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Research Article

The transcription factor Snf1p is involved in a Tup1p-independent manner in the glucose regulation of the major methanol metabolism genes of *Hansenula polymorpha*

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Abstract

Hansenula polymorpha is a methylotrophic yeast widely employed in biotechnology as a "protein factory". Most promoters used for heterologous protein expression, like *MOX* (methanol oxidase) and *DAS* (di-hydroxy acetone synthase), are involved in the peroxisomal methanol metabolism (C_1 metabolism) and are under strong glucose repression. Interestingly, the *MOX* promoter is subjected to glucose regulation also in *Saccharomyces cerevisiae*, a non-methylotrophic yeast in which this phenomenon is well studied. In this species, the transcription factor Tup1p plays an essential role in glucose repression of several genes. This effect is counteracted by the activator Snf1p when glucose is exhausted from medium. Therefore, to test whether this regulatory circuit has been conserved in *H. polymorpha*, Hp*TUP1* and Hp*SNF1* were partially cloned and disrupted. Deletion of Hp*TUP1* did not affect glucose repression of the major C_1 metabolism genes (*MOX*, *DAS*). Thus, though conserved, Hp*TUP1* does not seem to take part in a general glucose repression in *H. polymorpha*. In contrast, the deletion of Hp*SNF1* led to significant decreases in the activation of these genes in the absence of glucose. Therefore, the effect of Hp*SNF1* in transcriptional activation may be through an Hp*TUP1*- independent circuit.

Key words: TUP1, *SNF1*, *Hansenula polymorpha*, glucose repression, *MOX*.

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Introduction

Hansenula polymorpha (syn. *Pichia angusta*) belongs to a limited group of yeast capable of using methanol as the sole carbon and energy source (Veenhuis *et al*., 1983). The expression of the major enzymes accounting for peroxisomal methanol metabolism (C_1 metabolism), namely methanol oxidase (Moxp), catalase (Catp) and di-hydroxi acetone synthase (Dasp), is controlled at the transcriptional level. The genes encoding these three enzymes are fully induced when cells are grown on methanol, and repressed to different extents in the presence of glucose (Genu *et al.*, 2003; Janowicz *et al.*, 1985; Roggenkamp *et al.*, 1984). For instance, Moxp can represent up to 30% of the total soluble cellular proteins in continuous cultures growing on methanol (Giuseppin *et al*., 1998). In the presence of glucose, however, no methanol oxidase activity can be detected in crude extracts (Veenhuis *et al*., 1989). In view of this, the *MOX* promoter has been widely employed for heterologous protein expression, and *H. polymorpha* has been considered a "versatile protein factory" (Gellissen, 2000; van Dijk *et al*., 2000). Nevertheless, although it is a very suitable source of carbon for general industrial fermentation, glucose cannot be regularly employed for protein expression in *H. polymorpha*. It would therefore be a significant technological improvement if the *MOX* promoter could also function in glucose-containing media.

The *MOX* promoter has been studied in detail. Promoter mapping has revealed four *cis*-acting regulatory elements (Godecke *et al.*, 1994), but no *H. polymorpha* factor directly involved in *MOX* regulation has been identified to date. Remarkably, the *MOX* promoter can drive glucose-repressible expression of a reporter gene in *Saccharomyces cerevisiae* (Pereira and Hollenberg, 1996), a non-methylotrophic yeast. In this species, activation of the *MOX* promoter is dependent on Adr1p (Pereira and Hollenberg, 1996), a transcription factor that regulates the expression of a number of genes encoding peroxisomal enzymes (Simon *et al*., 1991). This finding indicates that mo-

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lecular mechanisms governing regulation by glucose may be conserved in both species.

In *S. cerevisiae*, the Mig1p-Ssn6p-Tup1p complex represses several genes involved in the utilization of different carbon sources (*e.g.* sucrose, galactose) when cells are grown in the presence of glucose (Bu and Schmidt, 1998; Rohde *et al*., 2000). When glucose is exhausted from the growth medium, the same genes need the protein kinase Snf1p to be properly derepressed (Gancedo, 1998; Ostling *et al*., 1996). Both the Mig1p-Ssn6p-Tup1p and the Snf1 complexes are highly conserved among several species and seem to contribute, in addition to glucose regulation, to many other processes (Cassart *et al*., 1997; Lo *et al*., 2001; Yamashiro *et al*., 1996; Zaragoza *et al*., 2000). As an example, the deletion of a *TUP1* homolog in *Candida albicans* causes failure in hypha development (Braun and Johnson, 1997).

