2-deoxy-D-glucose is an energy poison, rapidly depleting the cells of ATP. The comparative kinetics of uptake of glucose and these two analogs gave consistent results, as both analogs displayed the same kinase effect as the phosphorylatable sugars. The kinase dependency of high-affinity uptake cannot be explained on the basis of sugar metabolism alone. Another complicating factor concerns the role of sugar kinases in glucose repression. Loss of hexokinase PII results in constitutive depression of glucose-repressible functions, one of which is high-affinity glucose transport. Thus, one is never examining the "wild-type" condition in a hexokinase PII mutant background.

The impact of loss via mutation of other enzymes of glycolysis has also been investigated to determine if the observations made with loss of sugar kinase activity are specific to the sugar kinases or a consequence of a block of glycolysis. Loss of phosphoglucose isomerase *(pgi)* activity did not immediately impact the kinetics of glucose uptake[4,25,208]; however, incubation of the *pgi* cells in the presence of glucose did eventually result in decreased uptake activity.[4,208]

Hexokinases and glucokinase are catalytically irreversible steps of glycolysis, serving to phosphorylate sugars at the six position. Different enzymes, the sugar phosphatases, catalyze the reverse reaction in gluconeogenesis in those cells synthesizing free glucose. The end point of gluconeogenesis in *Saccharomyces* is glucose-6-phosphate. Two other steps of the glycolytic pathway are similarly irreversible: phosphofructokinase and pyruvate kinase. Loss of phosphofructokinase and pyruvate kinase activity does affect the kinetics of glucose uptake (Figure 1), having an impact similar to that observed in mutants lacking hexokinase and glucokinase activity. Phosphofructokinase is composed of two nonidentical subunits encoded by *PFK1* and *PFK2*.[59,60] Loss of both of these subunits dramatically decreases glucose uptake activity. Loss of pyruvate kinase has a similar, but less dramatic, effect.

In addition to *pgi,* transport of glucose has been investigated in mutants lacking other reversible steps of glycolysis: triosephosphate isomerase,

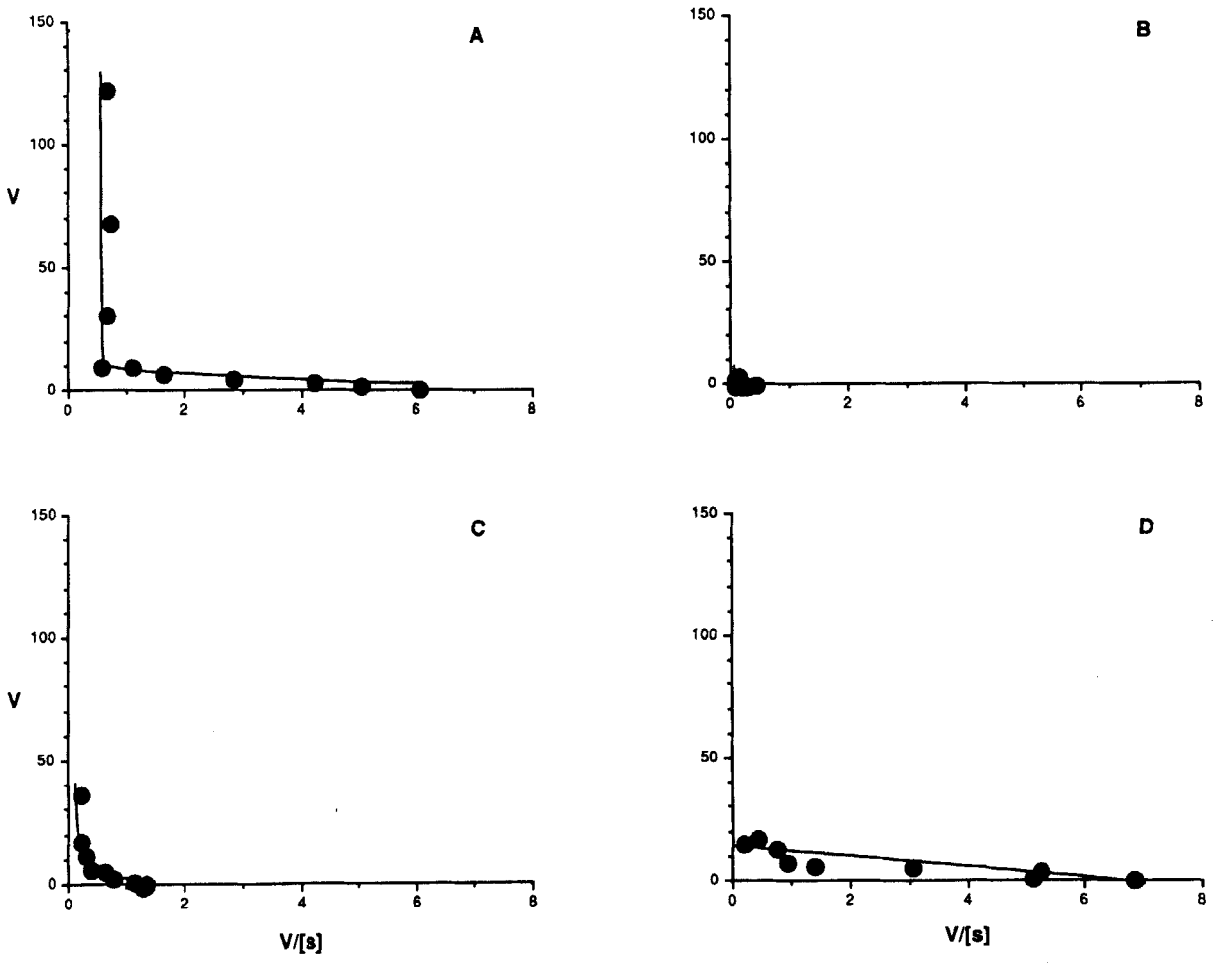

FIGURE 1. Kinetics of glucose uptake in lactate-glycerol grown cells of wild-type DFY1[27] (panel A), *pfk1 pfk2* (panel B), *pyk1* (panel C), and *gcr1* (pandel D) mutants. V, nmol/min/mg wet weight of cells, S, m*M*.

glycerophosphomutase, and phosphoglycerate kinase. Loss of any one of these enzymes had no impact on initial kinetics of glucose uptake and were indistinguishable from the wild-type or *pgi* mutant strains (Bisson unpublished observations). Thus, loss of any one of the irreversible steps of glycolysis, hexo(gluco)kinase, phosphofructokinase, or pyruvate kinase prevents expression of transporter activity, whereas loss of a reversible step does not have this effect. Regulatory mechanisms exist that prevent the expression of transport activity if a functional glycolytic vs. gluconeogenic pathway cannot be formed. The freely reversible enzymes do not immediately impact transporter kinetics. Because these enzymes are required for both glycolysis and gluconeogenesis, they might not be valuable cellular checkpoints of glycolytic functionality. Provision of glucose to cells carrying a block in glycolysis does lead to a reduction in transporter activity over time, reflecting regulation of transport by overall flux through glycolysis. Of the three irreversible steps of glycolysis, it is clear that loss of phosphofructokinase has the most dramatic impact, with loss of both high- and low-affinity uptake. Loss of sugar kinase activity seemed to affect high-affinity uptake preferentially.[25,26]

The kinetics of glucose transport were also examined in a strain carrying a *gcr1* mutation that carries a defect in a transcriptional regulator of glycolytic enzyme expression.[7,8,58,61,122,127] Loss of *GCR1* function confers glucose inducibility to the

reversible steps of the glycolytic pathway.[58] Glucose uptake was examined in a *gcr1* mutant grown on lactate and glycerol in the absence of glucose. In this case, high-affinity glucose uptake was readily apparent, but low-affinity transport was not observed (Figure 1). Glucose does induce higher levels of transport in this mutant, which may indicate that at least some transport component shares regulatory elements in common with the reversible enzymes of glycolysis.

Recently, Furhmann and colleagues[91,92,282,283] were able to demonstrate high-affinity glucose transport in plasma membrane vesicles purified from *Saccharomyces* grown under conditions of glucose limitation. When cells were grown under conditions of glucose excess, the vesicles obtained displayed only low-affinity kinetics of glucose uptake. Previous studies had been able only to demonstrate low-affinity uptake in vesicles.[203] The observation of high-affinity uptake in vesicles is significant in that the existence of both low- and high-affinity transport in an *in vitro* condition have now been demonstrated. This work also confirms *in vivo* studies that showed glucose repressibility of high-affinity glucose transport. The coexistence of both high- and low-affinity uptake has not been observed in any of the vesicle preparations.

These researchers also examined the kinetics of transport in vesicles purified from triple kinase mutants. These vesicles manifested high-affinity transport, in contrast to what is observed *in vivo*, indicating that high-affinity transport is, indeed, expressed in triple sugar-kinase mutants and by extension, probably in pyruvate kinase and phosphofructokinase mutants as well. This observation suggests that the failure to observe high-affinity uptake in sugar kinase mutants reflects posttranslational regulation of carrier activity, perhaps an inability to activate or alter transporter affinity once expressed. In this model, high-affinity transporters are made constitutively and are incorporated in the plasma membrane, but their activity is masked in the absence of sugar kinase function. Years ago, Serrano and DelaFuente[230] observed a change in kinetics of transport in yeast cells shifted from aerobic to anaerobic conditions, which they interpreted as evidence that the glucose carrier may exist in different kinetic states. The *in vitro* data suggest that the normal state of glucose transporters is to be "on" and that they are down-regulated by metabolic activities, not "off" and turned up or on by the presence of the irreversible steps of glycolysis.

An intensive mathematical modeling and analysis of the kinetic data obtained by other researchers was also performed.[91] The visual observation and calculation of kinetic constants from Eadie-Hofstee plots was found to grossly underestimate the putative K_m of the low-affinity transporter, which was reported to be actually in the molar range. The physiological significance of such a high-K_m system was questioned and the suggestion was made that this simply might be diffusion across the membrane.[91,92,282,283] It is important to remember, however that the natural yeast habitat, grape juice, is very rich in sugar. Concentrations in the range of 1 to 2 *M* are indeed typical. A transport system with an apparent K_m in this range certainly would be physiologically relevant. It is perhaps significant that other yeasts that are not normally found in such a glucose-rich environment display transport systems of much lower K_m values than even the high-affinity system of *Saccharomyces*. Yeasts that do not display a "Crabtree effect" (the inhibition of respiration at high sugar concentration) possess proton-symport glucose transporters of very high affinity.[263] Unlike *Saccharomyces*, these yeasts can concentrate glucose against a gradient.

Given the recent observations of a multigene family of transporters, a detailed kinetic analysis and interpretation is premature. Four genes that affect glucose uptake and have amino acid sequences resembling known transporters have been identified, and low stringency Southern analysis indicates that there are several others. Also, work in higher eukaryotic systems suggests that transporters may function as dimers,[62,63,115,131,209] raising the possibility of heterodimers as well as homodimers among the related genes. There is the potential to amplify the numbers and kinds of transporters in the membrane to a high and almost alarming degree. It is important to note that these kinds of kinetic studies, although informative for comparison of mutant and wild-type strains and of cells in different physiological conditions, may reveal little about the actual kinetics of the indi-

vidual transporters. Such information will only be obtained from analysis of purified transporters in reconstituted vesicle systems. We use Eadie-Hofstee plots routinely in our work, as do other researchers in the field, simply because the differences between strains and conditions are readily visible in this analysis. It has provided a convenient screen for an interesting set of mutants affecting glucose uptake. Kinetic constants obtained from such plots should be viewed with extreme caution. The shape of such plots should not be overinterpreted. In general, under many growth conditions, the Eadie-Hofstee plots are nonlinear, appearing to have at least two kinetic components, differing in affinity. There are, however, many alternative explanations for such an observation such as different states of the same carrier, uptake mediated by a "nonglucose" sugar carrier, and different components will only be revealed if the apparent K_m differs by at least one order of magnitude. Thus, several different transporters, operating with similar kinetics, will not be discernable using a biochemical kinetic analysis of transport by whole cells. It is probably more accurate in these studies to refer to kinetic constants as "consortium constants" as the genetic data clearly indicate that multiple putative transporters are being expressed simultaneously.

It can be argued also that *in vitro* studies using purified components, although informative as to the actual kinetics of transporter activity, do not allow detection of subtle, and perhaps not-so-subtle, influences of cellular metabolism and other activities on transport. Given the importance of uptake, the need for tight coordination with metabolism, and the global effects of glucose on cellular function, glucose transporters are probably highly "social" proteins in the plasma membrane, perhaps interacting and communicating with a variety of other membrane and cytoplasmic proteins to transmit a signal that glucose is present. Because protein-protein interactions may impact the kinetics of uptake, *in vitro* studies with purified systems may present only a small part of the *in vivo* picture. The technical limitations, however, are such that even with the most rapid *in vivo* assays, there is considerable metabolism occurring. This serves to keep the internal concentration of glucose low in those strains not deficient in glucose utilization. Internal glucose concentrations would be significantly higher in mutants unable to phosphorylate the substrate. There are numerous technical challenges to the definitive kinetic study of glucose transport in *Saccharomyces*.

Another potentially complicating factor concerns the well-known observation in mammalian systems that *in vitro* kinetic studies of GLUT1 always reveal a symmetric carrier with kinetic constants for influx equal to those for efflux, whereas *in vivo* work always suggests an asymmetric carrier with the kinetic constants being different for sugar translocation in different directions. Carruthers *et al.*,[44,45,116] obtained some evidence suggesting this was due to ATP levels, but that has recently been disputed by other researchers.[276] In any event, glucose uptake is a complex process, the kinetics of which are definitely not simple.

B. Regulation of Sugar Transport

Glycolytic flux and presence or absence of the irreversible steps of glycolysis are prime regulators of glucose transporter activity in *Saccharomyces*. Other factors also regulate glucose transport. High-affinity glucose uptake is glucose repressible and is not expressed at high glucose concentrations,[23,80] as is also observed in mammalian systems.[96,269] Mutations known to affect glucose repression either by causing constitutive expression of glucose repressible genes or by constitutive repression have the same effect on expression of high-affinity glucose uptake[23], that is, high-affinity uptake is constitutively expressed in mutants displaying constitutive expression of other glucose-repressed functions and is not expressed in mutants unable to depress glucose-repressible functions. Transport is also regulated posttranslationally, and differences in transporter protein half-lives with a change in growth conditions have been observed.[3] Galactose transport displays similar regulatory strategies to glucose uptake.[215]

The actual biochemical mechanism of sugar uptake has not been explored in *Saccharomyces* because sugar transporter proteins have not been

purified. Mechanistic studies have been done using the red blood cell glucose transporter, GLUT1, which is relatively easy to purify in amounts sufficient for detailed biochemical analyses. Several lines of evidence suggest that the transporter oscillates between two different conformations, alternatively exposing the substrate binding site to the cytoplasm and to the external milieu. The transporter is fixed in the lipid bilayer and does not flip or rotate a substrate binding site between plasma membrane surfaces.[6] Glucose is likely translocated across the membrane by passing through the protein via a series of hydrogen bond interactions.[265] The outward- and inward-facing glucose binding sites are not identical, as determined by interaction with inhibitors[6] and by photolabeling.[123] 4,6-Ethylidene-D-glucose preferentially binds to the extracellular binding site, and phenyl β-D-glucoside binds to the inward-facing site.[6]

When sugar transport by *Saccharomyces* is measured under certain conditions of very high sugar substrate concentrations (1 to 1.5 *M*), the phenomenon called "substrate inhibition" is observed.[177,178] This inhibition of uptake is specifically due to substrate and not to osmolarity, as an equivalent concentration of sorbitol has no impact or glucose transport (Bisson, unpublished observations). Substrate inhibition refers to a decrease in net uptake caused by excessive substrate concentration. The mechanistic explanation for this phenomenon is that the substrate has multiple points of attachment to an "open" substrate binding site.[189] At high substrate concentrations, more than one substrate molecule may attempt to bind at the same time, occluding the binding site and preventing translocation. Substrate inhibition could conceivably play an important physiological role, limiting sugar uptake if cells are suddenly switched to a very high substrate concentration. Such dramatic shifts between low- and high-substrate concentrations are the norm for *Saccharomyces* in grape juice.

Sugar transporter activity is also regulated by nitrogen availability and protein synthesis. If protein synthesis is blocked, there is an accelerated rate of turnover of glucose transporter activity.[36] Low- and high-affinity uptake are both affected.[36] Approximately 50% of the fermentation of sugars conducted by *Saccharomyces* when grown in grape juice is due to stationary phase cells.[24] Nitrogen limitation of cells during growth leads to nitrogen-depleted stationary-phase cells, which results in the decay of sugar transport activity leading to what is known as a sluggish or stuck fermentation.[173,221]

There is also some evidence that transport may be regulated differently under anaerobic vs. aerobic conditions.[177] These differences, however, may reflect the existence of a completely different population of transporters under these two growth conditions. One putative glucose transporter, HXT2, is highly homologous and likely identical to a gene identified as being controlled by the genetic state of the yeast mitochondrion, being maximally expressed under conditions limiting mitochondrial activity.[145] A regulatory interaction of mitochondrial activity and galactose transport has also long been postulated.[71,253] Thus, respiratory capacity appears to regulate transporter activity and may determine which transporters of the multigene family are expressed.

Glucose transport in *Saccharomyces* is also regulated by cell growth. The appearance of high-affinity glucose transport on shift of cells from high- to low-glucose concentration media requires a functional secretory pathway. Mutants temperature sensitive for secretion *(sec)* have been isolated in *Saccharomyces*.[199,200,224] When incubated at the nonpermissive temperature (37°C), cell growth ceases and cell surface proteins accumulate within the secretory pathway of the cell. If the cells are returned to permissive conditions and *de novo* protein synthesis arrested, the proteins accumulated within the cytoplasmic secretory pathway will be translocated to the cell surface. In the case of high-affinity glucose transport, transporter accumulation could only be demonstrated in a *sec1* strain, but not in any of the other *sec* mutants tested; *SEC1* is required for a very late step in secretion, fusion of secretory vesicles to the plasma membrane.[199] These results suggest that if the secretory pathway is blocked before the site of action of *SEC1*, transporter protein falls to accumulate and is unstable. The stability of fully expressed high-affinity transport in *sec* mutants at the nonpermissive temperature was therefore examined.[22] Transporter activity decayed rapidly on

arrest of cells at the nonpermissive temperature. Thus, this cessation of cell growth or secretion or both promotes the turnover and elimination of glucose transporter activity in the plasma membrane.

