Gts1p stabilizes oscillations in energy metabolism by activating the transcription of *TPS1* encoding trehalose-6-phosphate synthase 1 in the yeast *Saccharomyces cerevisiae*

Zhaojun XU, So-ichi YAGUCHI and Kunio TSURUGI¹

Department of Biochemistry 2, University of Yamanashi, Faculty of Medicine, 1110 Shimokato, Tamaho, Yamanashi 409-3898, Japan

We reported previously that Gts1p regulates oscillations of heat resistance in concert with those of energy metabolism in continuous cultures of the yeast *Saccharomyces cerevisiae* by inducing fluctuations in the levels of trehalose, but not in those of Hsp104 (heat shock protein 104). Further, the expression of *TPS1*, encoding trehalose-6-phosphate synthase 1, and *HSP104* was activated by Gts1p in combination with Snf1 kinase, a transcriptional activator of glucose-repressible genes, in batch cultures under derepressed conditions. Here we show that, in continuous cultures, the mRNA level of *TPS1* increased 6-fold in the early respiro-fermentative phase, while that of *HSP104* did not change. The expression of *SUC2*, a representative glucose-repressible gene encoding invertase, also fluctuated, suggesting the involvement of the Snf1 kinase in the periodic activation of these genes. However, this possibility was proven to be unlikely, since the oscil-

lations in both *TPS1* and *SUC2* mRNA expression were reduced by approx. 3-fold during the transient oscillation in *gts1* Δ (*GTS1*-deleted) cells, in which the energy state determined by extracellular glucose and intracellular adenine nucleotide levels was comparable with that in wild-type cells. Furthermore, neither the mRNA level nor the phosphorylation status of Snf1p changed significantly during the oscillation. Thus we suggest that Gts1p plays a major role in the oscillatory expression of *TPS1* and *SUC2* in continuous cultures of *Saccharomyces cerevisiae*, and hypothesized that Gts1p stabilizes oscillations in energy metabolism by activating trehalose synthesis to facilitate glycolysis at the shift from the respiratory to the respiro-fermentative phase.

Key words: *GTS1*, Snf1 kinase, *SUC2*, *TPS1*, transcriptional activator, ultradian oscillation.

INTRODUCTION

We have reported previously that the *GTS1* product, Gts1p, plays an important role in the regulation of heat tolerance in the yeast *Saccharomyces cerevisiae* both in the stationary phase of batch cultures [1] and in glucose-limited continuous cultures [2]. Recently we showed that heat tolerance was decreased in *gts1* Δ (*GTS1*-deleted) cells and increased in TMp*GTS1* (*GTS1*-overexpressing) cells under glucose-derepressed conditions, and that disruption of *SNF1*, which encodes a putative metabolic sensor and regulator of *S. cerevisiae* (for review, see [3]), diminished this effect of *GTS1* [4]. Under the derepressed conditions, intracellular levels of Hsp104 (heat shock protein 104) and trehalose, which were reportedly required for the acquisition of heat tolerance in the stationary phase of cell growth (for reviews, see [5,6]), were affected in both *GTS1* transformants roughly in proportion to the gene dosage of *GTS1* [4]. Further, we found that the mRNA levels of both *TPS1* and *HSP104* changed as a function of *GTS1* gene dosage, and that the Q-rich domain of Gts1p, consisting of 62 amino acids of which 48% are glutamine residues, was involved in the transcriptional activation. Furthermore, Gts1p was associated with subunits of Snf1 kinase *in vivo*, but it did not bind to DNA. Therefore we suggested [4] that *GTS1* increases heat tolerance by mainly activating the Snf1 kinase-dependent derepression of *HSP104* and *TPS1* under glucose-derepressed conditions, and that Gts1p functions as transcriptional modulator for Snf1 kinase, which then functions in the RNA polymerase II holoenzyme complex [7].