In this study, *H. polymorpha* genes highly homologous to *TUP1* and *SNF1* were cloned, disrupted and analyzed for their possible conserved roles in the regulation of genes involved in C_1 metabolism. The results show that mutants lacking Hp*TUP1* did not exhibit altered levels of *MOX* and *DAS* transcription when grown on methanol or glucose. On the other hand, mutants lacking Hp*SNF1* presented lower *MOX* and *DAS* induction on methanol, when compared to wild-type cells. Taken together, these results indicate that the participation of Snf1p in the regulation of the major *H. polymorpha* C_1 metabolism genes occurs in a Tup1p-independent manner, therefore implying a minor involvement of these conserved proteins in the mechanism of glucose repression of methanol metabolism.

Materials and Methods

Yeast strains and growth conditions

The *H. polymorpha* strain used to disrupt homologs of the *TUP1* and *SNF1* genes was isogenic to strain CBS 4732 (wild type – WT). YPD (1% yeast extract, 2% peptone, 2% glucose) was used as rich medium. SD (0.17% Yeast Nitrogen Base, 0.5% ammonium sulphate) was used as synthetic medium and was supplemented with 5% glucose or 1% methanol. Cells were grown at 37 °C.

Bacterial strains, plasmids and oligonucleotides

Escherichia coli strain DH10B (Invitrogen) was used for all DNA manipulations (grown at 37 °C in LB medium). The plasmid pF6A-kanmx4 (Wach *et al.*, 1994) was used for construction of the disruption modules (see below). pGEM-T (Promega) and pUC18 were used for routine cloning of PCR products. The oligonucleotides used in this work are listed in Table 1 and were synthesized by Operon.

Cloning of *H. polymorpha TUP1* and *SNF1* genes

The cloning strategy was based on degenerated oligonucleotides. We designed degenerated primers for conserved regions of the *TUP1* and *SNF1* gene sequences from *S. cerevisiae*, *Schizosaccharomyces pombe* and *C. albicans* (Table 1). These primers were used to conduct PCRs, using *H. polymorpha* genomic DNA as template. Fragments containing 600 bp and 800 bp were amplified using primers DTup1 and DSnf1, respectively. These DNA fragments were cloned into pGEM-T (Promega) and sequenced. The DNA sequences obtained were then analyzed by Blast X and found to present high similarities with *C. albicans TUP1* (4e-84) *and SNF1* (5e-54). The sequences of the 5' and 3' extremities of each fragment were used to design the specific primers shown in Table 1 (Stup1F, Stup1R; Ssnf1F, Ssnf1R). Each of these primers was used in combination with T7 or SP6 custom primers to amplify cDNA fragments from a *H. polymorpha* cDNA library, constructed in our laboratory using the vector pSPORT (Invitrogen). By this procedure, the putative Hp*TUP1* and Hp*SNF1* gene fragments were expanded. The predicted amino acid sequences encoded by the *H. polymorpha TUP1* and *SNF1* genes were compared with known homologs of other yeast species (see Figure 1) using the Multalin tool (www.prodes.toulouse.inra.fr/multalin/multalin.html).

Disruption and screening of Hp*tup1 and* Hp*snf1* strains

The Hp*TUP1* and Hp*SNF1* DNA sequences obtained as described above were also used to design chimerical disruption oligonucleotides. These oligonucleotides (see Ta-

Table 1 - Primers used in this work.