Additionally, transporter activity is highest in rich medium as compared with minimal or synthetic complete media. This does not appear to be strictly a function of nitrogen availability, but of the differences in cellular growth rates under the different environmental conditions. Glucose transport is regulated by many cellular factors, assuring an adequate supply of both carbon and energy. Accelerating the rate of transporter turnover in response to environmental or physiological stimuli allows the cell to assess the situation and adapt metabolism accordingly. There is evidence that transporter activity is regulated transcriptionally as well as posttranslationally. Posttranslational regulation is a complex mixture of protein activation and inactivation, changes in protein turnover rates, and sequestration in subcellular compartments.

C. Genetic Analysis of Glucose Transport

The previous discussion clearly indicates that the biochemistry of glucose transport in *Saccharomyces* is both complex and complicated by the existence of multiple putative carriers of unknown function. Therefore, a genetic analysis of transport is necessary. Early attempts to isolate mutants in *Saccharomyces* specifically defective in glucose transport were unsuccessful. Lobo and Maitra[158,159,168] screened over 1000 mutants insensitive to 2-deoxy glucose, hoping to find mutants resistant because of loss of sugar-carrier activity. No such mutants were obtained. However, these screens did yield the original triple kinase mutants. Failure to obtain transporter mutants could mean that loss of carrier activity is a lethal event or that there is tremendous redundancy in genes encoding glucose carriers, which now appears to be the case.

When early kinetic studies suggested the existence of two carriers of differing affinity, a search for mutants defective in glucose utilization at low- but not high-glucose concentrations was undertaken, the rationale being that such mutants would be specifically defective in high-affinity transport.[23] Indeed, such mutants were readily obtained and were deficient in high-affinity uptake. On further testing, these mutations conferred a pleiotropic phenotype, resembling previously isolated mutations defective in derepression of glucose-repressible functions. A test of mutants known to be defective in glucose repression and derepression indicated that high-affinity transport is under glucose-repression control.[23]

At the same time, a similar mutant screen was being conducted for strains that failed to grow on raffinose, a trisaccharide.[192] Strains of *Saccharomyces* can use raffinose completely only if they express both melibiase and secreted invertase. The wild-type strain used for the mutant analysis was deficient in melibiase activity and could therefore only use the fructose moiety of raffinose. Metabolism of this substrate provided the cells with a continual, but low, concentration of fructose. Most of the mutants isolated were defective in invertase expression. One class, *snf3* (*snf* for *s*ucrose *n*onfermenting), appeared to express normal to near normal levels of invertase, but still failed to grow on raffinose.[47] These mutants were found to be defective in high-affinity glucose (and fructose) uptake.[28] Although some of the point mutations appeared to have pleiotropic defects, null mutations at the *snf3* locus seemed to be specifically defective in sugar uptake and growth on low-substrate concentrations.[28,193] These strains did not possess observable high-affinity glucose uptake. Subsequent analysis of this gene revealed that its phenotype was genetic background-specific, as null mutations of the *SNF3* gene generated in other wild-type strains did not affect glucose uptake appreciably,[23] reflecting the existence of natural suppressors or redundancy of *SNF3* function in some strains.

The defect in growth on low-glucose medium was used to identify suppressors of the *snf3* mutation. Several genes capable of suppressing the *snf3* mutation when present only in high copy number (multicopy suppressors) were identified (described later). Chromosomal mutations called *RGT* for *r*estoration of *g*lucose *t*ransport have also been obtained[170] that suppress the growth and

transport defects of loss of *SNF3* function. One of these mutations, *RGT2*, is dominant and maps 38 c*M* from *snf3* on chromosome IV, whereas the other, *rgt1*, is recessive.[170]

The genetic investigation of glucose transporters in *Saccharomyces* has proven fruitful. Numerous putative transporters have been identified by complementation or suppression of the *snf3* mutant phenotype. There appears to be tremendous redundancy of transporter function. This redundancy may underscore the importance of transport to cell growth and metabolism or may reflect the need to control transporter activity under a broad diversity of physiological conditions. Further genetic and biochemical analyses are clearly required to define the roles and mechanisms of regulation of transporter expression and activity.

IV. THE YEAST MULTIGENE FAMILY OF GLUCOSE TRANSPORTERS

Twelve genes encoding solute transport proteins have been cloned and characterized from ascomycete fungi that share significant sequence and secondary structure similarity. Furthermore, this high degree of similarity suggests that these proteins share functional and higher order structural characteristics and that they are, in fact, homologous, with a common ancestral origin.

The 12 genes to be discussed in this section are listed in Table 1. Eleven of these genes are from yeast (nine from bakers yeast, *Saccharomyces cerevisiae*, and two from *Kluyveromyces lactis*), one is from the bread mold *Neurospora crassa*. The proteins predicted to be encoded by these genes have a wide range of substrate specificities: eight of them are implicated in sugar transport (six in monosaccharide uptake, two in disaccharide uptake), two in sugar alcohol uptake, one in the transport of quinate (1,3,4,5-tetrahydroxycyclohexane carboxylic acid), and one in the transport of phosphate. It is important to note that all of these genes have been identified by genetic means; their roles as transporters are largely inferred from the growth and transport phenotypes of strains with mutations in these loci and from homology of their predicted protein products with mammalian and bacterial sugar transport proteins. To our knowledge, none of these fungal transporters have as yet been purified and assayed for transport function. With the cloned genes in hand, however, these biochemical tests will surely follow in the near future.

This section includes brief descriptions of each of the transporter genes and their predicted protein products. The synopses include the evidence

TABLE 1
Yeast Transporter Genes

Cloned gene	Source	Remarks	Ref.
SNF3	*Saccharomyces cerevisiae*	Complements high-affinity glucose transport defect of *snf3* mutant	47
HXT1	*S. cerevisiae*	Complements high-affinity glucose transport defect of *snf3* mutant in multicopy	157
HXT2	*S. cerevisiae*	Complements high-affinity glucose transport defect of *snf3* mutant	145
HXT4	*S. cerevisiae*	Complements high-affinity glucose transport defect of *snf3* mutant in multicopy	
GAL2	*S. cerevisiae*	Complements galactose uptake defect of *gal2* mutant	237
ITR1	*S. cerevisiae*	Complements inositol transport defect of *itr1* mutant	197
ITR2	*S. cerevisiae*	Complements inositol transport defect of *itr1* mutant in multicopy	197
MAL61	*S. cerevisiae*	Complements maltose transport defect	51
PHO84	*S. cerevisiae*	Complements the constitutive synthesis of P_i-repressible acid phosphatase and the defect in high-affinity phosphate transport of *pho84* mutants	34
RAG1	*Kluyveromyces lactis*	Complements the glucose fermentation deficiency of *rag1* mutants	105
LAC12	*K. lactis*	Complements lactose transport defect of *lac12* mutants and confers lactose transport ability to *S. cerevisiae*	48
qa-y	*Neurospora crassa*	Occurs in *qa* gene cluster, responsible for quinate metabolism; mutants are defective in growth on quinate	102

that was used to identify and clone each gene and information on the transport phenotypes and regulatory properties of the gene. The primary sequence and secondary structure of each protein and the similarities among the proteins with respect to these characteristics are discussed in subsequent sections.

A. *SNF3*

The *SNF3* gene was first recognized in a series of mutant strains that are unable to use raffinose as a carbon source.[192] Subsequent analysis revealed that these mutants are defective for growth on low glucose and fructose concentrations as well, particularly in the presence of an inhibitor of respiration, antimycin A, or under anaerobiosis. The *snf3* point mutations were originally thought to be defective in regulation of invertase, explaining the defect in growth on raffinose. Expression of secreted invertase, encoded by the *SUC2* gene, is repressed in cells growing on high-glucose medium and is derepressed when glucose availability is low or nil. Invertase activity is required for the hydrolysis of raffinose to yield fructose, which occurs external to the cell. Thus, invertase activity on raffinose provides the cells with a steady but low concentration of fructose. Melibiose, the other product of the hydrolysis of raffinose by invertase, also can be broken down to glucose and galactose externally by the enzyme melibiase. However, the initial work with the *SNF3* mutations was done in a melibiase-deficient *(mel)* background. Some of the point mutations appeared to affect invertase activity, either reducing expression or making expression constitutive.[192,193] This inconsistency of phenotype indicated that the primary *snf3* defect perhaps was not in invertase levels or expression. Also, invertase is not needed for growth on low concentrations of fructose or glucose, suggesting that the *SNF3* gene is more globally involved in sugar utilization at low-substrate concentrations.

Analysis of sugar transport kinetics in *snf3* point and null mutants revealed a defect in the activity of the high-affinity, low-K_m transporter.[28] Mutants principally displayed only low-affinity uptake, but occasionally a very low level of high-affinity uptake was observed. Expression of plasmid-borne *SNF3* in a *snf3* null mutant restores high-affinity glucose uptake.[28]

The *SNF3* gene was cloned by its ability to complement the defect in raffinose utilization of a *snf3* mutant.[47] The *SNF3* gene encodes a protein of 884 amino acids.[47,169,193] The protein expressed from a fusion gene, composed of *SNF3* (codons 1–797) and *lacZ* (codons 8–1024), was targeted to the plasma membrane as shown by cofractionation of β-galactosidase activity with a membrane marker and by indirect immunofluorescence microscopy.[47,169,193] The *SNF3* gene is transcribed to produce a 3-kb mRNA that is expressed approximately fivefold higher in low-glucose medium when compared with expression in high substrate.[193] Expression of β-galactosidase activity by fusion of the *SNF3* promotor with *lacZ* confirms both the low absolute level of *SNF3* expression and its glucose repressibility. Furthermore, the codon usage of the *SNF3* open reading frame suggests that the *SNF3* protein expression is low (Kruckeberg and Bisson, unpublished observations).

Analysis of the predicted protein sequence of *SNF3* (Figure 2) displays the classic features of a member of the glucose transporter family with one striking exception. The carboxyl-terminal tail of the *SNF3* protein is unusually long, approximately 303 amino acids in length. Glucose transporter proteins display carboxyl termini that are generally around 30 to 50 amino acids in length. The *SNF3* tail sequence reveals some interesting characteristics. There is a repeated sequence of unknown function (boxed) and numerous putative phosphorylation consensus sites for both casein-kinase II-like activity and for a cAMP-dependent protein kinase. The strongest casein kinase II-like consensus sites lie within the repeated region. Preliminary *in vitro* work in our laboratory suggests that the *SNF3* tail can serve as a substrate for protein kinases.

To further define the role of the carboxyl-terminal tail of the *SNF3* protein, a series of deletions were generated, indicated in Figure 2. The ability of these deletions to complement both the growth and transport defects of a *snf3* null mutation have been assessed. Loss of 192 amino acids from the carboxyl-terminal domain of *SNF3* (p*SNF3*Δ15) results in loss of complementation of the growth defect of a *snf3* null mutant on low-

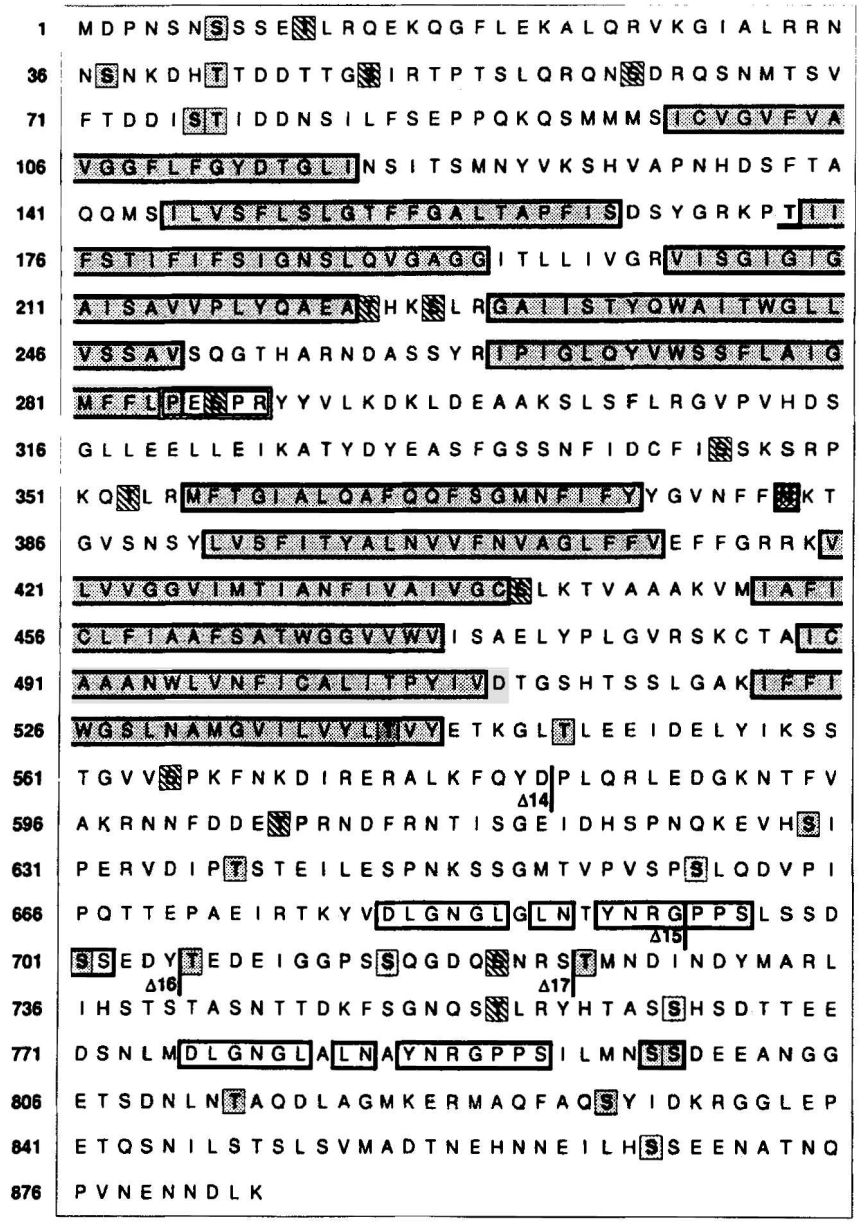

FIGURE 2. The predicted amino acid sequence of the *SNF3* protein. The following conventions are used to highlight regions of interest:

XXXXX - marks the putative membrane spanning domains.

S or T - represent casein kinase II consensus recognition sequences.

S or S - represent protein kinase C consensus recognition sequences.

T - marks the location of the cAMP-dependant kinase recognition site.

PESPR - shows the location of the conserved PESPR region.

- marks the consensus site for N-linked glycosylation.

XXXXX - outlines the amino acids involved in the 18 of 23 amino acid repeat.

XXX| - indicate the terminal residues of the noted deletion mutations.

substrate concentrations in the presence of antimycin A. A clone expressing a protein containing an additional 13 amino acids (p*SNF3*Δ16) retains the ability to complement fully; p*SNF3*Δ17 with an additional 18 amino acids beyond p*SNF3*Δ16 shows no greater degree of complementation and is indistinguishable from that due to full length *SNF3*. The p*SNF3*Δ15 deletion disrupts the second of the repeated amino acid sequences and narrowly defines the minimum sequence required for complementation. This deletion still retains 150 amino acids at the carboxyl terminus, in excess of the length of the carboxyl-terminal tails of the rest of the members of the glucose-transporter family. From analysis of the kinetics of uptake of this series of carboxyl-terminal tail truncation mutants, deletion beyond amino acid residue 213 results in a gradual decrease in high-affinity glucose uptake. Deletions Δ15 and Δ16 define the minimal region needed for *SNF3* protein function. The carboxyl-terminal tail is essential for *SNF3* function. The role of the tail within the transporter may not necessarily be catalytic, but could be involved in activation or stability of carrier activity.

A *SNF3-lacZ* protein fusion was constructed to determine site of localization of the *SNF3* protein and assess levels of expression.[47,169] The resulting fusion protein yielded β-galactosidase activity and complemented the growth defect imposed by loss of *SNF3* function. However, fusion protein activity and stability may not accurately reflect the activity and stability of native *SNF3*. To study *SNF3* promoter expression independently of *SNF3* protein, a fusion of the *SNF3* promoter to the *lacZ* gene was generated. β-Galactosidase activity in strains expressing this promoter fusion is very low, about 0.01 to 0.001% of the level of expression of β-galactosidase from a fully induced *GAL* promoter, depending on growth conditions. This low level of expression is inconsistent with a primary role as a catabolic sugar transporter (Coons and Bisson, unpublished observation). Reporter gene/promoter fusions give an indication of the level of expression of the native transporter gene but, obviously, reveal nothing about the actual protein levels of the transporter itself.

