Balancing between respiration and fermentation: Transcriptional regulation of respiratory glucose metabolism in yeast
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Chapter 6

General discussion

The different topics described in this thesis are positioned around the comparative analysis of carbon source dependent transcriptional regulation of respiratory function in *S. cerevisiae* and *K. lactis*, and aim at obtaining more insight in the molecular regulation of fermentative or oxidative glucose metabolism. Three central questions have been addressed:

i) How is transcription of the gene encoding the transcriptional activator subunit Hap4p regulated in the two yeasts and does a differential regulation bely the difference in function of the HAP complex in the two yeasts?

ii) If the HAP complex is not required for induction of respiratory genes in *K. lactis*, how is this induction then established?

iii) Do two *K. lactis* strains, genetically differing in glucose transport mechanisms, exhibit differences in transcriptional regulation of respiration?

Answers to these questions provided by work described in this thesis invoked new questions. Questions which not only demonstrate a need to increase our understanding of transcriptional regulation and carbon source signalling, but also raise issues that extend to others areas involving translational control, chromatin structure, nitrogen signalling, stationary growth, nutrient limitation. It is clear that the simplicity of yeast as an experimental organism belies the complexity of its regulatory network that has been honed by evolutionary pressures in order to allow it to adapt adequately and efficiently to environmental changes.
Transcriptional regulation of HAP4 expression in S. cerevisiae

Transcription of nuclear genes encoding mitochondrial proteins involved in the respiratory chain is repressed by glucose in S. cerevisiae. In this yeast, glucose repression is relieved and transcription is induced when glucose is limiting or when other, non-fermentable, carbon sources are present. The induction of transcription, and thus growth on non-fermentable carbon sources, is dependent on a functional activator complex, consisting of the proteins Hap2p, Hap3p, Hap4p and Hap5p. The actual activator subunit, Hap4p, is encoded by the HAP4 gene, and this gene is sensitive to glucose repression itself, whereas the genes encoding the other, DNA-binding, subunits are constitutively transcribed. Hap4p plays a pivotal role in the balance between fermentation and respiration, since overexpression of the HAP4 gene leads to a more respiratory glucose metabolism in S. cerevisiae. How transcription of HAP4 is regulated in response to changes in environmental conditions and, moreover, which signalling pathways are involved is the central issue addressed in chapter 2. What is evident from our results is that the regulation of HAP4 expression is extremely complex and cannot simply be described in a black-and-white manner. We have thoroughly studied a part of the promoter region, consisting of 265 bp ranging from -1006 bp to -745 bp upstream of the ATG start codon. This region is involved in the induction of the HAP4 gene when cells are grown on non-repressing sugars like galactose or non-fermentable carbon sources like lactate and ethanol/glycerol. Closer analysis revealed that the same extent of induction can be exerted by several subfragments of this region and suggested that a mosaic of cis-acting elements exists within this region, possibly constituting binding sites for a multimeric protein. The carbon-source dependent activation mediated by the 265 bp region, and, moreover, by a 30 bp element closely resembling a CSRE consensus binding site located within the 265 bp fragment, is dependent on the presence of a functional CAT8 gene. This involvement of Cat8p unveils a complex framework by which the expression of the HAP4 gene is coordinated. However, neither deletion of the 265 bp region from -1006 bp to -742 bp within the full-length promoter region nor 5'-HAP4 promoter deletions have any significant effect on activity of the reporter gene when placed in a chromosomal context, i.e. when integrated in the URA3 site of the genome. This probably reflects the fact that chromatin structure plays an important role in a higher level of HAP4 transcriptional regulation, for example in maintaining a specific nucleosomal context. Likewise, deletion of Cat8p might indeed not affect the transcriptional regulation in an in vivo context, but will affect specific Cat8p-dependent activation by subfragments when placed outside the chromosomal or full-length promoter context. Interestingly, whole-genome expression analysis of mutants affected in the Snf/Swi complex, controlling transcription and chromatin structure and involved in
remodeling nucleosomes, revealed that HAP4 transcription is reduced in these mutants [179]. A detailed study of the nucleosomal state at the HAP4 promoter under different conditions will possibly shed more light on a complete understanding of the carbon source dependent transcriptional regulation of the key component of the HAP2/3/4/5 complex in S. cerevisiae. Besides Cat8p, we have also found that Cat5p is involved in the specific activation by the 265 bp subfragment of the HAP4 5' non-coding region, as well as the 30 nt region within this fragment (figure 1). The CAT5 gene was identified as being required for the release of glucose repression of genes encoding enzymes of the gluconeogenic pathway [160]. In addition to this, the CAT5 gene was independently isolated as COQ7, a mitochondrial inner membrane protein required for the synthesis of ubiquinone [187]. Cat5p was proposed as a candidate connecting glucose derepression of gluconeogenesis and the respiratory state of the cell, since CAT5 expression strongly depends on SNF1, CAT8 and CAT5 itself, while CAT8 expression is decreased in cat5 mutants [160]. However, the inability of cat5 mutants to derepress gluconeogenesis upon glucose exhaustion appears to be a general consequence of a defect in respiration, since derepression of gluconeogenic promoters is also absent in other respiration deficient strains [103]. Although this makes Cat5p a less likely candidate for directly linking glucose derepression and respiration, it still seems conceivable that it is part of a check mechanism that allows glucose derepression only if respiration is also possible.