Primer	Sequence
Degenerated	
DSnf1.1	5'GGRRTTTCAATTRYRTCNGTRTCRAAGC3'
DSnf1.2	5'GCYTGNARRTGYAARTTYGCNTGRTGNGG3'
DTup1.1	5'GGGGTACCYTTCCADATNCKNGCYTTRCARTCN CC3'
DTup1.2	5' GGCTGCAGGGCNAYGARCARGAYATHTAYTC 3'
Disruption	
CdSnf1.1	5'TCACCATCAAGGAGATCATGGAGGACGAGT AGCTTGCCTCGTCCCCGCCGGGTC 3'
CdSnf1.2	5'GGTTGTATTTGGTTCCATTATTCGTCGACAG CAGTATAGCGACCAGC3'
CdTup1.1	5'TTTCTGGCTCAGAAGCCAGCCCAGTAAGATT TTGGAGCTCGCTCGTCCCCGCCGGGTC3'
CdTup1.2	5'CCCGACACCTCTCTTCTGGGTCATAACCGGT TCGACAGCAGTATAGCGACCAGC3'
Specific	
DasF	5'ACTTCAACAGAGACAGGTTTG 3'
DasR	5' AAATTGACAGACACAGACAGGTC 3'
Snf1.1	5' TCACCATCAAGGAGATCATGGAGG 3'
Snf1.2	5' GGTTGTATTTGGTTCCATTATTCG 3'
Tup1.1	5' CTTCGACTCAGCCAATGAAAGCG 3'
Tup1.2	5' TCTGATAACAGAGTTTCTGTGTCC 3'