In analyzing multicopy suppressors of *SNF3*, we identified a gene called ORF2 (for *o*pen *r*eading *f*rame) capable of suppressing the *snf3* null mutation growth defect on low-glucose concentration. A search of the nucleic acid data base revealed that this gene had been previously sequenced and named *AHT1* (for *a*ctivator of *h*exose *t*ransport by M. Ciriacy, Institute for Microbiology at Dusseldorf). In our hands, *ORF2* is able to suppress the growth defect of the *snf3* null mutation but does not affect glucose uptake (Theodoris *et al.*, submitted). This gene is located between the putative hexose transporters *HXT1* and *HXT4* described in the following section (Figure 3). Subsequent analysis revealed that *ORF2* suppression is mediated by the DNA sequence and not the coding sequence. Deletion analysis demonstrated that the region suppressing *snf3* is a stretch of nucleotides within the coding region. This suppression occurs in an *HXT4* null background and is probably due to the titration of a transcription factor or factors the removal of which results in stimulation of growth on low-glucose concentration (Theodoris et al., submitted). When overexpressed, *HXT1* and *HXT4* restore growth on low-substrate concentration as well as high-affinity glucose uptake to *snf3* null mutants. In the search for multicopy suppressors of *snf3*, a class of plasmids was obtained that did not restore transport but did restore growth on low-substrate

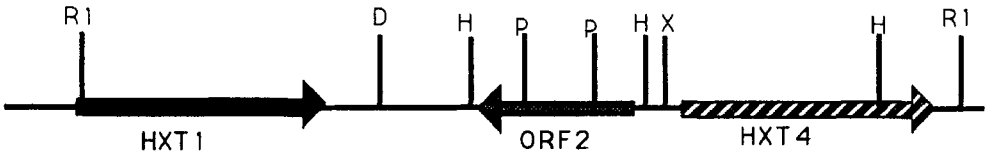

FIGURE 3. Restriction map of the *HXT1-ORF2-HXT4* region of *Saccharomyces cerevisiae*. Arrows denote direction of transcription. R1, EcoR1, D; Dra1; H, HindIII; P, Pst1; X, Xba.

concentrations.[28] These plasmids may define genes or regions of DNA similar to *ORF2*. We do not have any suppressors that seem to affect only transport and not growth, as these would not be detectable in the screens that have been used. It is also important to reemphasize the role of genetic background in expression of the *snf3* phenotype. Loss of *SNF3* seems to affect growth on low-substrate concentrations and high-affinity transport only in the S288C genetic background. In our experience, suppressors or modifiers of the *snf3* mutation arise frequently in the S288C background on continued cultivation, even under nonselective conditions, requiring repeated genetic characterization of these stains. This problem has also been reported in the literature.[169] The S288C may lack a natural functional redundancy of the *SNF3* activity present in other strains. Alternatively, these other strains may carry a cryptic suppressor of *SNF3* function. The ease with which strains carrying suppressors of *snf3* arise in mutant populations dramatizes the importance of this particular gene in glucose transport and metabolism.

B. The *HXT* Transporter Family

Three *HXT* genes were identified as yeast-genomic DNA fragments that are able to complement the defect in raffinose utilization of *snf3* mutants, when borne on multicopy plasmids.[28] The *HXT* transporter subfamily consists of proteins displaying high homology, between 60 to 85% identify. This family includes *HXT1*, *HXT2*, *HXT3* (R. Gaber, personal communication), *HXT4*, and *GAL2* all from *Saccharomyces* and the *RAG1* gene from *Kluyveromyces lactis*. This family contains transporters of differing substrate specificity and level of and timing of expression. *HXT1*, *HXT2*, *HXT4*, and *RAG1* are all able to complement the growth and transport defect of *snf3* mutations in *Saccharomyces*. *GAL2* is unable to do so. *HXT3*, recently identified in R. Gaber's laboratory, has not yet been examined in detail.

HXT1 encodes a protein of 569 amino acids.[157] Multicopy expression of *HXT1* in a *snf3* mutant strain restores glucose-repressible, high-affinity glucose transport. Under derepressing conditions, an *hxt1* null mutant has diminished high-affinity glucose transport when compared with a wild-type strain; under the same conditions, an isogenic *snf3* mutant has even less high-affinity glucose transport and the transport displayed by an *hxt1 snf3* double mutant is lower still. The *hxt1* mutation also affects high-affinity mannose (but not fructose) transport; the *snf3* mutation affects transport of all three of these sugars. Expression of *HXT1* was assessed by measuring the β-galactosidase activity produced by *lacZ* under control of the *HXT1* promoter. The level of β-galactosidase activity is low in stationary-phase cells and increases rapidly on transfer of cells to fresh medium. Levels decline after cells enter the logarithmic phase of growth and glucose begins to be depleted from the medium. The actual controlling nutrient appears to be nitrogen and not carbon. Thus, in terms of both substrate and pattern of expression, the *HXT1* gene product appears to play some specialized role in the cell.[157]

The HXT2 gene encodes a protein of 541 amino acids.[145] Multicopy expression of *HXT2* in a *snf3* mutant strain restores glucose-repressible, high-affinity glucose transport. Strains with a null *hxt2* allele display a partial defect in high-affinity glucose transport that, however, is not as pronounced as that in an isogenic *snf3* null mutant. The *hxt2 snf3* double mutant is severely defective in glucose transport.[145] No defect in fructose or mannose transport was detected in an *hxt2* mutant. Measurements of *HXT2* mRNA levels indicate that the gene is repressed by glucose and that it is expressed at moderately high levels on derepression.[145] This is confirmed by measurements of the β-galactosidase activity that is expressed by an *HXT2* promoter — *lacZ* fusion gene (Kruckeberg and Bisson, unpublished observations). The codon usage of the open reading frame of *HXT2* suggests that the protein can be expressed at moderate levels (Kruckeberg and Bisson, unpublished observations). Direct comparisons of *HXT2* and *SNF3* expression from promoter *lacZ* indicate that *HXT2* expression is significantly greater than that of *SNF3* (about 50-fold) when both are maximally expressed. It is remarkable, then, that *SNF3* has a stronger effect on glucose transport. It is possible that the primary substrate of *HXT2* has not been identified or

that its role is redundant and secondary to that of *SNF3*.

The *HXT4* gene encodes a protein of 576 amino acids. *HXT4* is tightly linked to *HXT1*; both occur on the same 4.5-kb fragment of yeast-genomic DNA (Figure 3). The *HXT4* gene is highly expressed as is *HXT2* (Theodoris *et al.*, unpublished observations). *HXT4* is able to restore growth on low concentrations of galactose to a *gal2* mutant (Theodoris *et al.*, to be reported). Overexpression of *HXT4* results in an increase in both high- and low-affinity uptake of glucose. *HXT4* is unique in being the only transporter identified so far that affects both glucose and galactose utilization.

The *GAL2* gene is required for galactose uptake in *Saccharomyces*. *S. cerevisiae* transports galactose via facilitated diffusion and has a low-affinity and a high-affinity transport system for this sugar.[55,71,215] Strains carrying the *gal2* mutation are defective in both high- and low-affinity galactose transport[55,71,215]; *gal2* null mutants display a Km for galactose uptake in excess of 200 m*M*[71] vs. 0.8 m*M* and 25 m*M* for high- and low-affinity *GAL2*-dependent transport,[215] respectively. Mutants lacking *GAL2* function accumulate galactose at a rate approximately 15-fold less than wild-type strains.[253] Galactose transport is inducible by galactose and repressible by glucose.

The *GAL2* gene was cloned by its ability to complement the defect in galactose use of a *gal2* mutant strain.[251] *GAL2* encodes a protein of 574 amino acids.[237] Galactose transport and expression of a *GAL2-lacZ* fusion gene (including the upstream regulatory region of *GAL2*) are both induced by growth of cells on galactose; galactose induction of the *GAL2-lacZ* fusion gene requires functional *GAL4* and *GAL80* proteins, demonstrating that *GAL2* expression is controlled by the same regulatory pathway as that of other *GAL* genes.[251] The β-galactosidase activity produced from the *GAL2-lacZ* fusion gene cofractionates with the yeast plasma membrane.[251] The *IMP1* gene, which also primarily affects galactose transport, has been shown recently to be allelic with *GAL2*.[71,253]

The *RAG1* gene of *K. lactis* was cloned by its ability to complement the *rag1* mutation,[105] a defect in growth on glucose in the presence of respiratory inhibitors.[104] *RAG1* encodes a protein of 567 amino acids.[105] It is able to complement the *snf3* mutation of *S. cerevisiae* when expressed in multicopy in *Saccharomyces* (P. Goffrini, personal communication). *RAG1* transcription is inducible by glucose[105]; *rag1* mutants are defective in low-affinity glucose uptake.[275]

C. *ITR1* and *ITR2*

Inositol (hexahydroxycyclohexane) is a sugar alcohol. It is an essential component of sphingolipids and phospholipids, including phosphatidylinositol. Inositol and its phosphorylated derivatives are involved in the regulation of phospholipid biosynthesis in yeast and in signal transduction pathways. *S. cerevisiae* is able to synthesize inositol from glucose-6-phosphate via inositol-1-phosphate. Inositol-1-phosphate synthase, the product of the *INO1* gene, participates in this pathway. Inositol biosynthesis is repressed, however, when the inositol level in the medium exceeds 50 μ*M*. Under these conditions, inositol transport from the medium supports normal growth.[277]

A strain defective in inositol transport was isolated.[196] This mutation, *itr1*, when combined with an *ino1* mutation, yields a strain deficient in both inositol uptake and inositol biosynthesis. This strain is unable to grow on low (11 μ*M*) inositol concentrations, but is able to grow on high (110 μ*M*) inositol concentrations. The inositol transport mutation was complemented by plasmids from a yeast-genomic library borne on multicopy plasmids. Two complementing genes, *ITR1* and *ITR2*, were recovered; the proteins encoded by them are similar in sequence (see the following).[197] Strains carrying only *ITR1* display higher specific inositol transport activity than do those carrying only *ITR2*; furthermore, *ITR1* is able to complement the transport mutation when carried on a single copy plasmid, whereas *ITR2* is not. An *itr1* null mutant displays less than 1% of wild-type inositol transport activity, whereas an *itr2* null mutant displays approximately 90% of wild-type activity. Inositol transport in an *itr1 itr2* double mutant could not be detected.[197] These data suggest that the two genes do not encode two subunits of a transporter complex. Rather, the genes probably encode separate transport systems,

with the system encoded by *ITR1* being the major one under the growth conditions used in the published studies.

ITR1 encodes a protein of 584 amino acids, and *ITR2* encodes a protein of 612 amino acids.[197] The apparent affinity for inositol of the two transporters is roughly equal (K_m = 100 μ*M* and 400 μ*M* for *ITR1*[196] and *ITR2*,[197] respectively). Inositol transport in wild-type yeast requires energy (glucose) and is inhibited by respiratory inhibitors.[196] Furthermore, transport in an *itr1* strain over expressing *ITR2* requires energy and is abolished by sodium azide.[197] Thus, inositol transport by both *ITR1* and *ITR2* is inferred to require an electrochemical gradient. The transport system in wild-type yeast is highly stereospecific for *myo*-inositol.[196]

Inositol transport activity is high in cells grown in medium containing glucose and low concentrations of inositol (≤11 μ*M*); activity is repressed approximately 50-fold when cells are cultured in high-inositol concentrations (110 μ*M*). On relieving cells of inositol repression, expression of the transport system requires *de novo* protein synthesis.[196]

ITR1 mRNA is much more abundant than *ITR2* mRNA. The level of *ITR1* mRNA is high in cells grown in glucose-containing medium and decreases in inositol- or choline-containing medium, whereas *ITR2* is expressed at comparable levels under these three conditions.[196,197] The pattern of *ITR1* gene expression is thus coordinate with that of phospholipid-synthesizing genes.

D. *MAL61* and *LAC12*

The yeast *S. cerevisiae* transports the disaccharide maltose into the cytoplasm, where it is hydrolyzed by maltase yielding two molecules of glucose.[229] Similarly, *K. lactis* is able to transport lactose; hydrolysis of this disaccharide within the cell by β-galactosidase yields glucose and galactose, which can be fermented.[260] Both transport systems are energy dependent and are inferred to involve H^+-cotransport.[229,236]

Maltose fermentation requires the presence of one of five polygenic loci, *MAL1*, *MAL2*, *MAL3*, *MAL4*, or *MAL6*.[11] Each locus encodes three genes; gene 2 of each locus encodes maltase, and gene 3 encodes a regulatory protein. In strains carrying only the *MAL6* locus, mutations in *MAL61* (i.e., gene 1 of the *MAL6* locus) abolish maltose transport.[50] Similarly, in a *MAL1* strain, *MAL11* is required for maltose transport.[51] The products of both genes mediate high-affinity (K_m = 4 m*K*) maltose transport; this activity is induced by maltose and is inactivated on shifting induced cells to glucose-containing medium.[51] The *MAL61* gene was cloned along with the other genes at the *MAL6* gene cluster; it encodes a protein of 614 amino acids[50] and is repressed by glucose and induced by maltose. The gene(s) responsible for constitutive low-affinity (K_m = 70 to 80 m*M*) maltose transport has not been identified. A recent report suggests that low-affinity maltose uptake is an artifact of trapping of this sugar in the yeast periplasmic space,[17] and a low-affinity transporter may not exist.

The activities required for lactose fermentation in *K. lactis* include lactose permease and β-galactosidase. *S. cerevisiae* is devoid of both of these activities. However, transformation of *S. cerevisiae* with a single 13-kb fragment of the *K. lactis* genome confers lactose-fermentation ability on the recipient strain.[236] This is due to the tight linkage of *LAC4*, encoding β-galactosidase, and *LAC12*, encoding lactose permease; both genes occur on this 13-kb *K. lactis* genomic fragment.[236] The lactose permease activity resulting from expression of *LAC12* in *S. cerevisiae* has similar kinetic properties to that of lactose transport in wild-type *K. lactis* cells: transport is saturable by lactose; the K_m in *S. cerevisiae* is about 1 m*M*[236] and about 0.7 to 2.8 m*M* in *K. lactis*.[69,236] In both species of yeast, lactose transport is strongly inhibited (90%) by the β-galactoside 3-*O* β-D-galactosyl-D-arabinose and is weakly inhibited by α-galactosides (1 to 10%) and by the monosaccharide galactose (25%).[236]

Interestingly, the lactose permease system in *K. lactis* is apparently able to transport galactose: mutations in the *LAC12* gene lead to defects in lactose and galactose transport. However, high residual galactose transport rates in *lac12* mutants suggest that *K. lactis* may have a separate galactose transport system.[216]

The *LAC12* gene was cloned from the 13-kb fragment described previously. The *LAC12* fragment is able to complement the lactose-transport defect of *K. lactis lac12* mutant strains and con-

fers lactose permease activity on *S. cerevisiae*.[216] These data argue strongly that lactose transport is solely due to the *LAC12* gene product. The *LAC12* DNA sequence contains an open reading frame, encoding a protein of 587 amino acids. A second open reading frame on the *LAC12* transcript has no role in lactose transport or in growth on lactose or galactose, as shown by disruption mutagenesis. The *K. lactis* lactose permease shows no sequence similarity to the *E. coli* lactose permease (encoded by *lac Y*).[48] Transport activity in wild-type *K. lactis* strains is induced 25-fold by lactose or galactose, and *de novo* protein synthesis is required for induction.[69] Furthermore, *LAC12* transcription is induced approximately 40-fold in cells grown in galactose.[216]

E. *PHO84*

The *PHO84* gene of *Saccharomyces* encodes a putative phosphate transporter related to the glucose transporter family. Inorganic phosphate is apparently taken up via two transport systems in *S. cerevisiae*.[241] One system has a low K_m (8 μM) and the other a high K_m (770 μM) for phosphate. Phosphate uptake is via symport with cations, sodium, potassium, magnesium, and ammonium and requires metabolism.[137,218,225,241] The low K_m system is repressed by high extracellular P_i levels, whereas the high K_m system is expressed constitutively. Repression of high-affinity uptake is governed by the same regulatory pathway as that controlling expression of repressible acid phosphatase, encoded by *PHO5*.[241]

Pho84 mutants display constitutive expression of repressible acid-phosphatase activity and are defective in phosphate transport.[34] The *PHO84* gene was cloned by its ability to complement the constitutive expression of repressible acid phosphatase; *pho84* mutants carrying *PHO84* on a single-copy plasmid or with the cloned gene integrated at the *pho84* locus only express acid phosphatase in low P_i medium. The cloned *PHO84* gene restores phosphate-repressible P_i-transport activity to a *pho84* strain.[34] Thus, *PHO84* appears to correspond to the low K_m phosphate transport system.

The *PHO84* gene encodes a protein of 596 amino acids. This protein shows no homology to the putative phosphate transporters of *N. crassa* or rat liver.[34] Null *pho84* mutants are viable and capable of growth in low-P_i medium.[34]

The *PHO4* protein is a transcriptional activator of the *PHO5* and *PHO8* genes; three *PHO4* binding sites (5′-CACGTG-3′) and a degenerate *PHO4* site (5′-CACGTT-3′) occur upstream of the *PHO84* TATA boxes. Northern blot analysis of *PHO84* expression showed that *PHO84* transcript is only detected in cells grown in low-P_i medium. *Pho4* mutants, as well as *pho2* and *pho81* mutants (which are both deficient in regulatory factors of the phosphate regulation pathway), do not produce *PHO84* transcript in high- or low-P_i medium, whereas a *pho80* mutant (which is defective in a negative effector of the phosphate regulation pathway) produced *PHO84* transcript constitutively. However, in the *pho84-1* point mutant, which is defective in phosphate transport, *pho84-1* transcript is detectable at moderate levels in low-P_i medium and at low levels in high-P_i medium.[34] Thus, *PHO84* is apparently regulated at the transcriptional level by the *PHO* regulatory pathway, but the role of *PHO84*-dependent phosphate transport in signaling the phosphate status of the medium is not clear.

Expression of *PHO84* in response to the extracellular P_i concentration was confirmed by measuring the β-galactosidase level expressed by a *PHO84-lacZ* fusion gene in cells growth on low- or high-phosphate media. β-Galactosidase activity was completely repressed in the high-P_i culture, but was derepressed in the low-P_i culture. The *PHO84* β-galactosidase fusion protein includes the amino-terminal half of *PHO84* (the first six transmembrane domains; see following); approximately 90% of the β-galactosidase activity expressed as this *PHO84*-β-galactosidase fusion protein in cells grown in low-P_i fractionated with total cellular membranes, suggesting the *PHO84* is targeted to the plasma membrane.[34]

F. *QA-Y*

Utilization of quinate as a carbon source by *N. crassa* is effected by the products of seven *qa* genes. These genes occur in an 18-kb gene cluster in the *N. crassa* genome and are coordinately regulated.[102] The *qa* genes are repressed by glu-

cose and induced by quinate; two of the *qa* genes are responsible for this regulation at the transcriptional level, and thus the *qa* gene cluster is autoregulated.[102] Quinate transport is inferred to be encoded by one of the *qa* genes because transport is dependent on *qa* regulatory gene function. Mutant *N. crassa* with the *qa-y* gene deleted are defective for growth on quinate and for *qa* gene regulation. The *qa-y* gene encodes a protein of 537 amino acids, with homology to the glucose transporter family.[102]

G. Summary

One intriguing aspect of these genes is that many of them occur in gene clusters. *HXT1* and *HXT4* are two tightly linked transporter genes, whereas *MAL11*, *MAL61*, *LAC12*, and *qa-y* are members of gene clusters that encode components of a single metabolic pathway. The gene clusters involved in maltose, lactose, and quinate metabolism are coordinately regulated and generally share common *cis*-acting transcriptional regulatory elements. The possible advantages of coordinate regulation of clustered genes in organisms with small genome sizes have been discussed.[102]

Another feature of these genes is their pattern of regulation. Some of them, including *SNF3*, *HXT2*, *ITR1*, and *PHO84*, are repressed by high extracellular levels of their substrates. This suggests that the cognate high-affinity transporters are responsible for scavenging their substrates from depleted media. Other genes, including *GAL2*, *MAL61*, *RAG1*, *LAC12*, and *qa-y*, are induced by their substrates. These transporters would then be involved in supplying their substrates to cellular metabolism under normal growth conditions when that substrate is plentiful. Most of the transporters of this type (*GAL2*, *MAL61*, and *qa-y*) are repressed when a preferred carbon source (glucose) is available.