![Beta-galactosidase activity](image)

**Figure 1: Involvement of Cat5p in transcriptional regulation of the ScHAP4 promoter.** Wild-type strain MC999 (Mat a; ura3-52; his3Δ1; leu2-3; 112trp1) and isogenic cat5 disruption strain CEN-MP3-1A (Mat a; ura3-52; his3Δ1; leu2-3; 112trp1; cat5::HIS3, [160], were transformed with pCZ containing either the 265 bpSmal HAP4 promoter fragment (-745/-1006) or the 28 nt region (-896 bp to −869 bp; see chapter 2). Data are obtained from two independent transformants of each construct. Dark bars, glucose-grown cells; light bars, lactate-grown cells.
A database search (Matlnspector [163]) for other known consensus binding sequences against a matrix of fungal consensus sites revealed the presence of consensus sites for the stress responsive proteins Msn2p/Msn4p and the pH responsive regulator Rim101p, as well as of four Dal80p and Gat1p consensus binding sites, in the HAP4 promoter. The latter two could possibly indicate a feed-back regulation with nitrogen regulatory pathways. This would be conceivable considering the cross-pathway regulation between nitrogen and carbon metabolisms connected by the HAP complex [44, 45]. Although a functional relevance of these sites is unknown yet, it does not alter the conclusion that the HAP4 gene possesses a complex promoter harbouring binding sites for independent and synergistically acting transcription factors that link the expression of the gene into other regulatory circuits controlling C-source responses, and possibly N-sources in S. cerevisiae.

Transcriptional regulation of HAP4 expression in K. lactis
Whereas the Hap2/3/4/5 complex is required for induction of respiratory function and growth of S. cerevisiae on non-fermentable carbon sources, the complex plays only a minor role in non-fermentative growth in K. lactis. Disruption of HAP2, HAP3 or HAP4 in K. lactis does not abolish growth on complete media containing non-fermentable carbon sources. Furthermore, transcription of KIQCR8, encoding a subunit of the mitochondrial respiratory chain, is not repressed by glucose. Although the KIQCR8 promoter region contains a functional binding site for the Hap2/3/4/5 complex and transcription of KIQCR8 genes is induced on non-fermentable carbon sources, the Hap2/3/4/5 complex is not required for the induction. It is, however, involved in maintaining a generally high basal level of expression on both fermentable and non-fermentable carbon sources. This led us to hypothesize a constitutive, not glucose repressed expression of the HAP4 gene in K. lactis, taking into account the strong carbon source dependent regulation of its homologue in S. cerevisiae and its role in balancing fermentative and oxidative glucose metabolism. Although the expression of HAP4 is indeed constitutive in exponentially growing steady-state batch cultures as shown in chapter 3, monitoring HAP4 transcript levels following the addition of a glucose pulse to glucose-limited steady state chemostat cultures led to new insights. This clearly demonstrated that HAP4 transcription immediately decreased after glucose has been added, and was strongly induced when glucose was finished and ethanol was nearly consumed. The carbon-source dependent transcriptional activation of KIHAP4 under these defined conditions suggest that KIHAP target genes respond to carbon limitation via the Hap2/3/4/5 complex. Interestingly, although the transcription of KIQCR8 remains rather constant during the first 240 minutes after the glucose pulse, not
resembling $KIHAP4$ transcript patterns, it becomes strongly induced at the end of the experiment, similarly to $KIHAP4$. The results are indicative of a model in which $KIHAP4$ does not affect transcription of $KIQC8$ under conditions of non-fermentative growth, but in which the HAP complex plays an important role when carbon sources are limiting.