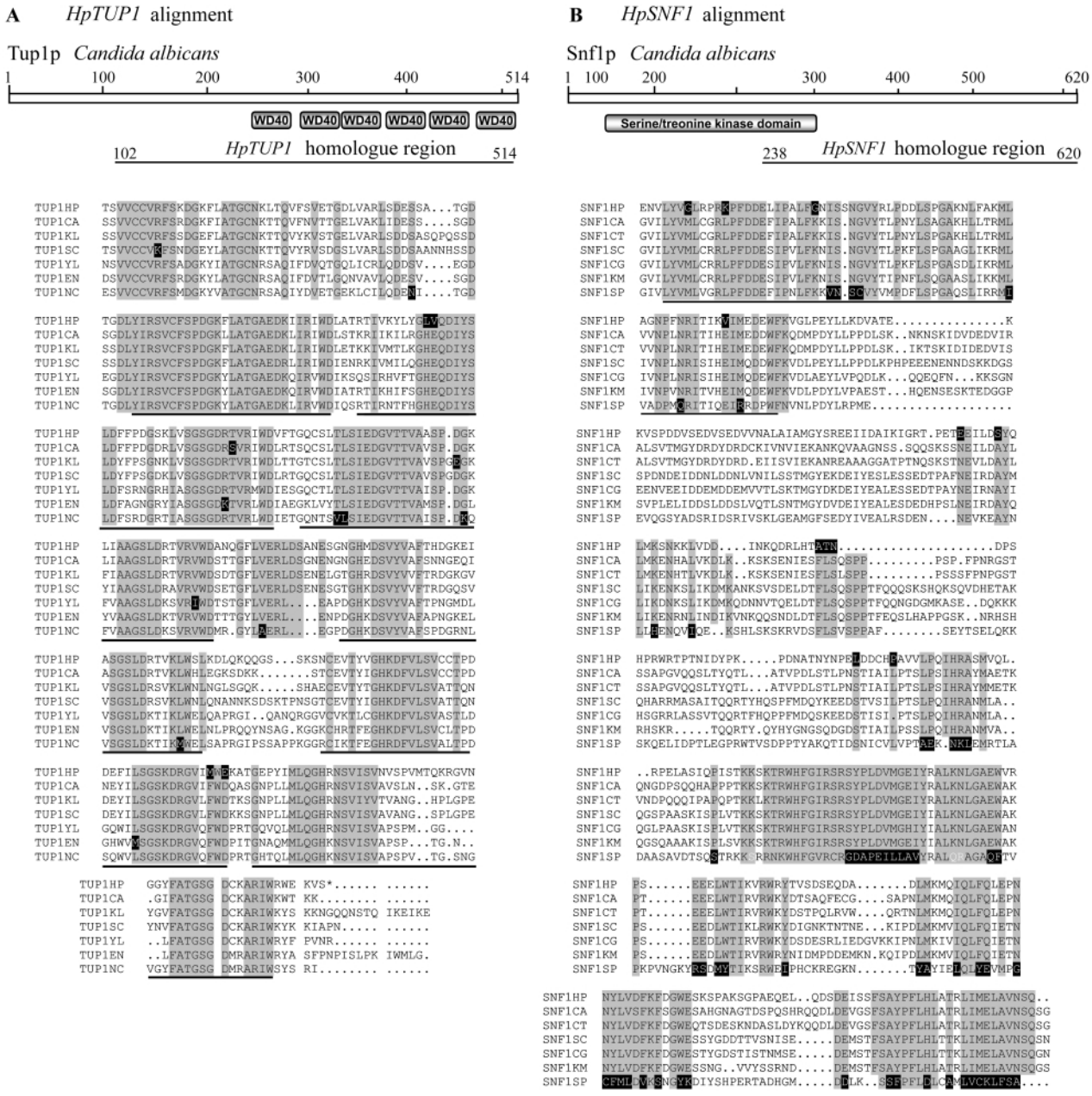

Figure 1 - Identification of Hp*TUP1* and Hp*SNF1* gene fragments. Alignments of the predicted HpTup1p (A) and HpSnf1p (B) proteins with other known homologs: HP - *Hansenula polymorpha*, CA - *Candida albicans*, CG - *Candida glabrata*, CT - *Candida tropicalis*, EM - *Emericella nidulans*, KL - *Kluyveromyces lactis*, NC - *Neurospora crassa*, SC - *Saccharomyces cerevisiae*, SP - *Schizosaccharomyces pombe*, YL - *Yarrowia lipolytica*. The known conserved domains identified in *C. albicans* proteins are schematically shown on top of the alignments (see text for details). Black boxes: single differences between species; gray boxes: highly conserved regions; underlined amino acids: essential functional domains. Alignments were done with the Multalin tool (www.prodes.toulouse.inra.fr/multalin/multalin.html), and the DNA sequences were deposited in the NCBI GenBank with the accession numbers: Hp*TUP1* - AY145087 and Hp*SNF1* - AY155202.

ble 1 and Figure 2) present ~30 bp of the genes to be disrupted in their 5' extremities; the 3' extremities are composed of ~25 bp complementary to the Multiple Cloning Site of the pF6A plasmid in which the kanmx4 disruption module was integrated (Wach *et al*., 1994). Specific *TUP1* and *SNF1* kanmx4 modules were constructed with these primers (CDTup1.1+CDTup1.2 and CDSnf1.1+CDSnf1.2, respectively) by standard PCR using the pF6A-kanmx4 plasmid as template. Either of these linear disruption modules bears sequences, at both the 5' and the 3' ends, which are homologous to *TUP1* and *SNF1* genes. These were used to transform *H. polymorpha* WT electro-competent cells (Cregg and Russell, 1998). The kanmx4 module confers resistance to the drug G418, used for preliminary screening.

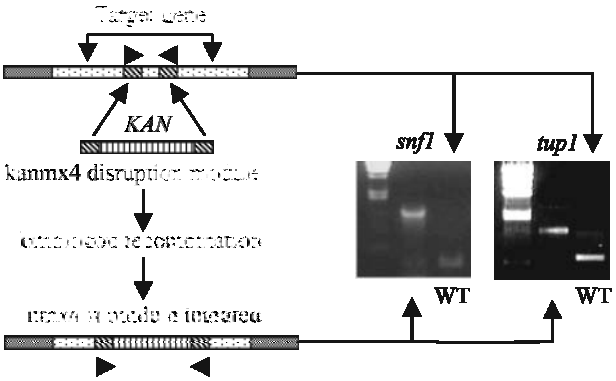

Figure 2 - Disruption strategy and screening of *H. polymorpha tup1* and *snf1* mutants. The specific kanmx4 disruption modules were constructed by PCR using chimeric oligonucleotides (see Materials and Methods for details). Screening for positive mutants growing in G418 was done by PCR, using specific primers for the Hp*TUP1* and Hp*SNF1* genes. These primers (STup1.1, STup1.2; SSnf1.1, SSnf1.2) amplify respectively 500 bp and 300 bp of the wild-type genomic copies of each gene. Mutants in which the genes were efficiently disrupted present a single 1.5 kb band as PCR product.