The fungal transporters can be divided into two functional classes. Transport dependent on the *ITR1*, *ITR2*, *MAL61*, *LAC12*, and *PHO84* gene products requires metabolic energy, and the transporters accumulate substrate against a concentration gradient. In contrast, transport of glucose in *S. cerevisiae* (dependent on *SNF3* and possibly *HXT1*, *HXT2*, and *HXT4*) and probably in *K. lactis* (dependent on *RAG1*) and of galactose in *S. cerevisiae* (dependent on *GAL2*) occur by facilitated diffusion.

The proteins range in length from 537 to 614 amino acids, with the exception of *SNF3*, which is 884 amino acids long. *SNF3* is unique in possessing a large carboxyl-terminal tail of 303 amino acids on the cytoplasmic face of the membrane. At least 150 amino acid residues of this tail are required for *SNF3* function.

V. COMPARISON OF YEAST SUGAR TRANSPORTERS

A. Overall Similarity

Figure 4 displays the aligned sequences of the twelve transport proteins, with a consensus sequence for the alignment included as the bottom line in each row. Before examining the aligned sequence in some detail, it is worthwhile to get an overall view of the similarity among these proteins. This is provided in Figure 5, which presents a plot of the similarity of the aligned proteins over their region of higher similarity (residues 60 to 700 of Figure 4). Ten or so regions of the alignment manifest similarity that is significantly greater than the mean similarity for the alignment as a whole (–0.53). These regions are dispersed throughout the sequences, suggesting that any conservation of structure or function is global and not limited to one or a few domains.

For ease of inspection, four highly conserved regions have been extracted from the alignment of Figure 4 and are shown in Figure 6. In the entire alignment, ten residues are conserved among all of the protein sequences (Figure 4); four of these are in the regions shown in Figure 6. Among those residues that are not perfectly conserved, some interesting kinds of divergence are evident; at some positions, all transporters but one have the consensus residue (e.g., the aspartate of region I and the first glutamate of region IV are conserved among all proteins except the quinate transporter, which has tyrosine and valine at those positions). Many times, the alternate residues at a position are chemically conserved (e.g., the last residue of region I is always arginine or lysine), and the alternate residues are often confined to a

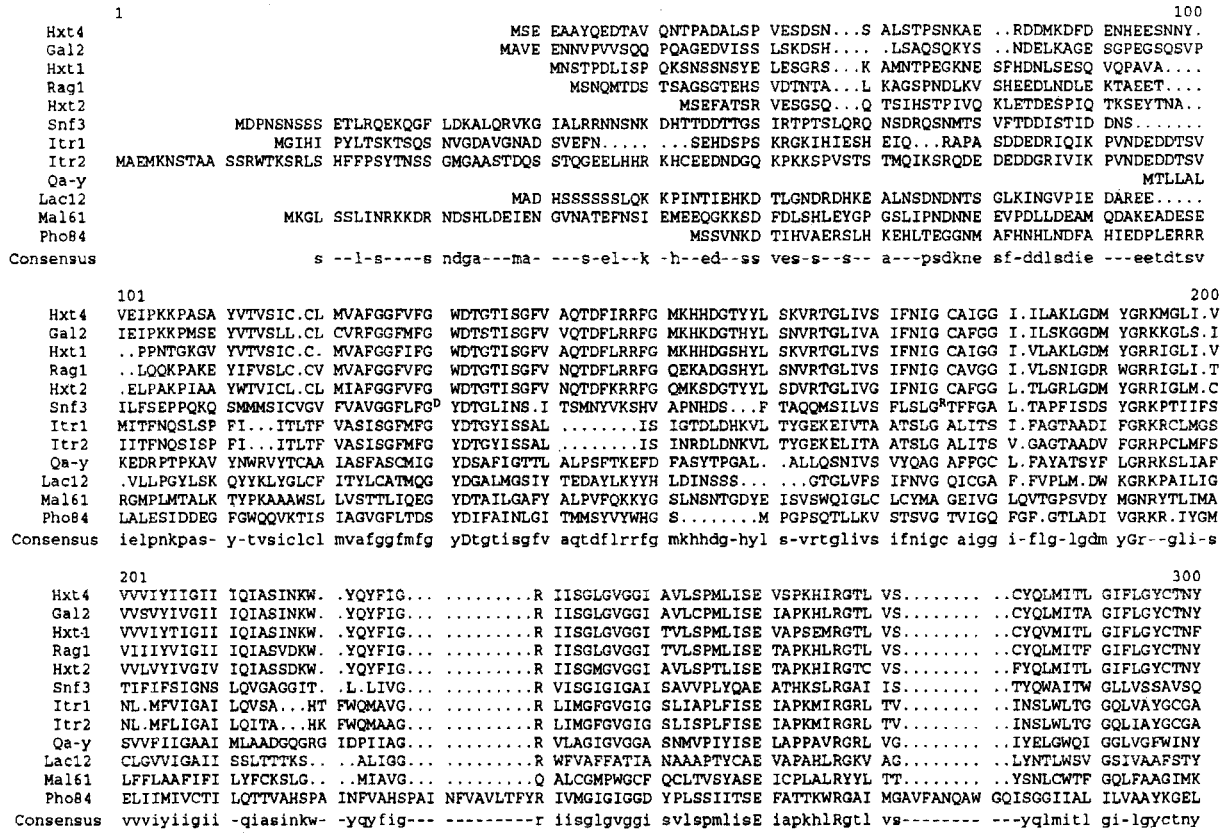

FIGURE 4. Part 1. The aligned sequences of the fungal transport proteins. The amino acid sequences of the transporters were deduced from the DNA sequences of the genes. The multiple sequence alignment was generated with the PILEUP program,[66] which uses a progressive alignment method based on that of Feng and Doolittle.[81] The gap weight was 3.0 and the gap length weight was 0.1. The consensus sequence was generated by the PRETTY program,[66] with a comparison threshold of 1.5 and a plurality of 2. The single letter amino acid code is used. Dots represent gaps. Dashes indicated that no consensus residue occurs at that position. Capitalized residues are those that are conserved among all transporters. Underlined residues in the Snf3 sequence have been mutated; the residue encoded at that position by the mutant allele is shown as a superscript.

transporter class (the conserved proline of region II is followed by glutamate-serine in all proteins except the two inositol transporters, which have aspartate-threonine). Sometimes, the alternate residue occurs in a protein that is a member of one of the transporter classes; for example, *RAG1* is one of the hexose transporters, yet it is unique among all twelve proteins in not having a second proline in region II. These examples typify the conservation and divergence among these proteins. It is reasonable to infer from this high degree of sequence conservation that the conserved residues play some structural or functional role in substrate translocation and that particular divergent residues are responsible for unique features of each transporter such as substrate specificity or proton cotransport.

Table 2A presents the percent similarity and identity between each pair of transporters. The highest levels of similarity and identity (87.2 and 80%, respectively) occur between the two inositol transporters, *ITR1* and *ITR2*. The levels of similarity and identity are high among the *HXT* family of transporters as well. Similarity ranges from 77.3% between *HXT1* and *HXT2* to 83.7% between *HXT4* and *GAL2* (the mean of pair-wise similarities among these five proteins is 80.9%). Identity ranges from 62.5% between *HXT1* and *HXT2* to 74% between *HXT1* and *HXT4* (the mean of pair-wise identities among these proteins is 68.1%). The relatedness of the *HXT* family to *SNF3* is considerably lower, averaging 56.1% similarity (range, 53.7–57.8%) and 30.7% identity (range, 29.4–31.3%). These values are not

```
          301                                                                                                  400
Hxt4      G..TKTYTNS VQ........ WRVPLGLGFA WALFMIGGMT FVPESPRYLV EVG...KIEE AKRSIALS.N .KVSADDPAV MAEVEVVQAT VE.AEKLAGN
Gal2      G..TKSYSNS VQ........ WRVPLGLCFA WSLFMIGALT LVPESPRYLC EVN...KVED AKRSIAKS.N .KVSPEDPAV QAELDLIMAG IE.AEKLAGN
Hxt1      G..TKNYSNS VQ........ WRVPLGLCFA WALFMIGGMM FVPESPRYLV EAG...RIDE ARASLAKV.N .KCPPDHPYI QYELETIEAS VE.EMRAAGT
Rag1      G..TKNYSNS VQ........ WRVPLGLCFA WAIFMVLGMM FVPESARFLV ETD...QIEE ARKSLAKT.N .KVSIDDPVV KYELLKIQSS IE.LEKAAGN
Hxt2      G..TKDYSNS VQ........ WRVPLGLNFA FAIFMIAGML MVPESPRFLV EKG...RYED AKRSLAKS.N .KVTIEDPSI VAEMDTIMAN VE.TERLAGN
Snf3      G..THARNDA SS........ YRIPIGLQYV WSSFLAIGMF FLPESPRYYV LKD...KLDE AAKSLSFL.R .GVPVHDSGL LEELVEIKAT YD.YEASFGS
Itr1      GLNYVNNG.. .......... WRILVGLSLI PTAVQFTCLC FLPDTPRYYV MKG...DLAR ATEVLKRS.Y ..TDTSEEII ERKVEELVTL NQSIPGKNVP
Itr2      GLNHVKNG.. .......... WRILVGLSLI PTVLQFSFFC FLPDTPRYYV MKG...DLKR AKMVLKRS.Y ..VNTEDEII DQKVEELSSL NQSIPGKNPI
Qa-y      GVNTTMAPTR SQ........ WLIPFAVQLI PAGLLFLGSF WIPESPRWLY ANG...KREE AMKVLCWI.R .NLEPTDRYI VQEVSFIDAD LERYTRQVGN
Lac12     GTNKNFPNSS KA........ FKIPLYLQMM FPGLVCIFGW LIPESPRWLV GVG...REEE AREFIIKY.H LNGDRTHPLL DMEMAEIIES FHGTDLSNPL
Mal61     NSQNKYANSE LG........ YKLPFALQWI WPLPLAVGIF LAPESPWWLV KKG...RIDQ ARRSLERILS GKGPEKELLV SMELDKIKTT IEKEQKMSDE
Pho84     EYANSGAECD ARCQKACDQM WRILIGLGTV LGLACLYFRL TIPESPRYQL DVNAKLELAA AAQEQDGEKK IHDTSDEDMA INGLERASTA VESLDNHPPK
Consensus glntknysns vq-------- wriplglqfa walfmiggmf fvPesprylv ekg-----ee A-rslaks-n -kv-pddp-i -aeleei-a- ves-eklagn

          401                                                                                                  500
Hxt4      ASWGEIF... STKTKVFQRL IMGAMIQSLQ QLTGDNYFFY YGTTVFTAVG L...EDSF.. .....ETSIV LGIVNF ASTF V.GIFLVERY GRRRCLLWGA
Gal2      ASWGELF... STKTKVFQRL LMGVFVQMFQ QLTGNNYFFY YGTVIFKSVG L...DDSF.. .....ETSIV LGVVNF ASTF F.SLWTVENL GRRKCLLLGA
Hxt1      ASWGELF... TGKPAMFQRT MMGIMIQSLQ QLTGDNYFFY YGTIVFQAVG L...SDSF.. .....ETSIV FGVVNF FSTC C.SLYTVDRF GRRNCLMWGA
Rag1      ASWGELI... TGKPSMFRRT LMGIMIQSLQ QLTGDNYFFY YGTTIFQSVG M...DDSF.. .....ETSIV LGIVNF ASTF F.ALYTVDHF GRRNCLLYGC
Hxt2      ASWGELF... SNKGAILPRV IMGIMIQSLQ QLTGNNYFFY YGTTIFNAVG M...KDSF.. .....QTSIV LGIVNF ASTF V.ALYTVDKF GRRKCLLGGS
Snf3      SNFIDCFISS KSRPKQTLRM FTGIALQAFQ QFSGINFIFY YGVNFFNKTG V...SNSY.. .....LVSFI TYAVNV[1]VFNV P.GLFFVEFF GRRKVLVVGG
Itr1      EKVWNTIKEL HTVPSNLRAL IIGCGLQAIQ QFTGWNSLMY FSGTIFETVG F...KNSS.. .....AVSII VSGTNF IFTL V.AFFSIDKI GRRTILLIGL
Itr2      TKFWNMVKEL HTVPSNFRAL IIGCGLQAIQ QFTGWNSLMY FSGTIFETVG F...KNSS.. .....AVSII VSGTNF VFTL I.AFFCIDKI GRRYILLIGL
Qa-y      GFWKPFL..S LKQRKVQWRF FLGGMLFFWQ NGSGINAINY YSPTVFRSIG ITGTDTGF.. .....LTTGI FGVVKM VLTI IWLLWLVDLV GRRRILFIGA
Lac12     EMLD..VRSL FRTRSDRYRA MLVILMAWFG QFSGNNVCSY YLPTMLRNVG MKSVSLNV.. .....LMNGV YSIVTW ISSI CGAFF.IDKI GRREGFLGSI
Mal61     GTYWDCV... ..KDGINRRR TRIACLCWIG QCSCGASLIG YSTYFYEKAG V.STDTAF.. .....TFSII QYCLGI AATF V.SWWASKYC GRFDLYAFGL
Pho84     ASFKDFCRHF GQWK..YGKI LLGTAGYWFT LDVAFYGLSL NSAVILQTIG YAGSKNVYKK LYDTAVGNLI LICAGS LPGY WVSVFTVDII GRKPIQLAGF
Consensus aswgelf--l stkp-vfrrl imgimlqs-q qltg-nyffy ygttif--vG ---t-dsf-- -----etsi- lgivnf astf v-alfttvdk- GRrkclliga

          501                                                                                                  600
Hxt4      ASMTACMVVF AS...VGVTR LWPNGKKNG. .SSKGAGNCM IVFTCFYLFC FATTWAPIPF VVNSETFPLR VKSKCMAIAQ ACNWIWGFLI GFFTPFI...
Gal2      ATMMACMVIY AS...VGVTR LYPHGKSQP. .SSKGAGNCM IVFTCFYIFC YATTWAPVAW VITAESFPLR VKSKCMALAS ASNWVWGFLI AFFTPFI...
Hxt1      VGMVCCYVVY AS...VGVTR LWPNGQDQP. .SSKGAGNCM IVFACFYIFC FATTWAPIAY VVISECFPLR VKSKCMSIAT AANWIWGFLI SFFTPFI...
Rag1      VGMVACYVVY AS...VGVTR LWPDGPDHPD ISSKGAGNCM IVFACFYIFC FATTWAPIAY VVISESYPLR VKGKAMAIAS ASNWIWGFLI GFFTPFI...
Hxt2      ASMAICFVIF ST...VGVTS LYPNGKDQP. .SSKAAGNVM IVFTCLFIFF FAISWAPIAY VIVAESYPLR VKNRAMAIAV GANWIWGFLI GFFTPFI...
Snf3      VIMTIANFIV AI...VGCS. .......... LKTVAAAKVM IAFICLFIAA FSATWGGVVW VISAELYPLG VRSKCTAICA AANWLVNFIC ALITPYIVDT
Itr1      PGMTMALVVC SIAFHFLGIK FDGAVAVVVS SGFSSWGIVI IVFIIVFAAF YALGIGTVPW Q.QSELFPQN VRGIGTSYAT ATNWAGSLVI A..STFLTML
Itr2      PGMTVALVIC AIAFHFLGIK FNGADAVVAS DGFSSWGIVI IVFIIVYAAF YALGIGTVPW Q.QSELFPQN VRGVGTSYAT ATNWAGSLVI A..STFLTML
Qa-y      AGGSLCMWFI GA...Y.IKI ADPGSNKAED AKLTSGGIAA IFFFYLWTAF YTPSWNGTPW VINSEMFDQN TRSLGQASAA ANNWFWNFII SRFTPQMFI.
Lac12     SGAALAL... ........TG LSICTARYEK TKKKSASNGA LVFIYLFGGI FSFAFTPMQS MYSTEVSTNL TRSKAQ.... ....LLNFVV SGVAQFVNQF
Mal61     AFQAIMFFII GGL....... ....GCSDTH GAKMGSGALL MVVAFF.... YNLGIAPVVF CLVSEMPSSR LRTKTIILAR NAYNVIQVVV TVLIMYQLNS
Pho84     IILTALFCVI GFAYH..... .......... ...KLGDHGL LALYVICQFP QNFGPNTTTF IVPGECFPTR YRSTAHGISA ASGKVGAIIA QTALGTLIDH
Consensus agmtac-v-- asafhvgvtr lwpng-dqp- -sskgagn-m ivficfyiff fattwapv-w v--sE-fplr vrsk-maia- aanwiwgfli afftpfit-l
```