On the other hand, we reported that *GTS1* facilitates the self-organization of oscillations in energy metabolism due to a periodic

change between the respiratory and respiro-fermentative phases [8]. The oscillation arises spontaneously in concert with cell division in continuous cultures at a DR [dilution rate; the rate of flow of medium (ml/h) through the culture vessel in proportion to the total volume of culture (ml)] lower than approx. 0.2 h^{-1} [9,10], in the presence of a high cell density ($\sim 5 \times 10^8$ cells/ml) [10]. We also reported that Gts1p is involved in the coupling of heat- and other stress-resistance oscillations with the energy metabolism oscillation [2,10,11], and that the oscillation in heat resistance is induced by a fluctuation in the trehalose level and not by the oscillatory expression of heat-shock proteins [12]. In *gts1* Δ , the oscillation of the trehalose level stopped together with that of heat resistance, suggesting that Gts1p induced the heat-resistance oscillation by regulating the trehalose level. On the other hand, a strong link between trehalose synthesis and the glycolytic pathway has been proposed, as mutations of Tps1p have been reported to prevent glucose influx for glycolysis [13,14]. The finding raises the possibility that Gts1p stabilizes the energy-metabolism oscillation by activating *TPS1* at an appropriate time. However, whether and how the expression of *TPS1* and *HSP104* is regulated during the oscillation has not been elucidated.

Snf1 kinase is a yeast homologue of mammalian AMPK (AMP-activated protein kinase), which is known to regulate various metabolic reactions depending on the energy state of the eukaryotic cell, both acutely through the phosphorylation of metabolic enzymes and chronically via effects on gene expression [3,15]. In yeast, Cortassa and Aon [16] reported that the onset of fermentative metabolism occurred at a DR value higher than 0.2 h^{-1} when glucose is relatively abundant; this was related to the glucose-repressive effect, as *snf1* Δ yeast started fermenting at DR

Abbreviations used: AMPK, AMP-activated protein kinase; DR, dilution rate; DO, dissolved oxygen; Hsp104, heat shock protein 104.

¹ To whom correspondence should be addressed (email ktsurugi@yamanashi.ac.jp).

values of 0.1 h^{-1} and above in continuous cultures, although the cultures were asynchronous for undefined reasons. These results suggested that the Snf1 kinase is required to maintain cells in a respiratory state at low DR values when the glucose supply is limited. However, whether Snf1 kinase has a function in the shift between metabolic phases in synchronous cultures has not been studied. In relation to this problem, the enzyme activity of invertase, encoded by *SUC2*, reportedly fluctuated in synchronous continuous cultures [17]. Among the products of many genes involved in the repression and derepression of *SUC2* [18,19], Snf1 kinase has a central role in the derepression mechanism by which the kinase removes the Mig1p repressor from the promoter of *SUC2* to the cytoplasm after phosphorylation (for review, see [20]). Thus it remains possible that Snf1 kinase is regulated in a periodic fashion in synchronous continuous cultures.

In the present study, we first found that the transcription of *TPS1* and *SUC2* was regulated in a periodic fashion in synchronous continuous cultures, while that of *HSP104* was not. Then we investigated whether Gts1p and/or Snf1 kinase are involved in the transcriptional activation of these genes, and found that the deletion of *GTS1* severely inhibited the expression of the genes, but obtained no evidence for the positive participation of Snf1 kinase. Therefore we suggest that the expression of *TPS1* and *SUC2* is regulated in an oscillatory manner by Gts1p in synchronous continuous cultures, and hypothesize that Gts1p facilitates energy-metabolism oscillation by activating *TPS1* at an appropriate time.

EXPERIMENTAL

Yeast strains and media

Strain W303-1A (*MATa SUC2 ade2 can1 his3 trp1 lue2 ura3*) of *S. cerevisiae* was used for the production of mutants and transformants, and strain S288C (*MAT α mal gal2 SUC2*) was used for continuous cultures. In the batch cultures, cells were incubated at 30°C in a synthetic medium consisting of a yeast nitrogen base without amino acids (Difco, Detroit, MI, U.S.A.) containing 2% (w/v) glucose with the required amino acids and bases. To collect the glucose-derepressed cells, cells pre-cultured overnight on 2% glucose were diluted 50-fold with the synthetic medium containing 0.2% (w/v) glucose and cultured for 18 h [4]. To obtain glucose-repressed cells, the pre-cultured cells were incubated for 8 h on 2% (w/v) glucose [4].

Production of *snf1 Δ* and *GTS1*-dosage transformants

An *SNF1*-disrupted mutant, *snf1 Δ* (*SNF1::LEU2*), *gts1 Δ* (*GTS1::URA3*) and Tmp*GTS1* were obtained by transforming W303-1A as described previously [21]. Tmp*GTS1 Δ Q* (a mutant expressing Gts1p lacking the Q-rich sequence comprising amino acids 316–396) was obtained as described previously [4].