Positive transformants growing in the presence of G418 (Amersham) were confirmed by colony PCR and also by PCR with genomic DNAs, using the specific primers STup1.1+STup1.2; SSnf1.1+SSnf1.2 (Table 1).

PCR conditions

All PCRs were performed in a final volume of 50 µL, using kits (Promega) and following instructions provided by the manufacturer. The reactions were carried out in a 9700 thermocycler (Applied Biosystems) using the following programs: i) for fragment amplification – 96 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 40 °C (degenerated primers) or 50 °C (specific primers) for 30 s, and 72 °C for 1 min; ii) for construction of the disruption modules - 96 °C for 4 min, followed by 30 cycles: 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 1.5 min.

Induction experiments and Northern Blotting

Cells were grown overnight in 100 mL YPD shaking at 280 rpm, harvested (centrifuged at 5000 rpm, 10 min) and inoculated in the appropriate medium at an OD_{600} = 2.0. After 6 h of growth, cells were collected for RNA extraction. Total RNA was prepared by the hot acid phenol method (Ausubel *et al*., 1998). Samples were resolved by formaldehyde gel electrophoresis, blotted onto a positively charged nylon membrane (Hybond N^+- Amersham Pharmacia) and fixed following standard protocols. The probes used were the following DNA fragments (numbers refer to position relative to each ORF start codon): *MOX* - a 2.3 kb *Eco*RV fragment (+21 to +2323), *CAT* - a 1.7 kb *Eco*RI/*Eco*RV fragment (+172 to +1695), and *DAS* - a 1.0 kb (+197/+1196) PCR fragment obtained with the primers Dasf and Dasr. All probes were labeled by random primer extension with α-^{32}P[dATP] (Amersham Pharmacia). Hybridization was carried out at 42 °C following standard protocols (Ausubel *et al*., 1998). rRNA bands in agarose/formaldehyde gels stained with ethidium bromide prior to blotting were used as internal loading control.

Growth characterization

The different strains were grown to log-phase in YPD, harvested and resuspended in the appropriate media (indicated in Results) to an OD_{600} = 0.01. Aliquots were collected each hour for 24 h and the A_{600} was measured. The results presented are the average of three independent experiments.

Patch growth assays

The assays were performed as described elsewhere (Demasi *et al*., 2001; Verdoucq *et al*., 1999).

Results

Identification of *H. polymorpha TUP1* and *SNF1* homologs

H. polymorpha DNA fragments were obtained by PCR, using degenerated primers for important regions of the *TUP1* and *SNF1* genes (Table 1 and Figure 1). The putative Hp*TUP1* fragment encompassed six WD repeats (Figure 1), a typical motif of this protein family, which is involved in protein-protein interaction and signalling (Smith *et al*., 1999). The putative Hp*SNF1* fragment bears a region with high similarity to other related proteins, including part of the serine/threonine domain which is present in all *SNF1*-like kinases (Hanks and Hunter, 1995) (Figure 1). Fragments of both genes presented the highest similarities with homologs from the *Candida genera*, which is phylogenetically close to *Hansenula*. Sequence comparison analyses led us to the conclusion that the DNA fragments obtained encompass the most important functional regions of *H. polymorpha TUP1* and *SNF1* genes.

Construction of *TUP1* and *SNF1 H. polymorpha* mutants by PCR

The Hp*TUP1* and Hp*SNF1* fragments were used to construct disruption modules, as described in Materials and Methods. *H. polymorpha* wild-type cells were transformed and positive colonies growing on G418 were selected. About 150 transformants for each gene were screened for the presence of the disruption module by PCR amplification using specific primers for the Hp*TUP1* and Hp*SNF1* gene fragments. Figure 2 depicts the disruption strategy and the PCR products of correct integrations, observed for 7% of the transformants, in comparison to amplification of the wild-type fragments. Growth abilities of *tup1* and *snf1* cells on plates containing G418 were also re-checked in comparison to wild-type cells (not shown).