The roles of the HAP complex: conserved and divergent

Is there a common, conserved role for the Hap2/3/4/5 complex of which the identified components are functional homologues in the two yeasts? Regarding the strong resemblance in carbon source regulation of $HAP4$ in response to the addition of excess glucose to glucose-limited steady-state chemostat cultures, this question can presumably be answered positively. It can be envisaged that yeast cells encountering conditions in which carbon sources are limiting, shift from active growth to growth arrest (stationary growth phase) and ultimately sporulate. This involves an extensive reprogramming of cellular metabolism, including increased mitochondrial function, and our results suggest that in $K. lactis$ the HAP2/3/4/5 complex might be involved in the regulation of this reprogramming via transcriptional regulation of $KIHAP4$. This is in line with the high $HAP4$ expression in $S. cerevisiae$ upon transition to stationary growth [72]. Besides a possibly common role in the stationary growth phase, the Hap2/3/4/5 complex in $S. cerevisiae$ has a function distinctive from its $K. lactis$ counterpart in non-fermentative growth. It is currently unknown how this function has diverged, whether the HAP complex contributes to respiratory glucose metabolism in $K. lactis$ and why the non-fermentative function is obviously dispensable in $K. lactis$. An explanation might be sought in the importance of the HAP complex to increase mitochondrial function on non-fermentable carbon sources in $S. cerevisiae$. Since respiratory function is already relatively high on glucose in $K. lactis$, a change towards non-fermentable carbon sources might involve less drastic reprogramming to increase mitochondrial function. Moreover, we have shown that another factor is involved in the induction of respiratory genes on ethanol/glycerol (see chapter 4 of this thesis), and this may have taken over the non-fermentative function of the HAP complex in $K. lactis$.

Besides differences in transcriptional regulation of the $HAP4$ gene in the two yeasts, post-transcriptional differences could also be involved. Whereas nothing is known yet of the 5' region of the $KIHAP4$ gene, the $HAP4$ gene in $S. cerevisiae$ contains a long leader of 280 bp. This long leader includes two upstream ATGs, initiating ORFs of nine and three residues, and these ORFs could be involved in translational regulation of $HAP4$ gene expression, as has been reported for other genes with upstream ATGs [1, 155, 199]. Furthermore, it was suggested that the cellular redox environment might be involved in regulation of HAP2/3/4/5 activity via
stabilization of Hap3p by glutaredoxin [207]. This would be similar to the mammalian heteromeric CCAAT binding complex, NF-Y, for which the cellular redox environment has also been reported to be an important post-transcriptional regulator of NF-Y subunit association and DNA binding activities [140]. Since *K. lactis* and *S. cerevisiae* display several differences regarding the maintainance of a redox balance (see chapter 1 of this thesis), it might be speculated that redox adjustment systems underlie the function of the HAP complex in non-fermentative growth of *S. cerevisiae*.

