Glucose transport in Saccharomyces cerevisiae effects on growth and metabolism
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Chapter 7

General discussion and perspectives

The work presented in this thesis is based on genetic and molecular biology approaches to alter the level of expression of transport proteins; on physiology and biochemistry approaches to determine the control coefficient of individual glucose transporters under various growth conditions at steady state; and on a cell biology approach to monitor the life history of selected transporters within Saccharomyces cerevisiae. The measured parameters in strains with modulated transporter expression include growth rate, glycolytic flux rate, glucose transport activity, glucose repression state, and of course transporter content per cell.

In this final chapter of my thesis, the following subjects, all taken together, will be addressed: conclusions derived from our findings; puzzles hidden in our data; problems encountered in the experiments; and suggestions for improvement and further development of the presented research.

Our data demonstrate that glucose transport is the major controlling factor of the glycolytic flux in HXT7-only strains under defined conditions, even maybe in wildtype yeast, if we assume that the elasticity of the various glucose transporters is identical. The control coefficient of glucose transport on the glucose flux has a value as high as 0.90. The control coefficient of glucose transport with respect to the growth rate is 0.54. Therefore, the growth rate of yeast on glucose medium could be improved by increasing the glucose uptake capacity. To this aim, a few proposals follow:

(i) Raising the HXT1 expression level in the bioengineered yeast strains by various approaches. Hxt1 is a low-affinity hexose transporter. Its expression is induced by a high concentration of glucose. The glucose transport capacity of Hxt1 is not influenced by the glucose repression. The rate of glucose fermentation can thereby be increased by high Hxt1 transporter activity and fast glucose transport. However, this approach may be limited by an as yet unknown mechanism (homeostased by glucose in?) for regulation of the transport capacity. In batch cultures the maximal rate of glucose transport ($V_{\text{max}}$) is fairly constant
during the various phases of growth, although different transporters are expressed (235). Also interactions among transporters or the capacity of the plasma membrane for integration of transporters may make it impossible to increase the transport capacity far above the wildtype level.

(ii) Using a heterologous promoter which is regulated by a gratuitous inducer to improve the expression level of hexose transporters. For instance, a rat gene promoter system (the rat glucocorticoid receptor), which is a latent transcriptional activator, has been used in in vivo expression systems (173, 198). The system is based on a mammalian steroid response. Upon binding of the hormone ligand, the receptor is translocated to the nucleus where it binds to short DNA sequences called glucocorticoid response elements (GREs) and activates the transcription of adjacent genes. In yeast its transcriptional activation potential is dependent on the presence of GREs in the context of a yeast basal promoter, and on the concentration of the steroid hormone deoxycortisone applied. Another is the promoter of the yeast metallothionein gene, which is responsive to copper (136, 141, 146). As an advantage of the above two promoters it has been claimed that the inducers are not metabolized by yeast, and are not toxic in the range employed. Recently, a hybrid tetO-CYC1 promoter has been used in a set of vectors for modulated gene expression in S. cerevisiae. Expression from this promoter can be induced up to 1000-fold by tetracycline or derivatives with a lacZ reporter system (72). These vectors may be very useful for overexpression purposes. On the other hand, by replacing the natural promoter with the inducible promoter to each of the HXT genes by simple DNA cloning techniques, the control exerted by various Hxt proteins can be estimated individually in matching conditions of cell growth and gene expression.

Our results show that fusion of GFP to yeast hexose transporters is a powerful tool to reveal some characteristics of this family of proteins. By making use of the Hxt::GFP fluorescence, we are not only able to visualize the subcellular localization of the Hxt proteins, but are also able to quantify the activities of the transporter proteins. The catalytic-center activities of Hxt2::GFP and Hxt7::GFP in vivo, which represent the maximum number of substrate molecules that 'turn over' to product per time unit, provide the examples. Although several HXTs among the 20 hexose transporter gene family have been identified, the relationships between them are still waiting to be established. It is likely that the different transporters are suited for different functions and are expressed in response to different environmental or cellular states. Therefore, by the GFP fusion technique, especially, by taking advantage of alleles of GFP with possible fluorescence resonance energy transfer, two or more transporters can be monitored simultaneously (88). In this case, the interactions between the Hxt proteins in living cells will become much clearer.
The present study was mainly to perform a glycolytic control analysis through control of the expression of one hexose transporter gene to different degrees, greater or lesser than normal. However, the truncated HXT7 promoter series generally demonstrated 2 levels of HXT7 transcriptional activity: positive or negative. A break occurs in the region of −495 to −346. Among the positive transcriptional activity mutants, the expression of Hxt7 is very similar: near, but below, the wild type level. The mutant 16 is an extraordinary case, in which an activator sequence chanced to form after ligation of the remaining ends after deletion. The integration of the gene at various copy numbers into the genome of an hxtΔl-7 strain increases the complexity of an analysis of the relationship between promoter size and glucose transport capacity.

For future research, two points deserve special attention:

(i) Creating adjustable and inducible promoters which cover a wide range of promoter activities in small steps of activity change. For example, the artificial yeast promoters (102, 103), constructed by using synthetic degenerated oligonucleotides and regulated by arginine, will be one of the candidates for replacing the HXT promoters to alter HXT expression level and to perform the experimental control analysis.

(ii) Controlling the single gene integration by using low concentrations of DNA (174) or strictly selecting the desired gene dosage by Southern blot analysis.