FIGURE 4. Part 2

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          601                                                                                                  700
Hxt4      ...SG..AID FYYGYVFMGC LVFSYFYVF. .FFVPETKGL TLEEVNTLWE EGVLPW..KS PSWVPPNK.R GTDYNADDLM HDDQPFYKKM FGKK*
Gal2      ...TS..AIN FYYGYVFMGC LVAMFFYVF. .FFVPETKGL SLEEIQELWE EGVLPW..KS EGWIPSSR.R GNNYDLEDLQ HDDKPWYKAM LE*
Hxt1      ...TG..AIN FYYGYVFMGC MVFAYFYVF. .FFVPETKGL SLEEVNDMYA EGVLPW..KS ASWVPVSK.R GADYNADDLM HDDQPFYKSL FSRK*
Rag1      ...TS..AIH FYYGYVFMGC MVFAFFYVY. .FFVPETKGL TLEEVNEMYS EGVLPW..KS SSWVPSSR.R GAEYDVDALQ HDDKPWYKAM L*
Hxt2      ...TS..AIG FSYGYVFMGC LVFSFFYVF. .FFVCETKGL TLEEVNEMYV EGVKPW..KS GSWISKEK.R VSEE*
Snf3      GSHTS..SLG AKIFFIWGSL NAMGVIVVY. .LTVYETKGL TLEEIDELYI KSSTGV..VS PKFNKDIRER ALKFQYDPLQ RLEDGKNTFV AKRNNFDDET
Itr1      QNITP..AGT FAF...FAGL SCLSTIFCY. .FCYPELSGL ELEEVQTILK DGFNIK..AS KALAKKRKQQ VARV...HEL KYEPTQEIIE DI*
Itr2      QNITP..TGT FSF...FAGV ACLSTIFCY. .FCYPELSGL ELEEVQTILK DGFNIK..AS KALAKKRKQQ VAEGAAHHKL KFEPTQEIVE S*
Qa-y      .......KME YGVYFFFASL MLLSIVFIY. .FFLPVTKSI PLEAMDRLFE IKPVQN..AN KNLMAELNFD RNPEREESSS LDDKDRVTQT ENAV*
Lac12     ATPKAMKNIK YWFYVFYVFF DIFEFIVIY. .FFFVETKGR SLEELEVVFE APNPRK..AS VDQAFLAQVR ATLVQRNDVR VANAQNLKEQ EPLKSDADHV
Mal61     EKWNWGAKSG FFWGGPCLAT LAWAVVD... ...LPETAGR TFIEINELFR LGVPARKFKS TKVDPFAAAK AAAAEINVKD PKEDLETSVV DEGRSTPSVV
Pho84     NCARDGKPTN CWLPHVMEIF ALFMLLGIFT TLLIPETKRK TLEEINELYH DEIDP..... ATLNFRNKNN DIESSSPSQL QHEA*
Consensus qnitsgkai- fyygyvfmgc lvfsffyvy- -ffvpetkgl tleevnelye egvlpw--ks ksw-pkskqr gaey-addl- hd-kp-yk-m -erks--d-v

          701                                                                                                  800
Snf3      PRNDFRNTIS GEIDHSPNQK EVHSIPERVD IPTSTEILES PNKSSGMTVP VSPSLQDVPI PQTTEPAEIR TKYVDLGNGL GLNTYNRGPP SLSSDSSEDY
Lac12     EKLSEAESV*
Mal61     NK*