Expression of C_1 metabolism genes in *tup1* and *snf 1* mutants

It has been established in several yeast species that Tup1p plays a key role in repressing genes involved in the utilization of a number of carbon sources in response to the availability of glucose in a growth medium (Bu and Schmidt, 1998; Rohde *et al*., 2000). Conversely, it is also well known that Snf1p accounts for relieving the repression caused by the Mig1p-Ssn6p-Tup1p complex at different promoters (Gancedo, 1998; Ostling *et al*., 1996). Since the major *H. polymorpha* C_1 metabolism genes are severely repressed by glucose, it was hypothesized that Tup1p and Snf1p homologous proteins might also be involved in the mechanism of glucose repression/derepression of these genes. To address this issue, the expression of the genes *MOX*, *DAS* and *CAT* was analyzed by Northern Blot in wild-type, *tup1* and *snf1* cells grown on glucose or methanol.

In *tup1* cells grown on either glucose or methanol, the transcription levels of the three genes were similar to those observed in the wild type (Figure 3). This indicates that the repression mediated by glucose on *H. polymorpha MOX*, *DAS* and *CAT* promoters is Tup1p-independent. Interestingly, *snf1* cells presented a significant reduction in the levels of *MOX* and *DAS* transcription in cells grown on methanol (Figure 3), suggesting that *SNF1* might be needed for activated transcription of these genes under this condition. These results indicate that Snf1p action in the derepression of the genes *MOX* and *DAS* happens through a different molecular mechanism (*e.g.*, different from relieving the Mig1p-Ssn6p-Tup1p complex repression at promoters). On the other hand, the levels of *CAT* transcription remained unaffected in *snf1* cells under all conditions tested (Figure 3), which indicates that the glucose repression/derepression mechanism acting on this promoter is also independent of Snf1p.

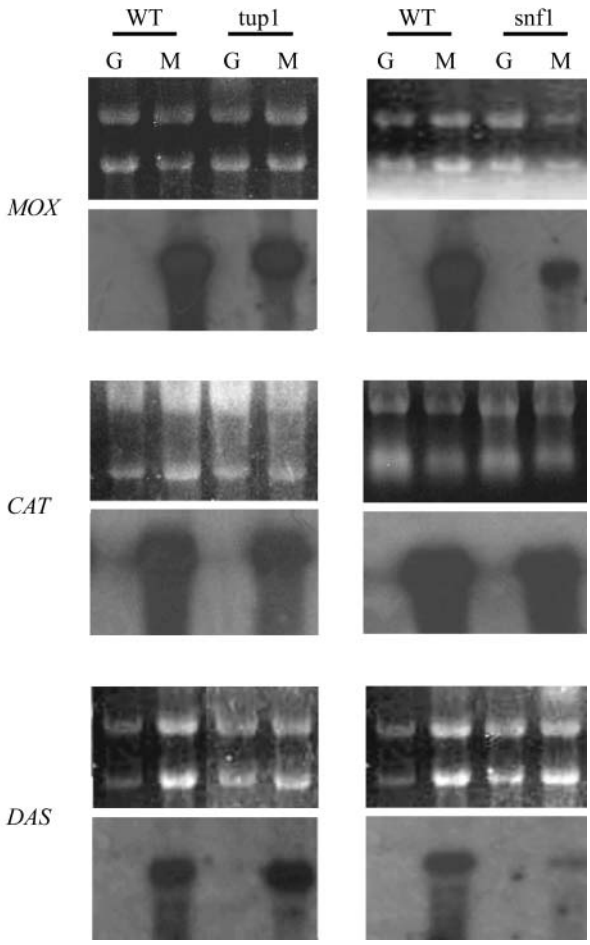

Figure 3 - Expression of *MOX*, *CAT* and *DAS* in WT, *tup1* and *snf1* cells. Northern blotting of samples collected after shifting cells from repressing (cells grown overnight in glucose 5% - time 0) to derepressing conditions (methanol 1%) for 6 h: G - glucose; M - methanol. rRNA bands of samples used in each filter are shown in agarose-formaldehyde gels stained with ethidium bromide prior to blotting. The probes used are described in Materials and Methods.