Concerning the HXT7 promoter analysis, as described in chapter 6, the HXT7 promoter-lacZ fusions with a promoter extending beyond −495 from the start codon (including the full promoter region) failed to give rise to β-galactosidase expression. We are not sure whether this lack of expression is caused by interference between the TATA box in the HXT7 promoter sequence and the TATA box in the minimal promoter of plasmid pLacZi (P_cyc1). We hope that this can be proven by the deletion of the TATA box in pLacZi in near-future experiments.

We found four Mig1-binding sites in the HXT7 promoter. Mig1 is a transcriptional repressor of many glucose-repressed genes. However, we did not find a significant glucose repression of HXT7 expression in the strains LYY0 – LYY7, LYY10 and LYY11, of which the promoters include a different content of Mig1 binding sites, relative to that in the strain LYY8 (lacking any Mig1-binding site). The phenomenon of partial derepression on high glucose has been observed for all HXT7-only strains. We propose that all HXT7-only strains are partly derepressed due to the low glucose transport capacity, and that the loss of the Mig1 binding site(s) can not induce a significant further derepression. To distinguish them, a test can be
made by comparing the β-galactosidase activities from the LYY7 promoter versus the LYY8 promoter in the wildtype strain MC996A at high glucose, in which no transport-dependent derepression is present, after the question of interference between two TATA boxes has been solved.

The 149 bp DNA region –495 to –346 in the HXT7 promoter plays an important role in HXT7 expression. We did not yet find any key transcriptional element located in this region. What kind of activator sequence is it? To find the answer, there are two routes that can be taken. First, narrow this 149 bp DNA region by using the PCR technique or oligonucleotide linkers and compare the level of the β-galactosidase activity produced by a specific region. Second, analyze this region by using the techniques currently used to characterize DNA-protein interactions, such as DNase I footprinting (16) and the mobility shift assay (21). The basis of footprinting is that the protein which binds to a singly end-labeled DNA fragment protects the phosphodiester backbone of DNA from DNase I-catalyzed hydrolysis. Binding sites are visualized by autoradiography of the DNA fragments that result from hydrolysis, following separation by electrophoresis on denaturing DNA sequencing gels. In the mobility shift DNA-binding assay, proteins that bind specifically to an end-labeled DNA fragment retard the mobility of the fragment during nondenaturing polyacrylamide gel electrophoresis, resulting in discrete bands corresponding to the individual protein-DNA complexes. To find the transcriptional activation element in this special 149 bp DNA region will be an interesting and exciting work. Other work still to be done is the identification of the transcription factors, which have been preliminarily screened by the HXT7 promoter analysis in chapter 6.

Teusink et al. (215) propose that intracellular glucose is a candidate signal molecule for glucose repression. Similar information can be drawn from a recent survey (47). Our results are also in agreement with this conclusion. This issue can be further tested by the following experiment: compare the glucose repression state under the conditions of the same external glucose concentration (for instance, 30 mM) and the same glucose flux, which is produced by either the high-affinity transporter Hxt7 or the low-affinity transporter Hxt1, with different gene dosage. Under 30 mM glucose condition, glucose transport in the HXT7-only cell ($K_m \approx 2$ mM) is saturated. While in the HXT1-only cell ($K_m \approx 100$ mM), glucose transport is proportional to the concentrations of external glucose and the Hxt1 protein. Therefore, the internal glucose concentration in the HXT1-only cell should be higher than that in the HXT7-only cell. If the intracellular glucose is indeed the signal molecule for glucose repression, a different effect of glucose repression should be observed between HXT7-only and HXT1-only cells. This experiment will also give the answer on the question whether the low-affinity carrier Hxt1 exerts the same control on the flux of glucose as does the high-affinity Hxt7.
In this study we noticed a remarkable phenomenon of an incomplete glucose repression in the HXT-only strains under conditions of high glucose concentrations. We presume it is the consequence of derepression at low internal glucose, since the HXT-only strains have lower glucose transport capacities than the wildtype strains. However, the actual mechanism remains to be answered. It is unknown whether glucose transport accompanies the Snf3 and Rgt2 signaling step. At low glucose concentrations (below 15 mM) a further induction occurs. This may be due to Snf3 sensing. What is the glucose concentration dependence of the signals emanating from Snf3? What determines the sensitivity of Snf3 sensing? Kruckeberg et al. put forward a proposal to measure the glucose sensitivity by fusing the Snf3 tail to the Rgt2 membrane-spanning domains and vice versa (126). Maybe the resulting chimeric proteins can provide the answer to the question whether Snf3 is a high-affinity sensor or not, and to reveal the sensitivity and specificity of the Snf3/Rgt2 system.

In our present work, the function of hexose transporters has been researched in mutants with an individual hexose transporter gene. It can detect and resolve the presence of multiple uptake systems with relative ease. Of course, transport is an enormously complex process that no doubt requires the participation of many gene products. When the understanding of each of the HXT genes has been greatly increased, these isolated components will be assembled together. It will be interesting to investigate the characteristics of both Hxt1 and Hxt7 combined in one strain and the like. How will the glycolytic pathway be controlled? What is the function of glucose signaling for glucose repression in these strains? Through these approaches, more precise explanations of the glucose transport mechanism will be achieved.

*S. cerevisiae* has been studied extensively for many years. In recent years, the application of genetic and molecular biology techniques provides a more complete global picture of this simple microorganism, but with a very sophisticated regulatory system. There are still many questions for which the answers have not been found yet. For example, in our study, Hxt2 demonstrates two-component kinetics. How can a protein function as a high-affinity and a minor low affinity transporter simultaneously? Why does Hxt7 show different kinetics from Hxt2? What is the relationship between them in the process of glucose metabolism? It is a long way to answer all the fundamental problems related to the glucose transport mechanism.