Snf3      TEDEIGGPSS QGDQSNRSTM NDINDYMARL IHSTSTASNT TDKFSGNQST LRYHTASSHS DTTEEDSNLM DLGNGLALNA YNRGPPSILM NSSDEEANGG
Snf3      ETSDNLNTAQ DLAGMKERMA QFAQSYIDKR GGLEPETQSN ILSTSLSVMA DTNEHNNEIL HSSEENATNQ PVNENNDLK*
```

FIGURE 4. Part 3

much greater than those between the *HXT* family and proteins with different substrates, for example, the quinate transporter and the inositol transporters (which have about 53% similarity and 28% identity with the *HXT* family). The disaccharide transporters do not appear to be highly related to one another: they display 44% similarity and 20.1% identity.

Table 2C lists the number of gaps introduced to optimize the pair-wise alignments. The same

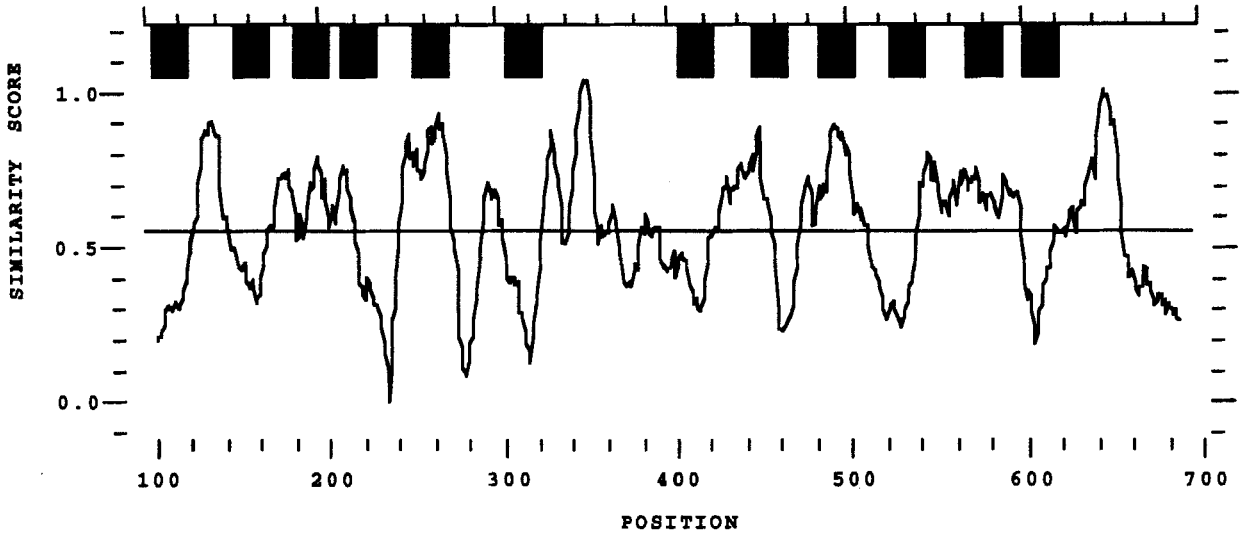

FIGURE 5. Similarity plot of the aligned fungal transporter sequences. The sequence similarity of the transporters was determined from position 60 to position 700 of the alignment shown in Figure 4. The program PLOTSIMILARITY[66] calculates the average value of pairwise residue comparisons at each position. Residue comparison values are 1.5 for identical residues, and lower values reflect greater evolutionary distance between residues. Values are based on the protein evolution data of Dayhoff et al.[65] The average similarity was determined within a sliding window of twelve positions. The mean of the similarity values for the whole region (~0.53) is plotted as a dotted horizontal line.

	I	II	III	IV
Consensus	dmyGrr k	Pesprylv	vdkfGRr i	vpetkgltleev
Hxt1	DMYGRR	PESPRYLV	VDRFGRR	VPETKGLSLEEV
Rag1	DRWGRR	PESARFLV	VDHFGRR	VPETKGLTLEEV
Hxt4	DMYGRK	PESPRYLV	VERYGRR	VPETKGLTLEEV
Gal2	DMYGRK	PESPRYLC	VENLGRR	VPETKGLSLEEI
Hxt2	DMYGRR	PESPRFLV	VDKFGRR	VCETKGLTLEEV
Snf3	DSYGRK	PESPRYYV	VEFFGRR	VYETKGLTLEEI
Qa-y	YFLGRR	PESPRWLY	VDLVGRR	LPVTKSIPLEAM
Itr1	DIFGRK	PDTPRYYV	IDKIGRR	YPELSGLELEEV
Itr2	DVFGRR	PDTPRYYV	IDKIGRR	YPELSGLELEEV
Lac12	DWKGRK	PESPRWLV	IDKIGRR	FVETKGRSLEEL
Mal61	DYMGNR	PESPWWLV	SKYCGRF	LPETAGRTFIEI
Pho84	DIVGRK	PESPRYQL	VDIIGRK	IPETKRKTLEEI

FIGURE 6. High sequence similarity among transporter sequence. Regions of the multiple sequence alignment of Figure 4 were excerpted as follows: region I: positions 195–200; region II: positions 348–355; region III: positions 498–504; region IV: positions 645–656.

Table 2A

	Hxt1	Rag1	Hxt4	Gal2	Hxt2	Snf3	Qa-y	Itr1	Itr2	Lac12	Mal61	Pho84
Hxt1		82.1	81.7	79.9	77.8	54.3	51.2	51.3	52.1	48.9	49.8	47.5
Rag1	71.1		81.1	81.9	78.7	55.9	52.7	52.7	56.0	50.1	49.2	49.0
Hxt4	73.2	68.3		83.7	81.6	56.4	53.7	52.4	50.9	50.7	47.7	48.8
Gal2	64.7	68.1	71.0		78.9	56.9	53.2	53.4	53.6	47.5	46.1	49.1
Hxt2	63.3	66.9	68.6	65.2		57.8	54.5	54.2	56.4	50.9	48.7	48.9
Snf3	28.9	30.7	31.3	31.0	31.1		54.6	51.1	53.0	47.9	49.5	49.5
Qa-y	26.9	27.1	27.5	27.9	28.5	28.4		49.8	52.5	53.0	48.1	50.6
Itr1	28.1	29.4	27.3	27.2	29.7	29.2	26.4		87.2	49.4	43.2	50.8
Itr2	27.7	30.9	27.6	26.2	29.7	28.0	28.5	80.0		49.4	43.6	50.9
Lac12	23.8	25.7	22.6	24.2	25.2	23.9	24.9	23.1	22.5		44.0	50.6
Mal61	22.2	21.5	21.3	21.4	21.6	21.6	25.0	20.9	22.6	20.1		47.7
Pho84	21.3	21.5	24.2	23.1	21.8	24.7	23.4	23.9	22.2	25.5	20.0	

(A)

Table 2B

	Hxt1	Rag1	Hxt4	Gal2	Hxt2	Snf3	Qa-y	Itr1	Itr2	Lac12	Mal61	Pho84
Hxt1		671	672	640	592	334	282	287	297	278	262	230
Rag1	171±5		651	641	605	352	282	297	300	273	263	234
Hxt4	171±5	173±5		681	615	351	300	305	306	276	265	230
Gal2	170±6	173±5	172±4		596	340	287	302	303	267	255	235
Hxt2	172±5	168±5	172±5	170±5		342	286	296	302	271	259	225
Snf3	186±5	191±5	189±5	191±4	184±4		286	305	308	271	268	236
Qa-y	168±6	167±5	168±4	168±5	165±4	184±6		269	270	278	226	222
Itr1	176±3	173±5	178±5	176±4	174±5	201±5	172±4		737	253	230	242
Itr2	178±7	177±5	178±4	178±6	177±6	205±4	170±5	188±6		255	236	239
Lac12	172±5	173±6	173±3	173±4	170±5	196±6	168±3	178±4	181±6		236	210
Mal61	172±4	176±6	177±5	176±4	170±3	201±4	171±7	182±5	182±6	179±6		209
Pho84	172±6	174±7	173±6	174±4	172±4	199±5	171±5	182±3	181±6	177±6	178±4	

(B)

TABLE 2
Similarity Metrics of the Fungal Transport Proteins

Each one of the 12 protein sequences was compared pairwise with all of the others by the GAP program,[66] which aligns pairs of sequences by the algorithm of Needleman and Wunsch.[190] The gap weight was 3 and the gap length weight was 0.1. **(A)** Above the diagonal, the percent similarity of each pair of transporters is given. Below the diagonal, the percent identity of each pair of transporters is given. **(B)** Above the diagonal, the quality of the alignment between each pair of transporters is given (quality is the value that is maximized by the alignment algorithm). Below the diagonal are shown the mean quality of alignments and the standard deviation of each mean from comparisons of one of the protein sequences to 20 randomized versions of the other protein sequence. **(C)** Number of gaps introduced into each alignment.

Table 2C

	Hxt1	Rag1	Hxt4	Gal2	Hxt2	Snf3	Qa-y	Itr1	Itr2	Lac12	Mal61	Pho84
Hxt1		2	3	2	2	12	11	16	15	16	19	18
Rag1			3	4	2	12	13	19	20	17	16	18
Hxt4				2	3	15	10	15	13	17	14	18
Gal2					1	16	11	20	15	18	15	19
Hxt2						11	12	17	17	17	13	19
Snf3							14	16	19	19	17	21
Qa-y								13	18	13	17	21
Itr1									3	20	18	19
Itr2										15	19	20
Lac12											16	29
Mal61												23
Pho84												

(C)

pattern that is evident in the relationships among these proteins from Table 2 emerges here, namely, that the *ITR* protein sequences are closely related, as are the *HXT* proteins; eight or fewer gaps are required to align the proteins in each set. All other pairs require ten or more gaps for optimal alignment.

B. Evolution

When the relative sequence similarities among the fungal proteins are portrayed graphically,[81] those proteins with similar substrates are seen to occur in clusters (Figure 7). The yeast inositol transporters are a closely related pair, and the disaccharide transporters, although not closely related, are more similar to one another than they are to any of the other proteins. The monosaccharide transporters form a single group of six related proteins, with *SNF3* less related than the five *HXT* family members. This dissimilarity between *SNF3* and the other monosaccharide transporters is not due to its unusually large size, as the plotted distance is calculated from the similarities of the aligned regions only. Instead, it reflects cumulative small differences between *SNF3* and the other five proteins throughout the aligned region (e.g., the replacement of tyrosine for leucine in region II of Figure 6). It is possible that this sequence

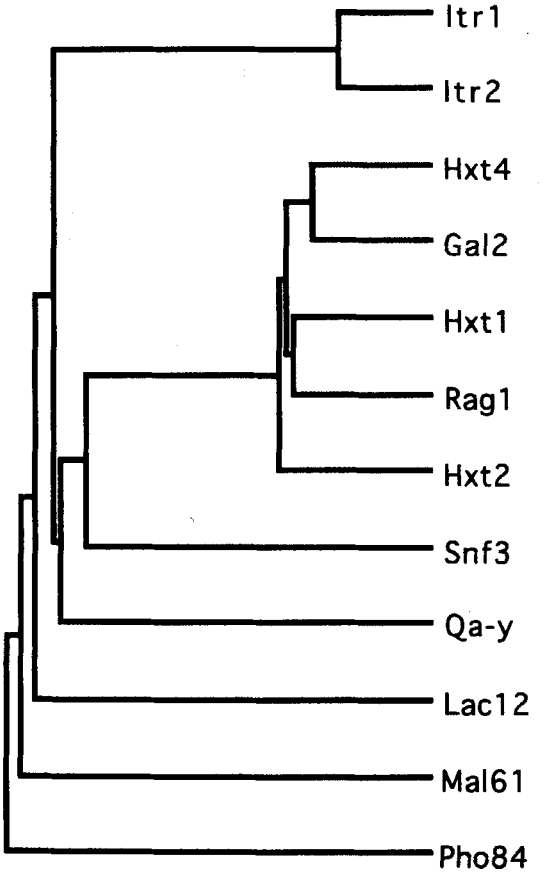

FIGURE 7. Dendrogram of similarity among the fungal transporters. Pairwise comparisons among the twelve proteins were made by the PILEUP program,[66] and the results of the comparisons ranked to determine the relative protein sequence similarities. Note that this plot is explicitly not an evolutionary tree.

divergence reflects differences in protein structure, regulation, or function. Alternatively, these may be biochemically silent differences. It is intriguing that the *HXT* subfamily of transporters is more closely related in sequence to the mammalian glucose transporters than is *SNF3* (data not shown; see the following).

The sequence conservation among these proteins suggests that they are evolutionarily related. An alternative hypothesis is that these highly hydrophobic, putative membrane proteins are similar because they are composed of an unusually high proportion of aromatic and aliphatic residues. These alternative hypotheses have been tested.[13] Determination of the pairwise alignments that generated the similarity and identity data of Table 2A involves maximization of the alignment quality.[190] The quality of each alignment is shown above the diagonal in Table 2B. For each alignment, one of the protein sequences was randomized 20 times, and the quality of the alignment of each randomized sequence vs. the unrandomized sequence of the other protein was determined. The mean quality of these 20 alignments and their standard deviations are shown below the diagonal in Table 2B. For each comparison, the quality of the alignment between the genuine sequences is many standard deviations higher than that of the random-sequence comparisons (ranging from 5.5 standard deviations above for the *LAC12-PHO84* comparison to 92 standard deviations above for the *ITR1-ITR2* comparison). These data strongly argue that these proteins are related by sequence similarity, not just by amino acid composition, and support a model of evolutionary relationship.

A family of sugar transporters from prokaryotes and other eukaryotes whose members share high sequence homology has been recognized.[9,107,117–119,232] It includes five glucose transporter isoforms from humans such as the transporter found in erythrocytes (GLUT1),[182] the transporter expressed in liver (GLUT2),[94] and the transporter expressed in insulin-responsive tissues such as muscle and adipose tissue (GLUT4);[93] cDNAs encoding functional homologs of these transporters have been cloned from a number of other mammals.[19,20,248] *Escherichia coli* possesses members of this family that transport xylose *(xylE),*[166] arabinose *(araE),*[166] and galactose *(galP).*[117] Genes or cDNAs encoding other homologous transporters have been cloned from lower and higher plants, cyanobacteria, and chicken.[222,223,278,279,287]

The sequence similarity of proteins in the yeast transporter family with this transporter superfamily is demonstrated in Figure 8. This figure presents an alignment of the same regions as those shown for the 12 fungal transporters in Figure 6; six *S. cerevisiae* transporters are aligned with six transporters from other organisms, including *E. coli, Arabidopsis thaliana,* and *Homo sapiens.* The degree of sequence conservation among these transporters from different phylogenetic kingdoms is comparable with that among the fungal proteins. For example, the four residues that are absolutely conserved among the fungal proteins are conserved here as well; surprisingly, this set of proteins contains two other absolutely conserved residues (a proline in region II and a glutamate in region IV; these residues are conserved among 11 out of the 12 fungal proteins). Again, many of the substitutions are highly conservative (the consensus glutamate and serine residues of region II, for instance, are only replaced with aspartate and threonine, respectively) or they occur only in one or a few proteins, which often have an atypical (i.e., nonmonosaccharide) substrate; for example, only the yeast inositol transporter has leucine replacing threonine in region IV.

By way of comparison with the values for homology among closely related fungal transport proteins presented in Table 2A, the human GLUT1 and GLUT4 isoforms are 81.5% similar and 65.4% identical (2 gaps). GLUT1 is 99.2% similar and 97.6% identical to its functional homolog from rat (no gaps).

The high degree of sequence similarity among these fungal transport proteins and between this family of proteins and a large family of eukaryotic and prokaryotic transport proteins strongly argues that the genes encoding the whole group are evolutionarily related and have descended from an ancestral transport protein. These transporters from bacteria, fungi, lower and higher plants, and vertebrates are considered to comprise a superfamily of homologous proteins.[117] Outside the

	I	II	III	IV
Consensus	d—Grk	PesPrylyv i	vd—GRr	vpEtkgltleei
Gal2	DMYGRK	PESPRYL.C	VENLGRR	VPETKGLSLEEI
Hxt2	DMYGRR	PESPRFL.V	VDKFGRR	VCETKGLTLEEV
Snf3	DSYGRK	PESPRYY.V	VEFFGRR	VYETKGLTLEEI
Itr1	DIFGRK	PDTPRY.YV	IDKIGRR	YPELSGLELEEV
Mal61	DYMGNR	PESPWWL.V	SKYCGRF	LPETAGRTFIEI
Pho84	DIVGRK	PESPRYQLD	VDIIGRK	IPETKRKTLEEI
Glut1	NRFGRR	PESPRFLLI	VERAGRR	VPETKGRTFDEI
Glut2	DTLGRI	PESPRYLYI	VEKAGRR	VPETKGKSFEEI
Glut4	QWLGRK	PESPRYLYI	VERAGRR	VPETRGRTFDQI
Stp1	RKFGRR	PDTPNSM.I	VDRWGRR	LPETKGIPIEEM
Galp	FKLGRK	PDSPRWF.A	VDRWGRK	VPETKHVSLEHI
Xyle	NRFGRR	PESPRWL.M	VDKFGRK	VPETKGKTLEEL

FIGURE 8. Sequence similarity among yeast transporter and those of other organisms, covering the same regions depicted in Figure 6.

fungi, the well-characterized members of this family are largely involved in monosaccharide transport.[118] The diversity of substrates among the fungal transporters is a puzzle. Presumably the genes encoding these proteins have evolved by gene duplication, followed by divergence in function of at least one of the gene products. This raises a number of interesting evolutionary questions: Have the fungi been particularly resourceful in their use of this transporter motif to solve a large number of substrate translocation problems? Do other classes of organisms have a greater array of this type of protein than the sugar transporters that have been found thus far? How many transporter genes of this type did the hypothetical ancestral fungus possess? What was the substrate specificity(s) of its product(s)? What is the evolutionary relationship of the genes encoding the yeast transporters?

The last question is amenable to some naive speculation with the data that is available. The *HXT* subfamily of monosaccharide transporters are all closely related; the obvious evolutionary affinity of *S. cerevisiae HXT1* and *K. lactis RAG1* for one another suggests that the gene encoding *HXT1* diverged from the ancestor of the other three *S. cerevisiae HXT* genes prior to the evolutionary divergence of *Saccharomyces* and *Kluyveromyces*. By the same argument, the *SNF3* gene probably diverged from the genes encoding the *HXT* proteins at an even earlier stage in the evolution of these fungi. A thorough understanding of the evolutionary history of these genes requires further studies, and will be aided by the identification of their products' true functional roles in the cell (Are the *HXT1*, *HXT2*, *HXT4*, and *SNF3* proteins functionally redundant? Have they been free to evolve without strict selective constraints?), the cloning of any remaining members of this subfamily from *S. cerevisiae*, and the characterization of this subfamily of genes from other, phylogenetically salient yeasts.

The recent evolutionary history of the other transporters might be more straightforward. The yeast inositol transporters are closely related in sequence and function, suggesting that they arose by gene duplication. This duplication could have been relatively recent or the gene sequences could have been maintained by strong selection pressures. The disaccharide transporters are most closely related to one another functionally and are clustered together when the 12 transporters are examined as a group (Figure 7). Their similarity,

however, is not nearly as high as that for the inositol or monosaccharide transporters. Perhaps disaccharide transport evolved early, and the divergence between *MAL61* of *S. cerevisiae* and *LAC12* of *K. lactis* has developed gradually over considerable evolutionary time (in contrast to the slight and possibly recent divergence between *HXT1* and *RAG1*. If so, then it is reasonable to speculate that other, intermediary disaccharide transporter genes (or pseudogenes) exist in these organisms.

The sequence divergence of the *S. cerevisiae PHO84* phosphate transporter is consistent with the dissimilarity of its substrate from those of the transporters already considered. Perhaps a protein with this function evolved a long time ago; if so, then it might be a common type of phosphate transporter (at least among fungi). Alternatively, the ancestor of the *PHO84* gene could have evolved rapidly if it was freed from evolutionary constraints on duplication. More intriguing is the quinate transporter: despite the chemical differences of its substrate and the evolutionary distance between *Neurospora* (order Pyrenomycetes) and the hemiascomycetous yeasts, this protein is more similar to the yeast monosaccharide transporters than are the yeast disaccharide and sugar alcohol transporters. Again, this could reflect either that considerable evolutionary time has passed since these three classes of sugar transporter genes diverged (perhaps predating the divergence of yeasts from other ascomycetes) or the rapidity at which they have evolved. A number of "missing link" transporter genes need to be characterized before this evolutionary history is clarified. No doubt, the transport of a number of other substrates will be shown to be mediated by proteins of this family.

C. Amino Acid Conservation and Function

The roles and functional importance of specific residues and regions in these proteins have not yet been studied in any detail. The value in recognizing the similarity among these proteins is that when a highly conserved residue or region is found to participate in a specific attribute of one protein (e.g., membrane localization or substrate affinity), then it is reasonable to predict that it will play a similar role in the homologous proteins. We are aware of studies on the functional consequences of mutations in the *SNF3* and GLUT1 genes; these will be described in the following discussion.

Three *snf3* missense mutations have been characterized.[169] The *snf3-142* allele causes substitution of aspartate for glycine at residue 112; the *snf3-72* allele produces a glycine → arginine change at 153; and the *snf3-39* allele produces a valine → isoleucine change at residue 402. The *snf3-142* and *snf3-72* alleles have strong phenotypes: growth on raffinose is abolished. In contrast, the *snf3-39* allele has a leaky phenotype: growth of a strain that has a null *snf3* allele at the chromosomal locus and that carries *snf3-39* on a low-copy plasmid is detectable after several days of incubation. The glycine codons that are mutated in *snf3-142* and *snf3-72* are conserved in 11 of the 12 fungal transporters (the exceptions being *PHO84* and *MAL61*, respectively). They are predicted to occur in the first and second transmembrane domains of *SNF3* (see the following). Indirect immunofluorescence microscopy indicated that *snf-142* and *snf-72* proteins fused to β–galactosidase are not localized in the plasma membrane, but were localized instead to intracellular cap-like structures *(snf3-142-lacZ)* or to discrete regions within the cells *(snf3-72-lacZ)*. These data suggest that a charged residue at either position interferes with proper targeting of this integral membrane protein.

The valine → isoleucine change of the *snf3-39* allele presents a different situation. The mutant protein is able to support cell growth on raffinose. Furthermore, a *snf-39-lacZ* fusion protein is localized in the plasma membrane. The valine-402 of *SNF3* is not at all conserved at this position in the alignment of Figure 4. The consensus residue at this site in the proteins is phenylalanine; *MAL61* has an isoleucine at this position. Residue 402 is predicted to occur in the eighth transmembrane domain. This is one of the domains that could form an amphipathic α-helix; such helices are hypothesized to create a hydrophilic channel for glucose translocation through the membrane (see the following).

An analogous study has been performed with the cDNA encoding the human erythrocyte glucose transporter (GLUT1).[202] A deletion mutant lacking 37 of the 42 carboxyl-terminal codons was constructed. When expressed in Chinese hamster ovary cells, the truncated protein was correctly localized in the membrane but was defective in 2-deoxy-D-glucose transport. The pattern of binding of a number of transport inhibitors demonstrated that, in this mutant, the glucose binding site on the extracellular face of the membrane is inaccessible to substrate. This suggests that the carboxyl-terminal portion of the protein is necessary for alternation between inward-facing and outward-facing conformations of the transporter.

The human erythrocyte glucose transporter (GLUT1) is reversibly inhibited by cytochalasin B.[29,243] The cytochalasin-B binding site is thought to be at or near the inward-facing glucose binding site of the protein,[67] and cytochalasin B is cross-linkable to a tryptophan residue.[37,68,207] This residue may be *Trp-363*, *Trp-388*, or *Trp-412*. Mutation of *Trp-412* to leucine reduces the intrinsic activity (turnover number/K_m) of GLUT1 to 15% of wild type, but does not abolish cytochalasin-B binding.[138] *Trp-412* of GLUT1 corresponds with position 595 of Figure 4, at which all fungal transporters except *LAC12*, *MAL61*, and *PHO84* also have a tryptophan. The conformation in the domain containing *Trp-388* is considered "dynamic" and, hence, is a candidate for the cytochalasin-B binding region; it may correspond to the gate of an aqueous glucose channel on the cytoplasmic face of the membrane.[232] GLUT1 *Trp-388* corresponds to position 571 in Figure 4, at which five of the fungal transporters have a tryptophan. Cytochalasin-B binding has not been demonstrated for any of these fungal proteins. Cytochalasin B does not inhibit glucose uptake in *Saccharomyces*, suggesting that yeast transporters are simply insensitive to cytochalasin B, are incapable of binding to this compound, or that any cytochalasin-B binding site is inaccessible to this compound *in vivo*.