Conditions for continuous culture

S288C cells were cultured at 30°C , pH 5.5, in a synthetic medium containing 1% (w/v) glucose as defined elsewhere [22] using an improved bench-top fermenter (MDL-6C; Marubishi Bioengineering, Tokyo) in a constant volume of 500 ml [2,10]. Continuous cultures were performed at a DR of 0.1 h^{-1} with aeration at 1 litre/h and stirring at 420 rev./min. The energy-metabolism oscillation was monitored by measuring the level of DO (dissolved oxygen) with an oxygen electrode. To collect cell samples, culture medium eluted from the fermenter was chilled on ice immediately after removal, and cells were collected by either centrifugation (3000 g, 5 min) or filtration in a cold-room.

Western blot analysis

A phosphorylated isoform of Snf1p was detected using anti-phospho-AMPK(Thr-172) antibody, which was purchased from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.). The cell lysate was prepared by shaking with glass beads in a lysis buffer in the cold-room, and Western blot analysis was performed according to the manufacturer's instructions. Western blots were visualized with a lumino-image analyser (RAS-1000; Fuji Film, Tokyo, Japan).

Determination of mRNA levels by Northern blotting

Total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) and 20 μg of RNA was electrophoresed on a 1% (w/v) agarose/formaldehyde gel. Probe labelling and hybridization were performed with the PCR DIG labelling mix and DIG system according to the manufacturer's instructions (Roche, Mannheim, Germany). Northern blots were visualized with a lumino-image analyser. mRNA levels were normalized to the level of *ACT1* mRNA as a control.

Determination of intracellular adenine nucleotides by HPLC

To extract intracellular nucleotides, cells precipitated from 1.5 ml samples of culture medium were suspended in 0.2 ml of ice-cold 0.4 M HClO_4 and vortex-mixed with glass beads. The glass beads were then removed by centrifugation (15 000 g, 10 min), and the supernatants were neutralized with 4.2 M KOH and kept at -80°C for 1–2 h to promote the precipitation of perchlorate. After another centrifugation (15 000 g, 10 min), the supernatants were passed through a $0.45 \mu\text{m}$ Millipore filter (Millipore Inc., Billerica, MA, U.S.A.). Samples were stored at -80°C until subjected to chromatography using an LC-800 HPLC system (JASCO, Tokyo, Japan). For HPLC, 15 μl of sample was applied to a $4.6 \text{ mm} \times 150 \text{ mm}$ column of TSK gel ODS-120T (Tosoh Inc., Tokyo, Japan) and eluted with 30 mM diethylaminoethanol, 20 mM phosphoric acid and 2% (v/v) methanol at a flow rate of 1 ml/min. Nucleotides were detected by measuring absorbance at 260 nm using a spectrophotometer (Waters, Milford, MA, U.S.A.). Intracellular concentrations (mM) of ATP, ADP and AMP were calculated by taking the average cell volume of S288C cells in the continuous culture to be $22 \mu\text{m}^3/\text{cell}$ [23]. Energy charge was calculated using the following equation:

$$\text{Energy charge (\%)} = ([\text{ADP}] \times 0.5 + [\text{ATP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}]) \times 100.$$

Assay of invertase activity

For the assay of invertase activity, cells were washed four or five times with 10 mM sodium azide and used as described by Goldstein and Lampen [24]. Invertase activity is expressed as μmol of glucose released/min per 100 mg of cells (wet weight).

RESULTS

Expression of *SUC2* under derepressed conditions in batch cultures

To investigate the effects of Snf1 kinase on gene expression during the energy-metabolism oscillation, we first planned to compare expression levels between wild-type and *snf1 Δ* cells. However, the growth of *snf1 Δ* cells was inhibited under glucose-limited conditions, consistent with a previous report [16], and *snf1 Δ* was unable to grow to the critical cell density [10] necessary

Figure 1 Effects of Gts1p and Snf1p on the expression of *SUC2* under glucose-repressed and derepressed conditions

(A) Invertase activity was assayed in wild-type (WT), *GTS1*-deleted (*gts1Δ*) and *GTS1*-overexpressing (TMpGTS1) cells, and in cells expressing Q-rich-domain-deleted Gts1p (TMpGTS1ΔQ) with (*snf1Δ*) or without (other columns) deletion of *SNF1*. Statistical analysis using Student's *t* test ($n=3$) indicated significant differences: * $P < 0.0001$, ** $P < 0.001$. (B) Northern blots of *SUC2* mRNA from WT, *gts1Δ*, TMpGTS1 and TMpGTS1ΔQ cells under derepressed conditions. S and I indicate the migration positions of mRNAs for secreted and intracellular isoforms of invertase respectively. Results are representative of three independent experiments.