Effects of *tup1* and *snf1* disruptions on glucose and methanol utilization

In *S. cerevisiae*, disruption of the genes *TUP1* or *SNF1* lead also to several pleiotropic phenotypes, in addition to transcriptional regulation mediated by glucose. For instance, a *TUP1* disruption caused cell flocculation (Lipke and Hull-Pillsbury, 1984), whereas a *SNF1* disruption led to a general slow growth (Schuller and Entian, 1987). Thus, to examine whether *H. polymorpha tup1* and *snf1* cells presented altered growth abilities on glucose or methanol, or showed any additional phenotype, we monitored the growth rates of cells from each strain in liquid media (Figure 4B). Surprisingly, no difference was observed among the wild-type, *tup1* and *snf1* cells grown in either glucose- or methanol-containing media. Moreover, we could not detect any *tup1* or *snf1* pleiotropic phenotype under the conditions tested. To confirm this observation, we carried out patch growth assays (Demasi *et al*., 2001; Verdoucq *et al*., 1999) on plates containing glucose or methanol as carbon sources (Figure 4A). Once again, we did not observe differences in either glucose or methanol utilization among the three strains. Taken together, these results indicate that, contrarily to *S. cerevisiae*, *tup1* or *snf1* disruptions failed to lead to any direct effect in the carbon utilization pathways of *H. polymorpha*.

Discussion

The transcriptional regulation of *H. polymorpha* C_1 metabolism promoters, especially the *MOX* promoter, has been intensively studied because of their potential in mediating industrial heterologous protein expression. The most important feature of these promoters is the strong glucose repression mechanism that functions when glucose is available in the growth medium, which makes this sugar useless as a carbon source for industrial fermentation of *H. polymorpha*. Thus, understanding the precise molecular

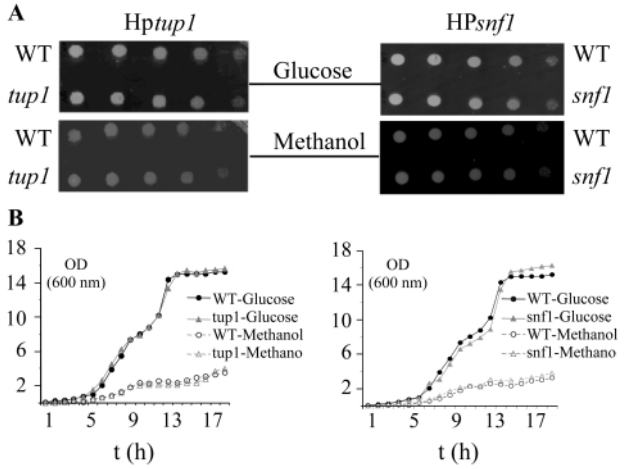

Figure 4 - Effects of *tup1* and *snf1* deletions on growth on glucose and methanol. **A:** Patch growth assays were performed with wild-type, *swi2* and *swi3* cells in agar plates containing 5% glucose or 1% methanol (Verdoucq *et al*., 1999; Demasi *et al*., 2001). Plates were photographed after 48 h of incubation at 37 °C. **B:** Growth rates of WT, *tup1* and *snf1* strains on liquid media containing 5% glucose or 1% methanol were determined by monitoring growth for up to 24 h. The results are the average of three independent experiments.

mechanisms governing glucose repression of C_1 metabolism promoters may be useful to expand their expression profiles and allow the utilization of these promoters under conditions where they are presently ineffective. For these reasons, we investigated possible contributions of the conserved *H. polymorpha TUP1* and *SNF1* genes in the glucose repression mechanism.