**Identification of an additional complex that binds to the KIQCR8 promoter**

Our first steps in the identification of additional elements involved in the induction of respiratory genes in *K. lactis* concerned protein binding to the KIQCR8 promoter in the presence and absence of binding sites for Abf1p, Cpf1p and Hap2/3/4/5p. We observed that Cpf1p-dependent, additional complexes were formed in gel shift assays using the region in the KIQCR8 promoter from −294 to −171 bp with a mutation in the Abf1p binding site (figure 2). The occurrence of various forms of Cpf1p-KIQCR8 protein-DNA complexes in the absence of a functional Abf1p binding site may be attributable to different versions of Cpf1p. In *S. cerevisiae*, Cpf1 proteins have been described which differ in their molecular masses [144]. Depending on nutritional and oxygen conditions during growth of the cells, specific processing and/or modification of the Cpf1 protein might occur, leading to different forms of Cpf1 within the nucleus. However, protein extracts from wild-type *K. lactis* cells grown under different culture conditions (glucose or glycerol as carbon source, aerobic and hypoxic cultures) show only a single KICpf1p-CYC1 protein-DNA complex in gel mobility shift assays [167]. The presence of different complexes is therefore unlikely to be attributable to modification of Cpf1p. Instead, this and the fact that the additional Cpf1p-KIQCR8 protein-DNA complexes are only observed in the absence of binding of Abf1p to its consensus binding site, might indicate that another factor is competing with Abf1p for binding to the KIQCR8 promoter. Like ScCpf1p, KICpf1p contains, C-terminal of the basic helix-loop-helix, a leucine-zipper which might be involved in dimerization of the protein. In *S. cerevisiae*, Cpf1p is involved in the formation of heteromeric complexes on the promoter region of the structural MET genes [10]. It is tempting to postulate that in *K. lactis* the binding of Cpf1p to the KIQCR8 promoter enhances the DNA-binding affinity of another factor, in the absence of Abf1p binding. This could be exerted via a direct interaction between Cpf1p and the unknown factor, the latter being tethered to the DNA through Cpf1p. Alternatively, the increase in DNA affinity might be the result of a modification of the DNA structure induced by the binding of Cpf1p to its site, a feature that also can be attributed to Cpf1p function. The fact
that no complexes were observed in gel mobility shift assays in the absence of Cpf1p binding, might be indicative of a dependence on Cpf1p for the DNA-binding of the unknown factor. Alternatively, it might bind in a low-affinity manner or involve a low-abundant protein. Closer analysis of the nature of the unknown binding factor was hampered by the lethal phenotype that cpf1 null mutants display in K. lactis [138], and therefore, we decided to continue our search for additional cis-acting elements in the KIQCR8 promoter by promoter deletion analysis as described in chapter 4.

Figure 2: An additional complex binds to the KIQCR8 promoter. A) schematic overview of the KIQCR8 promoter. B) gel shift experiment. Lane 1 to 3, wt 221 bp fragment; lane 4 to 6 221 bp fragment containing mutation in Abf1p binding site; lane 7 and 8 221 bp fragment containing mutations in Abf1p and Cpf1p site. Lane 1, 4 and 7 no protein extract added; lane 2, 3, 5, 6 and 8 5μg of total cell lysate from glucose-grown CBS2359 cells. Lane 3 competition with 200 ng of oligo containing Abf1p binding site; lane 6 competition with 200 ng of oligo containing Cpf1p binding site. When using a 124 bp fragment (probe A, figure 2A) containing the binding sites for Abf1p, Cpf1p and Hap2/3/4/5 in gel retardation assays, three complexes consisting of Abf1p and Abf1p/Cpf1p heterodimers can be observed as described previously [135]. Competition with an oligonucleotide containing the Abf1p binding site leads to the formation of three different complexes, two of which display a higher mobility than the Cpf1p/Abf1p heterodimer. The third has a lower mobility than the Abf1p monomer (figure 2, lane 3). The same pattern is observed upon mutation of the KIAbf1p binding site (ΔA, lane 5). The formation of the complexes is competed by an oligonucleotide harbouring the Cpf1p consensus site (lane 6). Furthermore, the formation of all three complexes is dependent on the presence of a functional KICpf1p binding site, as mutation of the Cpf1p site causes them to disappear (ΔAC, lane 8). The three complexes are supershifted with an antibody against Cpf1p, whereas a control antibody did not (data not shown). This indicates that Cpf1p is present in each complex, but it is unclear whether the three complexes consist of homodimers of KICpf1p or that heterodimers are formed between KICpf1p and another protein.