for the organization of energy-metabolism oscillation. Thus we decided to use the expression level of *SUC2* as a tentative indicator of the activity of Snf1 kinase. As expected, the expression of *SUC2*, as determined by measuring invertase activity, was fully dependent on *SNF1* under the derepressed conditions (Figure 1A), while it increased little in *gts1Δ* cells, for unknown reasons. However, invertase activity was increased 2-fold in TMpGTS1 cells and slightly decreased in the *GTS1* mutant lacking the Q-rich domain (Figure 1A). *GTS1* had the same effect on the mRNA level of *SUC2*, at least for the major isomer of secreted invertase, as determined by Northern blot analysis (Figure 1B). The result suggested that Gts1p might affect the expression of *SUC2* at the transcriptional level, although its effect was minimal in the wild type under derepressed conditions.

Figure 2 mRNA levels of *TPS1*, *HSP104* and *SUC2* during the energy-metabolism oscillation in wild-type cells

(A) Pattern of DO oscillation in a continuous culture of wild-type cells. Solid and dotted lines indicate when samples were collected for Northern blot analysis (B) and for the determination of adenine nucleotide levels (Figure 3) respectively. (B) Northern blots of *TPS1*, *HSP104*, *SUC2* and *ACT1* from samples of the continuous culture shown in (A). (C) Northern blot analysis was performed in duplicate using samples from three independent cultures, including the one shown in (A) and (B). mRNA levels of *TPS1* (○), *SUC2* (●) and *HSP104* (△) relative to that of *ACT1* are shown as means $\pm$ S.D. ($n=6$). The energy-metabolism oscillation is shown as a bold line. The relative levels of each mRNA were also normalized so that the lowest values (those at 0 h for *TPS1* and *HSP104* and at 3 h for *SUC2*) were given a value of 1.0. Statistical analysis using Student's *t* test indicated no significant difference over time for *HSP104* ($P=0.8$, $n=3$), but significant differences for *TPS1* and *SUC2* ($P < 0.001$, $n=6$).

Transcriptional activation of *TPS1*, *HSP104* and *SUC2* during the energy-metabolism oscillation

In continuous culture, the wild type showed oscillations in energy metabolism over a 4 h period composed of respiratory and respiro-fermentative phases with relatively low and high DO levels respectively, according to oxygen demand (Figure 2A). To examine whether the expression of *TPS1*, *HSP104* and *SUC2* fluctuated in concert with energy metabolism, we determined mRNA levels relative to that of *ACT1* by Northern blot analysis (Figure 2B). While the mRNA level of *HSP104* did not show significant fluctuation, that of *TPS1* increased approx. 6-fold, peaking in the late respiratory phase (Figure 2C). This result is consistent with the finding that oscillations in heat resistance

Table 1 Glucose concentrations of cells in batch and continuous cultures under various conditions

The culture conditions of batch cultures were as defined in [4]. Values for wild-type cells in continuous cultures are given. u.d., under the detectable level (< 0.1 mM).

Conditions	[Glucose] (mM)	
	Extracellular	Intracellular
Batch cultures		
2 % (w/v) Glucose, 8 h (repressed)	22	0.887 ± 0.179
0.2 % (w/v) Glucose, 8 h (derepressed)	0.27	0.982 ± 0.045
Continuous cultures		
Maximum	u.d.	1.821 ± 0.260
Minimum	u.d.	1.178 ± 0.103
Average	—	1.546 ± 0.131

are induced by fluctuations in the trehalose level, and not by oscillatory expression of Hsp104 [12]. The *SUC2* mRNA level also oscillated, increasing by a maximum of 6-fold approx. 90° in advance of the peak in the *TPS1* mRNA level during the respiratory phase (Figures 2B and 2C). The change in the activation of *SUC2* suggested a possible role for Snf1 kinase in the expression of *TPS1* during the energy-metabolism oscillation.