TUP1 mutations eliminate or reduce glucose repression of many *S. cerevisiae* promoters. In addition, they induce several other phenotypes, like flocculation, failure to sporulate, and sterility of MAT-α cells (Williams and Trumbly, 1990). Surprisingly, no apparently abnormal phenotype was observed in *H. polymorpha tup1* cells. These cells were indistinguishable from the wild-type by microscopic analysis, and no variations in growth behavior or in expression of the C_1 metabolism genes were observed. It is important to note that, although the deletion achieved does not encompass the complete ORF, the removed region bears the WD40 repeats, which are essential and sufficient for *TUP1* repressing function in *S. cerevisiae* (Sprague *et al*., 2000; Williams and Trumbly, 1990). These results were unexpected, since clear effects of *TUP1* mutations have been demonstrated in other yeast species closer to *H. polymorpha* than *S. cerevisiae*. For example, *Candida albicans TUP1,* which works in a complex similar to that identified is *S. cerevisiae*, is involved in general regulation of metabolism, cellular morphogenesis and stress response (Murad *et al*., 2001). Although *TUP1* is not essential in this species, its deletion led to female sterility, reduced growth rates and failure to turn on filamentous growth (Braun and Johnson, 1997; Yamashiro *et al*., 1996).

With regard to proper *SNF1* function in *S. cerevisiae*, it is required for appropriate derepression of several glucose-repressible genes, such as *SUC2* (encoding invertase), *GAL1* (encoding galactokinase) and *ADH2* (encoding alcohol dehydrogenase 2) (Abrams *et al*., 1986; Denis and Audino, 1991; Flick and Johnston, 1990). The *snf1* mutation leads to pleiotropic effects, such as a petite-like phenotype and a generally slow growth, which gives the mutant cells a "sick" appearance (Carlson *et al*., 1981). One of the main roles of Snf1p in *S. cerevisiae* is to relieve repression mediated by the Mig1p-Ssn6p-Tup1p complex, by phosphorylating the Mig1p protein and releasing the complex from promoters (Ostling and Ronne, 1998). Additionally, Snf1p is also involved in the operation of transcription factors like Adr1p (Young *et al*., 2002), and possibly other still unidentified factors (Gancedo, 1998). For example, disruption of the *Candida glabrata SNF1* homolog resulted in the loss of the ability to utilize trehalose, indicating that even in an organism with a limited carbon utilization spectrum, the regulatory mechanism governing catabolite repression is preserved (Petter and Kwon-Chung, 1996).

Consistent with the possibility of playing a role in derepression in *H. polymorpha*, the deletion of *SNF1* led to a significant reduction of the *MOX* and *DAS* expression in cells growing in methanol. This effect seems to be specific for some genes, since *CAT* expression remained unaffected by the deletion. These results reinforce the view that *MOX* and *DAS* are tightly co-regulated, and that possibly Snf1p plays a role in their regulatory circuits. However, this role may not be due to the inactivation of the Mig1p-Ssn6p-Tup1p complex, as has been reported for several *S. cerevisiae* genes (Gancedo, 1998). As described above, Hp*TUP1* does not seem to repress either *MOX* or *DAS* expression. Probably, Hp*SNF1* exerts its effect by interacting with a putative Adr1p-like factor that is believed to regulate *MOX* (Pereira and Hollenberg, 1996). In *S. cerevisiae*, several genes, including some that encode peroxisomal proteins, are regulated by both factors (Denis and Audino, 1991; Navarro and Igual, 1994; Simon *et al*., 1992). Moreover, ScSnf1p has been recently demonstrated to promote binding of Adr1p to chromatin (Young *et al*., 2002). Taken together, these facts indicate that both factors (Snf1p and Adr1p) act in conjunction in the regulation of some genes, a situation that might be conserved in other yeast species.

However, despite the reduction in *MOX* and *DAS* expression, deletion of Hp*SNF1* did not lead to any obvious external phenotype, in contrast to what has been observed in *C. albicans* (Petter *et al*., 1997) and *C. tropicalis* (Kanai *et al*., 1999), species which are phylogenetically close to *H. polymorpha*. Another interesting observation was that the *snf1* growth rate on methanol-containing media was indistinguishable from wild-type cells, indicating that the reduction in *MOX* expression was not sufficient to create a bottleneck in peroxisomal methanol metabolism.

In summary, our work indicates that, in contrast to the observations made for other yeast species, *TUP1* and *SNF1* do not play essential roles in carbon source regulation in *H. polymorpha*. Nonetheless, Snf1p may participate in the activation of genes repressed by glucose, but at least in the case of the genes *MOX* and *DAS*, its mechanism of action differs from counteracting the repression mediated by the Tup1p-containing complex.

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