Mutation of GLUT1 *Asn-415* to aspartate markedly decreases the intrinsic activity of the transporter; the mutant protein also displays reduced cytochalasin-B binding but normal levels of ethylidene-glucose binding (ethylidene glucose binds only to the external glucose-binding site of GLUT1).[128] Asparagine is conserved among all mammalian glucose transporters at this position. Among the fungal proteins, however, it is only found in *SNF3*, the quinate transporter, and *LAC12* at this position; *MAL61* has glutamine. These biochemical studies on GLUT1 suggest the utility of mutagenesis in associating specific residues with specific functions. It is anticipated that similar studies on the fungal transporters, taking advantage of the powerful genetics of these organisms, will be forthcoming.

D. Oligomerization

A heptad repeat of leucine residues ("leucine zipper") in mammalian sugar transporters has been observed to occur in a conserved location in these proteins.[280] These motifs have been shown to mediate protein-protein interactions in other systems by formation of a coiled-coil structure.[1,151] A leucine zipper motif is found in many of the fungal transporters as well, conserved in position with that of the vertebrate proteins.[145] The sequence in this region is shown in Figure 9. The leucine zipper motif is rather degenerate in some of the proteins and is absent from *MAL61*. In the *HXT* family and the inositol transporters, however, it is quite similar to functional leucine zippers from other proteins. It is not known if this motif has any function in these transporters, but its high degree of conservation, both among the fungal proteins and among eukaryotic transporters in general, is suggestive. It is noteworthy that homology among the proteins is not otherwise high in this region (Figure 5).

Radiation inactivation,[62,131] freeze-fracture electron microscopy (cited in reference 209), size exclusion chromatography,[115] and sucrose gradient ultracentrifugation[115] of the human erythrocyte glucose transporter suggest that it occurs as a homodimer and/or a homotetramer. When hybrid proteins consisting of the amino-terminal portion of GLUT1 and the carboxyl-terminal portion of GLUT4 are immunoprecipitated by anti-GLUT4 carboxyl-terminus antibodies, full-length (endogenous) GLUT1 coprecipitates.[209] This dem-

Hxt1	Leu-Xaa$_6$-Leu-Xaa$_6$-Ile-Xaa$_6$-Ile
Rag1	Leu-Xaa$_6$-Leu-Xaa$_6$-Ile-Xaa$_6$-Ile
Hxt4	Leu-Xaa$_6$-Leu-Xaa$_6$-Ile-Xaa$_6$-Ile
Gal2	Leu-Xaa$_6$-Leu-Xaa$_6$-Ile-Xaa$_6$-Ile
Hxt2	Leu-Xaa$_6$-Leu-Xaa$_6$-Ile-Xaa$_6$-Ile
Snf3	Phe-Xaa$_6$-Ile-Xaa$_6$-Leu-Xaa$_6$-Leu
Qa-y	Leu-Xaa$_6$-Ile-Xaa$_6$-Gly-Xaa$_6$-Phe
Itr1	Leu-Xaa$_6$-Ile-Xaa$_6$-Leu-Xaa$_6$-Ile
Itr2	Leu-Xaa$_6$-Leu-Xaa$_6$-Leu-Xaa$_6$-Val
Lac12	Leu-Xaa$_6$-Val-Xaa$_6$-Phe-Xaa$_6$-Trp
Mal61	(NONE)
Pho84	Met-Xaa$_6$-Leu-Xaa$_6$-Val-Xaa$_6$-Phe

FIGURE 9. *"Leucine zipper" region of the fungal transporters.* The region shown corresponds with positions 166 to 187 (qa-y: 165 to 186; LAC12: 173 to 196) of Figure 4. The three-letter amino acid code is used.

onstrates that the amino-terminal region of GLUT1 is involved in strong homomeric interprotein interactions. Furthermore, interactions between GLUT1 and the Band 3 anion-exchange protein (an integral membrane protein)[130] as well as hexokinase[188] and glyceraldehyde-3-phosphate dehydrogenase[148] have been proposed. On the other hand, GLUT1 transporters reconstituted at low-dilution levels into lipid vesicles may function as monomers, unassociated with cytosolic proteins.[182] The question of whether GLUT1 or other transport proteins form homomeric or heteromeric structures *in situ* is thus not resolved, and the role of the leucine zipper motif in mediating any oligomer formation remains a hypothesis.

E. Glycosylation

Many secreted and integral membrane proteins of yeast are glycosylated on asparagine residues. This modification occurs posttranslationally in the endoplasmic reticulum (ER).[224] The domains of integral membrane proteins that will ultimately be on the extracellular face of the membrane are the only portions of the proteins that face the ER lumen and, thus, are the only substrates for asparaginyl *(N-)*glycosylation.[224] The consensus site for *N*-glycosylation is Asn-Xaa-Ser/Thr[146,242]; in yeast, threonine appears to be the preferred residue at the third position.

The GLUT1 human erythrocyte glucose transporter is extensively glycosylated on a single asparagine residue at the site Asn$_{45}$-Gln-Thr.[232] This site is predicted to occur between transmembrane domains 1 and 2 on the extracellular face of the membrane.[183] All of the other mammalian glucose transporters have an *N*-glycosylation site in the same topological region, although the sequence of the site may vary. Glycosylation of GLUT1 appears to be required for normal transport activity both *in vivo* and on reconstitution of the transporter into proteoliposomes.[83,84,113,114,165] The glycosylation state of other GLUT proteins has been assessed,[31] and GLUT3, GLUT4, and GLUT5 also appear to be glycosylated.

Two yeast glucose transporters, *HXT2*[145] and *RAG1*,[105] have an *N*-glycosylation site identical to

that of GLUT1 at the same topological region of the protein (position 147 in Figure 4, see the following). Interestingly, other members of the *HXT* family have sequences in this region that differ only by substitution at the asparagine (i.e., Xaa-Gln-Thr). The *SNF3* protein has an *N*-glycosylation site at residue 383 (position 458 in Figure 4) between transmembrane domains 7 and 8. This position would be topologically "homologous" to the sites previously described if the transporter genes arose by internal duplication[166]; in any case, the *SNF3* site may also lie on the extracellular face of the membrane. The role of *N*-glycosylation in yeast sugar transport has been tested by studying the effect of the glycosylation inhibitor tunicamycin on the expression of sugar transport activity,[27,149] but the results are equivocal. Biochemical means of determining the glycosylation state of individual transport proteins will be valuable in clarifying the issue.

Some of the other transporters (*GAL2*,[237] *HXT1*,[157] and see *ITR1* and *ITR2*, position 105 in Figure 4) also have *N*-glycosylation sites, but these are not predicted to occur on the extracellular face of the membrane and, thus, would not be exposed to the enzymes involved in *N*-glycosylation. Whether the sites are *N*-glycosylated or not will require further study.

F. Phosphorylation

A common theme of this review is that heuristic inferences drawn from studies on fungal transport and transporters can be buttressed or weakened by drawing analogies from the extensive investigations on mammalian glucose transport. Applying this approach to the occurrence and role of phosphorylation of the fungal transporter family will be tantalizing at best, given the data that are currently available.

Sugar transport systems in yeast undergo catabolite inactivation; in other words, when cells are shifted from derepressing to repressing conditions, transporter activity is rapidly lost, presumably due to proteolysis.[36] Catabolite inactivation of glucose and galactose transport is dependent on cyclic AMP-dependent protein kinase (cAPK) activity.[214] Furthermore, proteolytic degradation of some of these proteins has been hypothesized to be accelerated by phosphorylation of weak PEST regions (regions enriched for proline, aspartate, glutamate, serine, and threonine residues). Phosphorylation would convert these to strong PEST regions (strong PEST regions are a feature of many proteins with short half-lives (see reference[237] and references therein). A number of yeast sugar transporters contain consensus sites for phosphorylation by cAPK and by casein kinase II. Whether these fungal transporters are protein-kinase substrates and whether phosphorylation affects their stability or activity have not been determined.

Phosphorylation has been implicated in regulating glucose transport in insulin-responsive mammalian tissues. Insulin plays a key role in maintaining mammalian blood-glucose homeostasis by stimulating glucose transport across the membrane of target cells (e.g., skeletal muscle, adipose tissue).[233] The mechanism of insulin action is complex and manifold. Briefly, stimulation of the insulin receptor recruits the GLUT4 glucose transporter to the plasma membrane from an intracellular vesicle pool; inhibition of adenylate cyclase and stimulation of protein phosphatase activity have been implicated in this process.[233] The rat insulin-responsive glucose transporter, homologous to GLUT4, is phosphorylated near its carboxyl terminus by cAPK *in vitro* and in response to the β-adrenergic agonist isoproterenol *in vivo*.[153] Phosphorylation appears to inhibit insulin-stimulated glucose transport, perhaps because of internalization of the transporter.[154] However, the primacy of this phenomenon in regulating glucose transporter abundance in the plasma membrane in response to insulin has not been demonstrated. Phosphorylation has not been implicated in regulation of the other mammalian glucose transporters.

G. Secondary Structure

The secondary structures of the 12 transport proteins have been predicted from their amino acid sequences. The hydrophobicity profiles of these proteins reveal a common pattern, as shown in Figure 10. Each protein has two sets of about

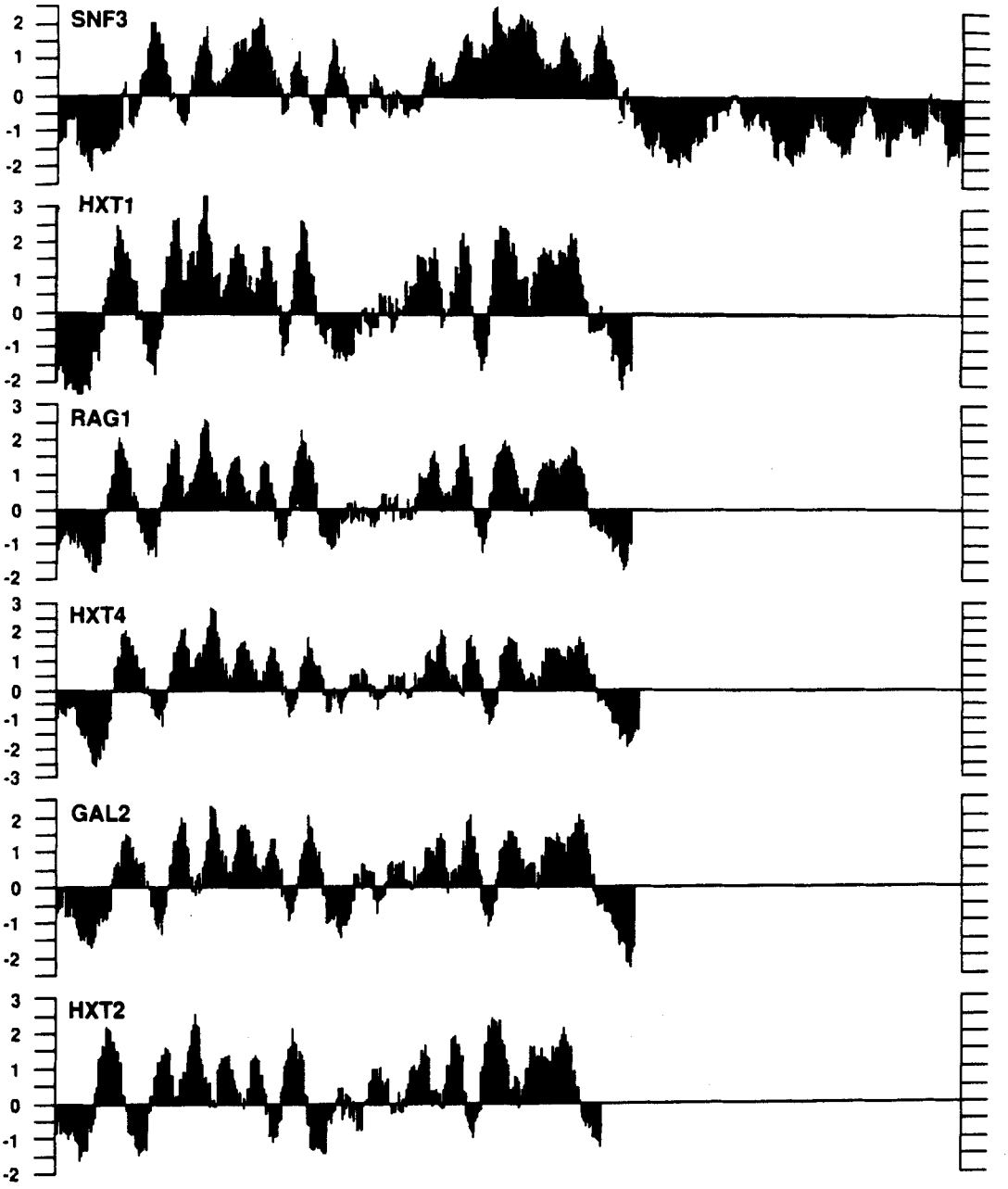

FIGURE 10. Part 1. Hydrophobicity plots of the fungal transporters. The Kyte-Doolittle[147] hydropathy index was calculated for a window of 20 residues at each position in the protein sequences, and these values were plotted vs. residue number. Values above the abscissa indicate hydrophobic regions, and values below the abscissa indicate hydrophilic regions.

six highly hydrophobic segments. Each hydrophobic segment is approximately 20 residues in length; these hydrophobic segments are separated by hydrophilic segments of varying lengths. The sets of six hydrophobic segments are separated by a long hydrophilic region of approximately 60 to 100 residues. The amino-carboxyl termini are also hydrophilic.

The role of transmembrane solute transport constrains these proteins to reside in the mem-

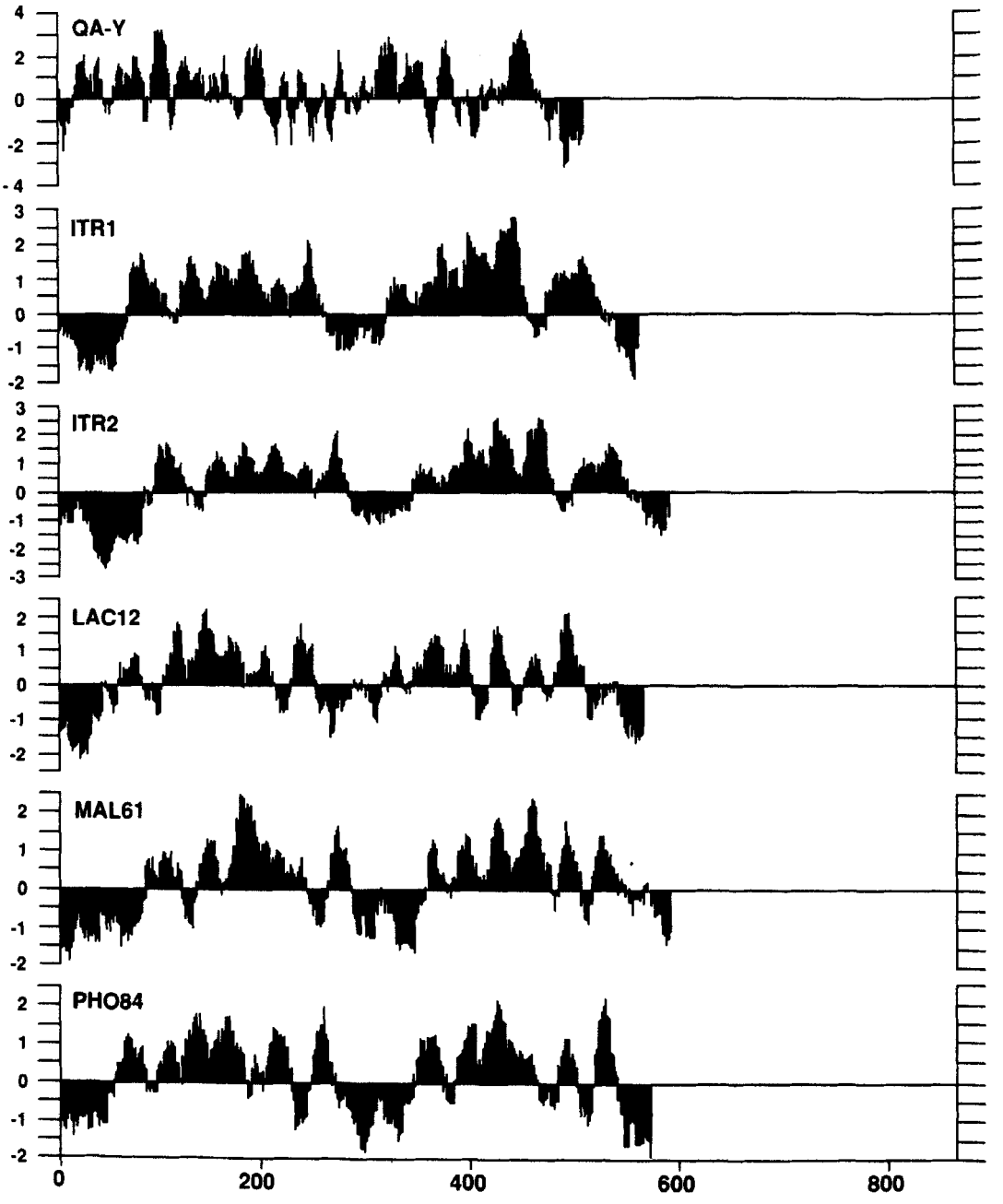

FIGURE 10. Part 2

brane, and it is proposed that the hydrophobic segments are membrane-spanning α-helices, or "transmembrane domains." The hydrophilic segments between the transmembrane domains are thus interdomain loops. The disposition of the transmembrane domains of the human erythrocyte glucose transporter has been characterized in detail by immunological, protease accessibility, and chemical derivatization studies,[37–39,63,64] and it has been shown that its amino and carboxyl termini are on the cytoplasmic side of the membrane. A cartoon of the two-dimensional topology of one of the fungal transport proteins within the membranes, based on the erythrocyte glucose transporter model, is shown in Figure 11.

To date, four of the yeast glucose transporters have been localized to the plasma membrane:

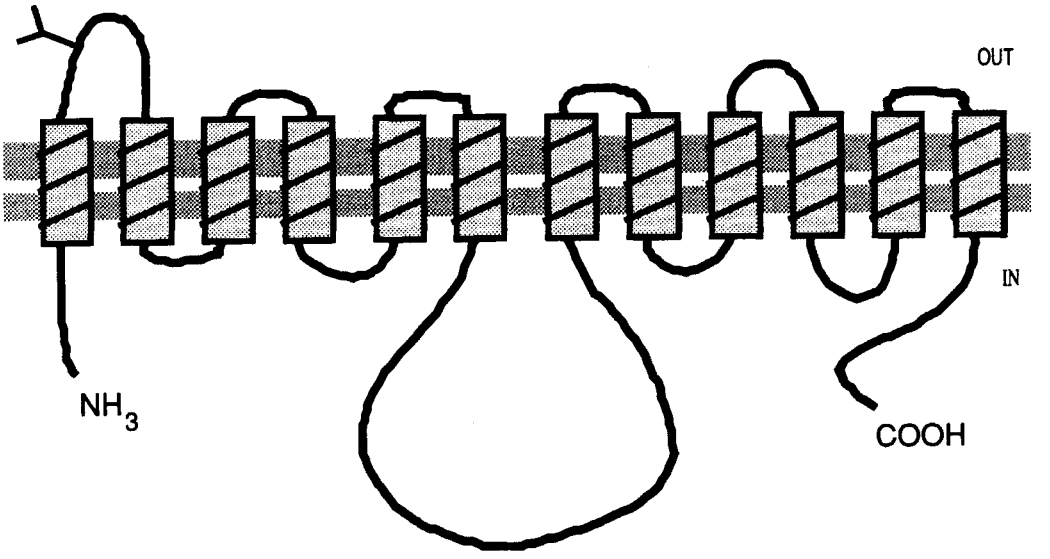

FIGURE 11. Two-dimensional model of the *HXT2* transporter in the plasma membrane, based on the model of Mueckler *et al.*[183] Twelve transmembrane domains are shown as helices. The putative glycosylation site between transmembrane domains I and II is indicated.

SNF3[47] (fused to β-galactosidase), *HXT1* (Lewis and Bisson, unpublished observations), *HXT2* (Wendell and Bisson, unpublished observations), and *GAL2*[251] (fused to β-galactosidase). This confirms that these highly hydrophobic proteins are membrane-associated proteins. The model shown in Figure 11 remains speculative, however. As pointed out by Lodish,[160] a protein with multiple alpha-helical segments that are oriented perpendicular to the plane of the membrane need not expose all of them to the lipid bilayer; some of the transmembrane domains could be in contact solely with other segments of the protein. In this context, it should be noted that it is difficult to rationalize 12 transmembrane domains in three of the fungal transporters, based on their Kyte-Doolittle[147] hydrophobicity profiles: the region of hydrophobic domain 7 of *HXT1* and *RAG1* and domain 11 of the *Neurospora* quinate transporter are not hydrophobic over a sufficient length to qualify as a membrane-spanning domain.

The algorithms that predict secondary structure are based on correlations of the primary sequence and secondary structure (as determined by X-ray crystallography) of soluble proteins and, therefore, should be applied with skepticism to these hydrophobic proteins. For example, a number of the transmembrane domains of the fungal transport proteins, even those that are predicted to be α helical, contain proline and glycine residues. Helix-breaking proline residues are relatively abundant in the transmembrane domains of many transport proteins. These residues are proposed to play a role in substrate translocation,[30] either by permitting domain motion via *cis-trans* isomerization of peptide bonds on the carboxyl-terminal side of this amino acid or by the reduced electronegativity of the peptide carbonyl in Xaa-Pro bonds. The actual structure of the fungal transport proteins and the dynamics of substrate translocation will require biophysical analysis.

The secondary structure of the human erythrocyte glucose transporter has been determined by circular dichroism and infrared spectroscopy.[52,53] The protein is predominantly α helical, but proteolytic digestion of membrane-inserted protein (which cleaves away hydrophilic domains) increases the proportion of β-sheet structure. These data suggest that the models of these proteins that postulate 12 membrane-spanning α helices may be simplistic.

H. Tertiary Structure and Substrate Translocation

Speculations on the tertiary structure of these proteins are limited by the inadequacy of our knowledge about their secondary structure. By

virtue of their ability to translocate hydrophilic substrates across phospholipid bilayers, however, the occurrence of a hydrophilic channel through the proteins is widely accepted. Five of the putative transmembrane α helices of GLUT1 would be amphipathic, and it has been suggested that these (particularly domains 7, 8, and 11) line this hydrophilic channel and mediate substrate translocation.[183] Similar amphipathic α helices are found in the putative transmembrane domains of many of the fungal transporters. Other domains in some of these proteins contain many hydrophilic residues, but are isotropic; these domains could line a hydrophilic channel on one face and be involved in protein-protein interactions (as well as protein-lipid interactions) on the other.

VI. THE MULTIGENE SUGAR KINASE FAMILY OF *SACCHAROMYCES*

No review of the sugar transporters of *Saccharomyces* and their role in sugar transport would be complete without simultaneous discussion of the sugar kinase family. *Saccharomyces* possess three sugar kinases, hexokinase PI, PII, and glucokinase. The kinetic differences of these proteins have been described previously. The presence of any one of the three kinases is sufficient for growth on glucose[60,97,158,159,168] and of either of the two hexokinases for growth on fructose.[60,97,158,159,168] The respective physiological roles of these kinases are largely obscure. Mammalian cells also possess a multigene family of sugar kinases, hexokinases I, II, III, and glucokinase.[181] These enzymes can be distinguished on the basis of kinetic parameters, tissue specificity, and product inhibition.[181]

Null mutants lacking each of yeast sugar kinases have been constructed and the effect of loss of these genes analyzed.[177,272] The hexokinase PII protein, encoded by the *HXK2* gene, appears to be constitutively expressed.[97,186] Mutations of the *HXK2* gene do not result in any apparent defect in growth on glucose or fructose[60,159]; however, loss of hexokinase PII activity results in the loss of glucose repression. Early models suggested a regulatory role for hexokinase PII in the repression of glucose-repressible genes.[74,76,78,161,180,272] Recent data analyzing mutants partly defective in the sugar phosphorylating activity of hexokinase PII indicate a strong correlation between residual hexokinase PII enzymatic activity and the level of glucose repression.[161,163] In addition, hexokinase PI can substitute for hexokinase PII in glucose repression if it is expressed under the same conditions of high sugar concentration as hexokinase PII.[219] Hexokinase PI is not normally expressed under conditions of high substrate.[97,186] Interestingly, glucokinase was not able to substitute for hexokinase PII in glucose repression.[219] Thus, hexokinase PII activity is required for glucose repression. If this were simply because of provision of glucose-6-phosphate, one would expect glucokinase to be capable also of glucose repression.

In addition to a catalytic role in the phosphorylation of sugar, hexokinase PII has been shown to have a protein kinase activity capable of autophosphorylation and of phosphorylation of other protein substrates.[120,268] This activity has been shown to have a substrate specificity similar to a casein-kinase-II-like protein kinase. There is a strong correlation between the extent of hexokinase PII phosphorylation and the expression of high-affinity glucose transport.[268] Hexokinase PI is highly homologous in sequence to PII and might also possess a protein kinase activity. Glucokinase is more divergent and might not be a protein kinase. Hexokinase PI and PII may be interchangeable in bringing about glucose repression because of the putative protein kinase activity. However, a protein kinase activity for hexokinase PI has not been described.

The *SNF3* carboxyl-terminal tail displays several classic casein-kinase-II-like consensus sites for protein phosphorylation (see Figure 2). It is tempting to speculate that the *SNF3* protein may be a natural substrate for hexokinase PII protein kinase activity. We are currently testing this hypothesis to further define the interactions between the sugar transporters and the sugar kinases. There is, indeed, evidence for the phosphorylation of mammalian transporters,[198] although the physiological function of the phosphorylation is not known.

VII. THE ROLE OF GLUCOSE TRANSPORTERS IN GLUCOSE SENSING AND SIGNAL TRANSDUCTION

Eukaryotic cells are on the "glucose standard," meaning that glucose is their primary and preferred form of capital to invest in the formation of new cells and to spend on metabolic activities via the generation of ATP. It is therefore not surprising that cells have evolved mechanisms both for detecting the presence as well as concentration of glucose and for adapting metabolic machinery to optimally use available substrate. Glucose functions as a first messenger, analogous to a hormone, and triggers a cascade of events in glucose-responsive cells. How cells detect or sense glucose and transduce the glucose signal is a fundamental, and as yet unresolved, biological question. No one has identified a "glucose receptor" — a cell surface protein analogous to hormone receptors — that would bind glucose and generate an appropriate response. Nutrient sensing may be a cascade involving several proteins and activities. There may not be a classic sensor protein but a sensor complex or sensing pathway. Current data, to be discussed later, indicate that the sensing function may be mediated in part by the glucose transporters themselves and not involve a distinct set of glucose receptor proteins. This model is appealing because it is physiologically simple yet readily explains the complexity of the glucose response.

Cells not only need to be able to detect glucose, but they need to determine if it can be utilized by the cell and to what degree. It is not surprising that several lines of evidence suggest a role for the sugar kinases in glucose sensing or at least in mediating the cellular response to glucose. In some cells such as yeast, determining the concentration of available glucose is also critical, as this will direct metabolism in favor of fermentation vs. respiration if the substrate concentration is sufficiently high.

In many respects, use of sugar transport apparatus and subsequent phosphorylatability of the sugar to determine the amount of sugar present and direct cellular activities accordingly is physiologically relevant. Unlike the case with hormones, there would be no need to evolve a distinct mechanism for sensing glucose. The glucose transporters are themselves located on the cell surface and could communicate directly with any necessary components of the signal transduction machinery, also typically localized to the plasma membrane.