Fluctuation of adenine nucleotide levels during the energy-metabolism oscillation

It is generally accepted that the activity of Snf1 kinase is regulated in response to the cellular energy state, which is correlated with the extracellular glucose level or the intracellular [AMP]/[ATP] ratio as a candidate for the glucose signal [15,20,25]. Thus we first determined the concentration of glucose in the medium and found that it was below the detectable level (< 0.1 mM) throughout the oscillation (Table 1), during which Snf1 kinase is reported to be fully activated [25]. The result thus suggested that the culture is in a derepressed state probably due to a severe limitation of glucose. It should be added that the intracellular glucose level during the energy-metabolism oscillation did not show significant fluctuation (Table 1), although a small peak was observed in the late respiratory phase, probably corresponding to trehalose degradation [12] (results not shown).

Next we determined adenine nucleotide levels in order to determine the [AMP]/[ATP] ratio (Figure 3A). The ATP level increased steadily to a maximum of 1.4-fold through the respiratory-fermentative phase, and then decreased at the onset of the respiratory phase, reaching a minimum at the end of this phase (Figure 3A). Consequently, the [ADP]/[ATP] and [AMP]/[ATP] ratios increased by approx. 1.7-fold and 2.6-fold respectively during the respiratory phase, accompanying a decrease in energy charge of approx. 20 % (Figure 3B). At first glance, it seemed strange that the ATP level decreased relative to the ADP and AMP levels in the respiratory phase, during which the rate of ATP production is increased. However, this may be interpreted to mean that the [ADP]/[ATP] ratio is increased to stimulate the citric acid cycle, probably due to consumption of ATP and/or its conversion into storage compounds such as polyphosphate. The [AMP]/[ATP] ratio, although it fluctuated between 0.13 and 0.34, was much lower throughout the oscillation than under the derepressed conditions, and rather similar to that in the repressed cells (Table 2). Furthermore, the ATP level and the energy charge were higher throughout the oscillation than under the glucose-repressed conditions [4] (Table 2). The discrepancy in the results

Figure 3 Adenine nucleotide levels during the energy-metabolism oscillation in wild-type cells

(A) Levels of ATP (○), ADP (●) and AMP (△) were determined from samples from three independent cultures, including that shown in Figure 2(A), and are given as means $\pm$ S.D. (B) [ADP]/[ATP] (○) and [AMP]/[ATP] (●) ratios and the energy-charge level (△) were calculated from the data shown in (A). The energy-metabolism oscillation is shown as a bold line.

Table 2 Energy states of cells in batch and continuous cultures under various conditions

Culture conditions of the batch cultures were as defined in [4]. Cases 1 and 2 of *gts1Δ* cultures indicate those with long and short DO oscillations respectively.

	[ATP] (mM)	[AMP]/[ATP]	Energy charge (%)
Batch cultures			
2 % (w/v) Glucose, 8 h (repressed)	2.52 ± 0.02	0.46	63
0.2 % (w/v) Glucose, 8 h (derepressed)	0.16 ± 0.01	13.1	25
Continuous cultures			
Wild type			
Max.	2.40 ± 0.016	0.34	80
Min.	1.63 ± 0.019	0.13	67
Amplitude (max./min.)	1.47	—	1.19
<i>gts1Δ</i>			
Case 1			
Max.	1.90	0.50	75
Min.	1.35	0.20	62
Amplitude	1.41	—	1.20
Case 2			
Max.	1.75	0.43	69
Min.	1.42	0.29	62
Amplitude	1.23	—	1.11

Figure 4 mRNA levels of *SNF1* and phosphorylation of Snf1p during the energy-metabolism oscillation in wild-type cells

(A) Pattern of DO oscillation and Northern blots of *SNF1* and *ACT1* mRNAs during the DO oscillation. The maximal difference in the *SNF1* mRNA level relative to that of *ACT1* among the samples was calculated to be 43%. Results are representative of two independent experiments. (B) Western blot of phosphorylated Snf1p (72 kDa) in lysates of wild-type cells in continuous culture reacted with the anti-phospho-AMPK(Thr-172) antibody. The pattern of DO oscillation (not shown) was similar to that in (A), and samples at 0 and 3 h are from the respiratory phase. CIP indicates the sample at 0 h treated with calf alkaline protein phosphatase as a control. Results are representative of three independent experiments. (C) Western blot of lysates from wild-type (WT) in continuous culture and *snf1Δ* under derepressed conditions (left panel), and from wild-type cells under repressed (rep.) and derepressed (derep.) conditions using the anti-phospho-AMPK(Thr-172) antibody (right panel). Cells were collected by either centrifugation (cent.) or filtration (filtr.).

regarding glucose and adenine nucleotide levels may be because the continuous culture is highly aerobic and respiratory, resulting in increases in the ATP level despite a severe limitation of glucose.