Present or lost?
As mentioned before, KIQCR8 is two- to three-fold induced when cells are shifted from glucose to media containing ethanol/glycerol. In sharp contrast to S. cerevisiae, the Hap2/3/4/5 complex is not required for this induction. This raises the question how this induction is accomplished. If not by the action of the Hap2/3/4/5 complex, are additional factors involved in carbon source dependent regulation of the QCR8 gene in K. lactis? This dilemma is addressed in chapter 4.
Promoter deletion analysis of the *KIQCR8* promoter reveals a 30 bp region, ranging from -144 bp and -113 bp from the ATG start codon, that is involved in carbon source dependent activation. Activation, as well as specific protein binding to this region, is furthermore dependent on a functional *KICAT8* gene. Although KICat8p is a functional homologue of Cat8p in *S. cerevisiae*, their functions, like the respective Hap2/3/4/5 complexes, diverge between the two yeasts. Whereas ScCat8p is involved in activation of genes that are strongly derepressed under non-fermentative growth conditions, in particular gluconeogenic genes, the function of KICat8p is restricted to the derepression of genes involved in the glyoxylate pathway, for growth on C2-sources. Our results now imply that, in *K. lactis*, KICat8p is involved in transcriptional regulation of respiratory function. However, although the consensus binding site (CSRE) for Cat8p is well conserved in both yeasts, no consensus sequence is present within the 30 nt region of the *KIQCR8* promoter that is involved in induction. In fact, no other known consensus sites are present except for a TA-rich element, which may harbour the TATA box docking site for RNA polymerase holoenzyme, and an AAGAAAT motif corresponding to the consensus sequence for Med8p, a subunit of the mediator RNA polymerase II carboxy-terminal domain (CTD) complex in *S. cerevisiae* [31]. This raises the possibility of carbon source dependent regulation of respiration by a subunit of the RNA polymerase holoenzyme in *K. lactis*. Besides promoter deletion analysis as a tool to identify cis-acting elements in the *KIQCR8* promoter, one could also apply genetic screens to find trans-acting factors required for the induction of respiration in *K. lactis*. Despite numerous attempts involving a double selection of dimished growth and dimished reporter activity, we were not successful in identifying which gene product plays a role in the mechanism of HAP-independent induction of respiration in *K. lactis*.

Irrespective of the identity of the factor(s) responsible for induction of the *KIQCR8* gene, an intriguing question remains whether the factor(s) is also present and functional in *S. cerevisiae*, or whether it is lost during the evolution of this yeast from an ancestral oxidative metabolism still exhibited by the present-day *K. lactis*, towards a specialized type of anaerobic metabolism. The observation that the additional cis-acting elements, able to activate transcription of the *KIQCR8* gene in a carbon dependent manner, are not functional in *S. cerevisiae*, as shown by reciprocal exchange of the *KIQCR8* gene to *S. cerevisiae* [135] would be in favour of the latter hypothesis.

**The power of genome-wide heterologous hybridization**

Since the complete sequence of the *S. cerevisiae* genome has become available, rapidly developing functional-genomic technologies are being applied to understand the behaviour of *S. cerevisiae* in response to physiological changes, to reveal new gene functions and interacting
pathways within the cell. The *K. lactis* genome has not been completely sequenced yet, hampering genome-wide studies to reveal molecular divergences underlying physiological differences within this species. However, studies of this kind would ultimately contribute to our understanding how yeast cells outbalance their metabolism, fermentative or oxidative. Therefore, we performed a pilot study on the use of gene filters containing the protein-coding sequences of the *S. cerevisiae* genome as a means of monitoring transcript levels in *K. lactis*. Unfortunately, deterioration of the gene filters used limited the number of hybridizations necessary to perform a full statistical analysis. Furthermore, high background levels complicated the analysis as well. Nevertheless, results described in chapter 5 demonstrated that it is feasible to use cross-hybridization with *S. cerevisiae* to analyse transcript levels in another, closely related, yeast. Not only were new homologues of *S. cerevisiae* genes identified in *K. lactis*, but also differences in transcript patterns between different carbon sources could be monitored and were in agreement to what was expected. Genes involved in glycolysis were more abundantly present on the filter when cDNA from glucose-grown cultures was hybridized compared to ethanol/glycerol-grown cultures. Furthermore, comparison of transcript patterns of *K. lactis* strains CBS2359 and JA6, exhibiting different sensitivities to glucose, provides support for previous statements that JA6 is a fermentative strain. Glycolytic transcripts were more abundant in the latter strain than in the less glucose-sensitive CBS2359, which, in turn, showed higher abundance of respiratory transcripts. Our results provide an encouraging basis for the optimization of heterologous hybridization conditions for other yeast species. The continuous refinement of techniques for genome-wide analysis in *S. cerevisiae* will without doubt be substantially useful to this. It should however be noted that homologous hybridization is preferred when comparisons are to be made between different yeast species. In those cases, since hybridization is dependent on DNA sequence-identity, results will be biased in favour of the most conserved genes. Furthermore, and obviously, genes that are unique within one particular yeast species will not be revealed by heterologous hybridization. For example, random sequencing of the *K. lactis* genome revealed 25 potentially new genes with no known homologues in *S. cerevisiae* [19]. Nonetheless, the results obtained in our pilot study underline the potential power residing in heterologous hybridization of *S. cerevisiae* ORFs as a tool for the identification of highly conserved homologues and for monitoring global transcript patterns in other yeast species that have not yet been completely sequenced.