Cells also need to be able to detect the concentration of substrate, which is easily accomplished by having transporters of differing substrate affinities that would therefore be activated at different substrate concentrations. Manipulation of the amount or number of species of transporters in the membrane would control the rate of metabolism and, therefore, the rate of generation of the signal. There simply would be no need to evolve a glucose binding site on a receptor that is distinct from the binding site of the transporter, as transportability of the sugar is critical for the cell and the response of cellular metabolism and growth. The proteins ultimately responsible for translocation of the sugar serve as the sensors for that sugar. This model is simple in concept, but it guarantees to the cell that there will be no mistake in the generation of the glucose response, no easy means of uncoupling the sensor function from the ability to utilize substrate. This model also accounts for the fine tuning of the glucose response in the detection of different concentrations of substrate through the use of sensors with different kinetic properties. If differential control of expression of carriers and regulation of their levels at the cell surface is considered, this sensing mechanism permits a rapid, sensitive, and accurate physiological response to glucose, which is precisely what is observed *in vivo* throughout the eukaryotic kingdom.

This model of glucose sensing also explains the need for a multigene family of glucose transporters in unicellular eukaryotes such as *Saccharomyces*, as transporters may be required to interact with different downstream signal-transduction pathways. In multicellular organisms, the glucose transporter-sensing pathway would be the default pathway, with hormonal controls superimposed over this sensing mechanism for those cells with a lower priority for glucose consumption. Although this model is intriguing and seductive, definitive proof has yet to be obtained. However, numerous studies in both mammalian cells

and yeast to be detailed in the next section suggest its validity.

A. The Case in Mammalian Cells

There has been much scientific interest in determining the mechanism of glucose sensing and signal transduction in higher eukaryotic cells, as defects in glucose sensing and response have severe and significant medical implications. The diseases collectively known as diabetes are primarily disorders of glucose sensing and subsequent metabolism and affect a large proportion of the population.[54,90,135,194,249,254,266] Glucose/galactose maladsorption is caused by a defect in the sodium-dependent glucose transporter.[252]

Six glucose transporters have thus far been described in mammalian cells. One of these, GLUT6, appears to be an expressed pseudogene.[139] GLUT1, GLUT2, and GLUT3 can be distinguished on the basis of kinetic properties and specificity for substrate.[109] GLUT4, a low K_m transporter, is the insulin-regulatable glucose transporter.[19,288] GLUT1 is expressed primarily in fetal tissues, but occurs at low levels in many adult cells[20,86,183,238,248]; GLUT2 is found in the pancreatic β cells, liver, and kidney[94,204,248]; GLUT3 is widely expressed, found in the adult brain[140]; GLUT4 is found in insulin-responsive tissues, muscle, and fat[19,49,93,129]; and GLUT5 occurs in the small intestine[139] and spermatozoa[35] and appears to be a fructose transporter.[35] GLUT2 can also transport fructose.[109] In addition, mammalian systems also express a sodium-dependent glucose transporter, SGLT1,[284] involved in the concentrative uptake of glucose from the intestine for subsequent translocation to the rest of the body. The same transporter also appears to function in the kidney for the recovery of glucose. Cells may express more than one GLUT gene at the same time, often to differing levels.[143,179,267] The GLUT genes appear to be regulated by the same factors known to control expression of the yeast transporters.[96,114,142,143,172,285]

The mechanism of GLUT4 regulation by insulin is fairly well known (reviewed in 233, 254) and is a combination of recruitment of transporters from cytoplasmic sites of storage to the cell surface on exposure of the cells to insulin as well as an increase in the transcriptional expression of GLUT4 and an activation of transporters in the plasma membrane.[40–42,57,103,125,164,172,201,270,288] GLUT1 is also expressed in insulin-responsive cells, but appears to be regulated independently of GLUT4, controlled not by insulin, but by glucose concentration.[143]

GLUT gene expression is regulated by growth factors.[217] GLUT1 and GLUT3 have been shown to be elevated in human cancers.[286] GLUT1 expression is increased on cellular transformation with a variety of viruses[21,87] and is elevated in response to stress.[274] These observations have been taken as evidence that GLUT1 is actively controlled by oncogenes. These studies were principally done with established cell lines that already highly expressed GLUT1. Somewhat different conclusions were reached with the investigation of GLUT1 expression *in vivo*,[240] suggesting a danger in overinterpretation of the results obtained from mammalian cells in culture. Mammalian cells in culture are perhaps best viewed as being analogous to yeast, a model system for the events occurring *in vivo* in a multicellular organism, which, although providing important clues to the properties and mechanisms of glucose sensing and signal transduction, might not be directly extendable to cells of the whole organism.

The clearest case in mammalian systems for a role of glucose transporters in glucose sensing comes from the study of GLUT2. GLUT2 is a low-affinity glucose transporter located in the liver and in the insulin-producing pancreatic β cells. Underexpression of GLUT2 in these cells appears to be correlated with the appearance of non-insulin-dependent diabetes.[135] Experimental evidence exists that underexpression of GLUT2 results in loss of the response of insulin secretion to glucose concentration,[249] but the role of transporters in sensing remains controversial. Perhaps the most compelling indication of a role of GLUT2 as a glucose sensor has come from conferring glucose-stimulated insulin secretion to AtT-20ins cells (derived from the anterior pituitary gland) by transfection with GLUT2 cDNA.[126]

In addition to GLUT2, the pancreatic β-cell glucokinase has been implicated as a component of the glucose-sensing apparatus.[174] The data im-

plicating glucokinase in the sensing process are more conclusive than that of the transporters. A variety of experimental data suggest a role of glucokinase in glucose sensing. As with GLUT2, a correlation between glucokinase levels and ability to secrete insulin in response to glucose has been observed.[204,248,249] The glucokinase inhibitor, alloxan, inhibits glucose-stimulated insulin secretion (Ref. 156 and references therein). A nonsense mutation of the human glucokinase gene has been linked to early-onset non-insulin-dependent diabetes.[90,266] AtT-20ins cells used in the analysis of the role of GLUT2 in glucose sensing already express mammalian glucokinase.[126] Glucokinase expression in different cell types of the pancreas is also consistent with a role on sensing: it is more highly expressed in those cells responding to the glucose signal.[134] Glucokinase gene expression appears to be regulated by insulin.[164]

Although the data implicating GLUT2 and glucokinase as components of the glucose-sensing apparatus in islet β cells are strong, confirmation of this model will require reconstitution of the signal pathway in a genetically defined and simple system or *in vitro*. Expression of GLUT genes in *Xenopus* oocytes is possible and has proved useful in determining some of the kinetic properties of the GLUT proteins,[108,109,141,264] but is limited in terms of definitive studies on glucose sensing, as the oocytes express their own transporters and presumably also have a mechanism of some sort for the detection of glucose.

B. The Case in Yeast

Signal-transduction pathways display a remarkable degree of conservation among eukaryotes, particularly evident in comparison of *Saccharomyces* with mammalian cells.[32,244] In contrast to bacteria, evolution in eukaryotic systems seems to involve the differentiation and specialization of cell types, not the generation of cells using novel regulatory and metabolic mechanisms and strategies. Because of this fact, yeast can serve as an excellent model system for the dissection of fundamental biological processes common to all eukaryotes. However, yeast have been subjected to the same degree of evolutionary pressure as all other eukaryotic cells, so it is important to distinguish when yeast are responding as a typical eukaryote and when they are responding as yeast.

With respect to the mechanism of glucose transport and sensing, it has already been demonstrated that *Saccharomyces* displays multigene families of both transporters and sugar kinases, as is observed in mammalian cells. Both high- and low-affinity transporters are found, with approximately equivalent K_m values. Although differential expression in cells and tissues can explain the physiological roles of the GLUT genes in mammals, there is as yet no clear explanation for the multitude of transporters observed in *Saccharomyces*. The *HXT* genes are all expressed and appear to be expressed to differing levels and under different growth conditions. Four *HXT* genes have been characterized to date, in comparison with five GLUT genes. However, many more *HXT* genes await isolation and analysis, as evidenced from low- and medium-stringency Southern analysis, using the cloned *HXT* genes as probes. The *HXT* genes examined so far affect glucose *(HXT2)*, galactose *(GAL2)*, glucose and mannose *(HXT1)*, or glucose, fructose, and galactose *(HXT4)* uptake; however, none have been identified specifically affecting fructose. With the exception of *GAL2*, members of the *HXT* family were identified as suppressing in multicopy simultaneously the growth and transport defect imposed by mutation of the *SNF3* gene.

Does the plethora of transporters in this simple eukaryote imply that many other glucose transporters await detection in mammalian systems? Perhaps, but it is important to remember that the ecological niche of *Saccharomyces* displays dramatic swings in glucose concentration, from 2 *M* down to undetectable, and that this organism shifts from fermentative to respiratory growth, depending on substrate concentration and the availability of molecular oxygen. As yeast cells adapt to increasing concentrations of ethanol, from undetectable to 17% (v/v) or more, striking changes in plasma membrane lipid composition occur to confer ethanol tolerance, requiring that transporters function in a different microenvironment. These factors may necessitate a large array of

sugar transporters that can be expressed under different conditions of growth. Alternately, if the sugar transporters indeed play the primary role in detection of glucose, yeast must detect glucose over a much broader concentration range than do mammalian cells.

The evidence implying a role of glucose transporters in glucose sensing in yeast is largely circumstantial and not nearly as compelling as the data obtained in mammalian systems. Exposure of yeast cells to glucose triggers the formation of a variety of second messengers, affecting the levels of just about every known eukaryotic second messenger. Excellent reviews on this topic have appeared recently.[245-247] The kinetics of the glucose response are identical to those of the low-affinity glucose transporter and occur in *snf3* mutants that fail to express high-affinity glucose uptake.[18] Because sugar phosphorylation, but not subsequent metabolism, is required for most of the glucose-induced regulatory effects, sugar kinases are also believed to be a component of the glucose-sensing machinery in yeast.

A strong advantage of yeast as an experimental model system is the powerful genetic analyses that may be performed. Numerous mutations affecting glucose regulation of cellular activities have been isolated. Analysis of the complex phenotypes of these mutations is in its infancy but has begun to shed light on the mechanism of glucose control in *Saccharomyces*.

Several interesting genes have been identified via mutant isolation that appear to affect early steps in the glucose sensing pathway[10,231] (reviewed in Ref. 98,246). The *fdp* mutation was identified as leading to impaired growth on sugars. This mutant fails to inactivate fructose-1,6-bisphosphatase, phosphoenolpyruvate carboxykinase, and malate dehydrogenase on glucose exposure,[10,14,99,205,256-258] and it has recently been shown that the *fdp1* mutant is deficient in generation of the cAMP signal in response to glucose.[257] On glucose exposure, this mutant fails to accumulate fructose-2,6-bisphosphate and fails to stimulate potassium uptake and plasma membrane H^+ ATPase activity.[246,257] Strains carrying the *fdp1* mutation will transport and phosphorylate fermentable sugars, leading to the depletion of cellular ATP levels and eventual cell death. Not only are all known glucose-induced regulatory responses defective in *fdp1* mutants, but glycolytic flux and transport of sugar appear to be completely uncoupled.[246] *FDP* stands for *f*ructose *di*phosphate inactivation and, given the recent data implying much broader phenotypic effects, a change in designation to *GGS* for *g*eneral *g*lucose *s*ensor has been proposed.[246] From analysis of the predicted amino acid sequence of the *GGS1* gene, the *GGS1* protein is not expected to be localized to the cell surface, as it contains no putative membrane-spanning regions or sequences associated with transiently membrane-localized proteins. The current working hypothesis is that the *GGS1* protein may be a component of the glucose-sensing apparatus, part of a complex of the glucose transporter and sugar kinase.[245-247]

Other genes affecting glucose repression that may help define the glucose sensing and signal-transduction process have also been identified. Mutation of *GRR1* gene results in loss of glucose repression and can confer glucose inducibility to the *SUC2* (invertase) gene.[85] The *GRR1* protein is localized to a particulate fraction in yeast crude extracts. The *grr1* mutants display reduced growth rates on glucose. Both high- and low-affinity glucose uptake are greatly deficient in strains carrying the *grr1* mutation (Coons and Bisson, unpublished observations), explaining the reduced growth rates. However, the physiological role of the *GRR* protein is far from clear. It could be intimately involved in transmission of the glucose signal and control of the glucose response or it could play only a secondary role via the disruption of expression of the glucose transporters that then causes the defects in glucose control.

The effect of loss of hexokinase and glucokinase activity on glucose-uptake kinetics has been described in detail earlier and may be interpreted as evidence of a regulatory relationship between sugar phosphorylation and sugar uptake that may impact glucose sensing. However, loss of the other two irreversible steps of glycolysis, phosphofructokinase and pyruvate kinase, similarly inhibit glucose transporter activity. The sensing function of the transporters may be impacted by loss of these steps of glycolysis without those enzymes being directly involved in the sensing process.

There is experimental evidence suggesting that the glycolytic enzymes are present *in vivo* as a multienzyme complex[33,111,271] and may interact with the plasma membrane[133] or protein components of the membrane.[148,187] Glucokinase has been shown to be associated with a membrane fraction in brain.[206] Phosphofructokinase has been found to affect hexokinase activity.[195] The enzymes of the entire glycolytic pathway may be physically associated with the glucose transporters forming a metabolic complex. Formation of this complex may be necessary for activation of transporter activity. This model would explain the impact of loss of one of the irreversible steps of glycolysis on sugar uptake if these three enzymes were necessary and sufficient to activate transporters. Cells growing gluconeogenetically, by extension, would possess a gluconeogenic complex not necessarily interacting with transporters except in those cells where glucose would then be excreted into the bloodstream to feed other cells and tissues.

VIII. PERSPECTIVES AND CONCLUSIONS

Yeast and higher eukaryotes display multigene families of sugar transporters, highly related in protein sequence and predicted structure and topology. Prokaryotic transporters have been identified that also belong to the same superfamily. Members of this superfamily differ greatly in substrate specificity and mechanism of transport, with some being facilitated diffusion systems and others proton or ion symporters. Within the superfamily, there are more highly related subfamilies such as the yeast *HXT* family. The six members of this group are between 60 to 75% identical in primary amino acid sequence.

The mechanism of glucose sensing and signal transduction in yeast and in mammalian cells is an area of intense scientific investigation. Downstream steps of nutrient sensing have been genetically and biochemically defined in yeast, but the very early steps of the pathway are unknown. Mutant analyses have been informative but not definitive.

HXT subfamily members display differing substrate specificities, but all appear to function via facilitated diffusion. The yeast transporter family has by no means been saturated; other related genes exist awaiting further characterization. The regulation of these genes is complex, influenced by glucose concentration, nitrogen availability, medium composition, and the stage of growth. Some (*HXT2, HXT4,* and *GAL2*) are expressed at high levels consistent with a role as catabolic transporters, whereas expression of others (*HXT1* and *SNF3*) is barely detectable. Clearly, we have only just begun the investigation of the kinds, numbers, and nature of yeast sugar transporters.

Of special note is the *SNF3* paradox. *SNF3* was originally identified as a high-affinity glucose, fructose, and mannose transporter on the basis of the following criteria: (1) *snf3* null and point mutations do not display high affinity uptake in kinetic assays; (2) *snf3* null and point mutations prevent growth on low-substrate concentrations; (3) analysis of the predicted amino acid sequence of *SNF3* indicates the protein is a member of the transporter family; (4) *SNF3*-β-galactosidase fusions are localized to the plasma membrane. More recent data suggest a different physiological role for *SNF3:* (1) growth and transport defects caused by mutation of *SNF3* are separately suppressible; (2) β-galactosidase activity is not highly expressed from fusion of the *lacZ* gene to the *SNF3* promoter under conditions where the protein is predicted to be highly active according to mutant phenotypes; (3) preliminary investigations using an epitope-tagged *SNF3* protein confirm the low level of expression observed with the *lacZ* fusion; (4) the unusually long carboxyl-terminal tail appears to be required for both transport and growth functions of *SNF3*. No other known transporter encodes a tail near this length.

Why does a unicellular organism like *Saccharomyces* possess a large family of glucose transporters? That these transporters are regulated differently at the transcriptional level implies that different proteins are required for specific growth conditions. The reason that different transporter proteins are necessary may reflect the need for different regulatory properties. Quick response to changing environmental conditions may dictate rapid cessation and turnover of glucose transporters, but some glucose-uptake capacity will al-

ways be necessary. It may be biochemically more feasible to have different transporter proteins, one that is programmed to be rapidly degraded and the other that is stable, rather than to try to achieve the same effect with a single polypeptide species. Oftentimes a rapid, accurate response to changing glucose concentrations is necessary. For example, under a given set of conditions, in response to a signal of low energy the cell may wish to reduce transport by 50%. This may be best achieved by complete elimination of one polypeptide, a transporter species that is responsible for 50% of the total or "consortium" uptake, rather than trying to reduce the activity of a single polypeptide by 50%. There may be many such requirements for physiological regulation or biochemical modification of transporter activity necessitating a large family of transporters.

Alternatively, some of these transporters may not be transporters at all, but serve a regulatory or glucose-sensing function. As detailed in this review, provision of glucose to glucose-starved cells of *Saccharomyces* triggers secondary messenger cascades, which are independent of glucose catabolism. The transporter is thought to be a component of the sensing apparatus, acting early in the secondary messenger pathway. However, the proteins serving this regulatory function might not be actual transporters at all but simply be sensors. Sensors may have retained close structural similarity to transporters to assure accurate detection of glucose. The diversity of the glucose transporter family in yeast may be due to the need of glucose to impact many cellular activities simultaneously. The answer to the question of "Why so many transporters in yeast?" will have to await further physiological characterization of the transporter family in *Saccharomyces* and other yeasts.

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