State of Snf1 kinase during the energy-metabolism oscillation

To investigate further whether and how Snf1 kinase is regulated during the oscillation, we first determined the *SNF1* mRNA level by Northern blot analysis (Figure 4A). There was no significant fluctuation in the mRNA level during the oscillation, ruling out regulation of Snf1p at the transcriptional level. Then, as Snf1p is known to be activated by phosphorylation of the conserved

threonine residue at position 210 [26], we determined the level of the phosphorylated isoform of Snf1p during the oscillation. For the purpose, we used an antibody raised against the peptide containing the phospho-threonine (Thr-172) of human AMPK, as the sequence of 20 amino acid residues around Thr-172 of AMPK is fairly similar (~80% identity) to that around Thr-210 of Snf1p. A reactive band with a molecular mass corresponding to that of Snf1p was detected in the cell lysate from the wild type in continuous culture; however, there were no significant fluctuations in band density during the oscillation (Figure 4B). Reportedly, phosphorylation of Snf1p can be induced when cells are precipitated by centrifugation at the room temperature [25]. Thus, to test the possibility that the phosphorylation of Gts1p was caused by centrifugation in the cold-room, the reactivity of the protein with the antibody was compared between cells collected by centrifugation and filtration (Figure 4C). The result showed that there was no significant difference in band densities, suggesting that the phosphorylation level was not affected by centrifugation in the cold-room and that the signal was not detected in *snf1Δ* under the derepressed conditions (Figure 4C). Furthermore, the cells under repressed conditions did not show any signals around the molecular mass of Snf1p with the anti-phospho-AMPK(Thr-172) antibody, irrespective of the method used for cell collection (Figure 4C). Thus these results suggested that the phosphorylated isoform of Snf1p was detected with the antibody, and that Snf1 kinase is not regulated at the level of either transcription or phosphorylation during the energy-metabolism oscillation. This appeared to rule out the possibility that the kinase is directly involved in the periodic expression of the genes.

Transcription of *TPS1* and *SUC2* in *gts1Δ* cells

To examine whether Gts1p is involved in the transcriptional activation of *TPS1* and *SUC2* during the energy-metabolism oscillation, the mRNA levels of these genes were determined during the transient oscillation in *gts1Δ* cells. As we reported previously, the effect of deleting *GTS1* on the energy-metabolism oscillation varied among experiments [2]. In about half of the *gts1Δ* cultures, the DO oscillation faded within 24 h, but, in rare cases, the oscillation continued for as long as 2 days. Thus we determined mRNA levels of *TPS1* and *SUC2* in cultures with both long and short transient oscillations.

In the culture with a long oscillation, the DO oscillation continued for 2 days, showing significant regular waves; the wavelength was approx. 3 h on average, approx. 1 h shorter than that of the wild type (Figure 5A). Northern blot analysis showed that both *SUC2* and *TPS1* mRNA levels fluctuated with amplitudes of 2-fold or less (Figures 5B and 5C); the size of the fluctuations was reduced to approx. one-third of that in the wild type, but they peaked at the same phases as in the wild type (compare the peak value at the late respiratory phase in Figure 5C with that in Figure 2C). On the other hand, the adenine nucleotide levels and amplitudes of the [ADP]/[ATP] and [AMP]/[ATP] ratios (1.5- and 2.5-fold respectively) were only slightly decreased compared with the wild type (Figure 6 and Table 2). These results suggest that the expression of *SUC2* and *TPS1* mRNAs was significantly inhibited even in the culture of *gts1Δ* cells where the energy state was not greatly affected.

In the *gts1Δ* culture with a short oscillation, the DO oscillation ceased after a few waves (Figure 7A), and neither the *SUC2* and *TPS1* mRNA levels (Figure 7B) nor the levels of adenine nucleotides showed significant fluctuations in concert with the transient DO oscillation (Figure 7C and Table 2). Thus we suggest that the regulation of expression of *TPS1* and *SUC2* is dependent predominantly on Gts1p, whereas the two genes were expressed

Figure 5 mRNA levels of *TPS1* and *SUC2* during the energy-metabolism oscillation in *gts1Δ* cells

(A) Pattern of the DO oscillation in a continuous culture which showed oscillation for 2 days. Solid and dotted lines indicate when samples were collected for Northern blot analysis (B) and for the determination of adenine nucleotide levels (Figure 6) respectively. (B) Northern blots of *TPS1*, *SUC2* and *ACT1* from samples of the continuous culture shown in (A). (C) Northern blot analysis of *TPS1*, *SUC2* and *ACT1* was performed in duplicate using samples from two different oscillatory waves in the culture shown in (A), and the relative mRNA levels of *TPS1* (○) and *SUC2* (●) to that of *ACT1* are given as means $\pm$ S.D. The energy-metabolism oscillation is shown as a bold line. The relative levels of each mRNA were also normalized so that the lowest values (those at 3 h for *TPS1* and at 2 h for *SUC2*) were given a value of 1.0. Statistical analysis using Student's *t* test ($n=4$) indicated significant differences at $P < 0.001$ for relative *TPS1* and *SUC2* mRNA levels (*).

at low levels independent of Gts1p when energy metabolism oscillated on a scale comparable with that of the wild type.

DISCUSSION

Here we show for the first time that *TPS1* is transcriptionally activated in a periodic manner, peaking in the early respiro-fermentative phase when activation of the glycolytic pathway is required to resume after exit from the respiratory phase. Tps1p is a regulatory enzyme of trehalose synthesis and is known to play a key role in the control of glucose influx during glycolysis [13,14]. Yeast mutants defective in Tps1p cannot grow on glucose or rapidly fermentable sugars, and accumulate sugar phosphates, particularly fructose 1,6-bisphosphate, rapidly consuming ATP. It has been proposed that a metabolic function of Tps1p is to

Figure 6 Adenine nucleotide levels during the energy-metabolism oscillation in *gts1Δ* cells

(A) Levels of ATP (○), ADP (●) and AMP (△) were determined by averaging data from two different waves in the culture shown in Figure 5(A). (B) [ADP]/[ATP] (○) and [AMP]/[ATP] (●) ratios and the energy-charge level (△) were calculated from the data shown in (A). The energy-metabolism oscillation is shown as a bold line.

restrict the flux of glucose for glycolysis by inhibiting the early steps of the glycolytic pathway, which is designed based on the 'turbo' principle [27]. Thus the increase in the Tps1p level should facilitate the metabolic shift from the respiratory to the respiro-fermentative phase. We have also shown for the first time that this activation of *TPS1* is dependent predominantly on Gts1p, suggesting that Gts1p facilitates the metabolic transition during the energy-metabolism oscillation by activating *TPS1* [2]. However, the finding that *TPS1* was still expressed in a periodic manner at low amplitude in *gts1Δ* cells in the presence of a nearly full-scale energy-metabolism oscillation suggested that activation of *TPS1* is partly dependent on an oscillation-dependent and Gts1p-independent mechanism. Thus it is also possible that Gts1p facilitates the function of the oscillation-dependent mechanism. On the other hand, the expression of *HSP104* was not activated during the energy-metabolism oscillation, suggesting that Gts1p as a transcriptional activator functions in continuous cultures in a different way from that under derepressed conditions, at least with regard to the expression of *HSP104*.

We show here that the expression of *SUC2* was also regulated in a periodic manner during the energy-metabolism oscillation, and that it was markedly decreased in *gts1Δ* cells. *SUC2* is a representative glucose-repressible gene and is known to be derepressed via a mechanism involving Snf1 kinase as a key component

Figure 7 mRNA levels of *TPS1* and *SUC2* during the energy-metabolism oscillation in *gts1Δ* cells

(A) Pattern of DO oscillation in a continuous culture with a short energy-metabolism oscillation. Solid and dotted lines indicate when samples were collected for Northern blot analysis (B) and for the determination of adenine nucleotide levels (C) respectively. (B) Northern blot analysis of *TPS1*, *SUC2* and *ACT1* was performed in duplicate using samples from the culture shown in (A), and the relative mRNA levels of *TPS1* (○) and *SUC2* (●) to that of *ACT1* were determined. The relative levels of each mRNA were also normalized so that the lowest values (those at 3 h for *TPS1* and at 2 h for *SUC2*) were given a value of 1.0. (C) ATP (○), ADP (●) and AMP (△) levels during the energy-metabolism oscillation. The energy-metabolism oscillation is shown as a bold line in (B) and (C).

[20]. However, the results presented here indicated that Snf1p is constitutively expressed and phosphorylated in synchronous continuous cultures. Furthermore, the activation of *SUC2* was first observed following the increase in the ATP level, in advance of an increase in the [AMP]/[ATP] ratio (Figure 3C), ruling out the direct participation of the kinase in the periodic expression of *SUC2*, although it is still possible that Snf1 kinase functions in combination with Gts1p. Thus we suggest that Gts1p predominantly regulates the expression of both *SUC2* and *TPS1* during the energy-metabolism oscillation. Consistent with this, the Gts1p level increases over the respiratory phase, corresponding to the activation of *TPS1* and *SUC2* in-phase [2]. On the other hand, the constitutive activation of Snf1 kinase is consistent with the finding that the culture is highly respiratory, as Snf1 kinase

inhibits acetyl-CoA carboxylase, allowing acetyl-CoA to enter the citric acid cycle [28].

Very recently, Klevecz et al. [29], using microarray analysis, reported that a continuous culture system in which a yeast diploid strain exhibited a short-period (~ 40 min) DO oscillation largely independent of the cell-division cycle [30–32] showed a genome-wide oscillation in transcription. The mRNA levels of almost all genes fluctuated, peaking at three nearly equally spaced intervals in the DO oscillation, with an average amplitude of approx. 2.0 [29]. These workers suggested that transcription is functionally organized into the three clusters in response to the redox state of cell during the DO oscillation, and that the oscillation in transcription gates DNA replication and the cell cycle in a constant fraction ($< 10\%$) of the population at 40-min intervals. However, the mechanism whereby the change in the redox state regulates the transcription of genes has not been elucidated. As the redox state is closely connected to the state of energy metabolism, it is likely that the latter primarily, if not directly, regulates gene transcription. The transcription pattern in our culture with a long-period (4 h) oscillation would be expected to be similar to that in the short-period one shown by Klevecz et al. [29]. However, of the genes that we analysed by Northern blotting, the mRNA levels of genes such as *TDH2*, *TDH3* [33], *ACT1* [2,33], *HSP104* and *SNF1* were constitutively expressed, while those of *GTS1* [2], *TDH1* [33], *TPS1* and *SUC2* fluctuated markedly (> 5 -fold) during the oscillation. Furthermore, the mRNA levels, especially those of *GTS1* and *TDH1*, fluctuated slowly with broad peaks relative to the period-length compared with those in the short-period oscillation [2,33]. Thus the transcriptional pattern in the long-period oscillation may be more complex than that in the short-period one. It should be mentioned that Gts1p has been reported to stabilize the short-period oscillation [34], suggesting that it plays a role in the organization of this oscillation, possibly as a transcriptional activator. Recently we found in a preliminary experiment that Gts1p interacted with the Srb-mediator-protein complex that associated with RNA polymerase II holoenzyme (S.-i. Yaguchi and K. Tsurugi, unpublished work), suggesting that Gts1p influences the transcription of many genes other than those examined here and in the previous report [4].

Finally, we hypothesize here that Gts1p stabilizes the self-organization of the energy-metabolism oscillation by activating *TPS1* at the beginning of the respiro-fermentative phase. However, it should be mentioned that this may not be the only function of Gts1p in stabilization of the metabolic oscillation. We showed previously that Gts1p interacts with glyceraldehyde-3-phosphate dehydrogenase [4,33], which participates in regulation of the cytoplasmic redox potential by producing NADH, and that Gts1p is involved in the oscillation of cAMP levels during the energy-metabolism oscillation [12]. As cAMP activates neutral trehalases, it is likely that Gts1p is also involved in the degradation of trehalose. Further studies are required to resolve these issues.

REFERENCES

- Yaguchi, S., Mitsui, K., Kawabata, K., Xu, Z. and Tsurugi, K. (1996) The pleiotropic effect of the *GTS1* gene product on heat tolerance, sporulation and the life span of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **218**, 234–237
- Wang, J., Liu, W., Mitsui, K. and Tsurugi, K. (2001) Evidence for the involvement of the *GTS1* gene product in the regulation of biological rhythms in the continuous culture of the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **489**, 81–86
- Hardie, D. G., Carling, D. and Carlson, M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* **67**, 821–855
- Yaguchi, S. and Tsurugi, K. (2003) Gts1p activates *SNF1*-dependent derepression of *HSP104* and *TPS1* in the stationary phase of yeast growth. *J. Biol. Chem.* **278**, 29760–29768

- 5 Piper, P. (1997) The yeast heat shock response. In *Yeast Stress Responses* (Hohmann, S. and Mager, W. H., eds.), pp. 75–99, Landes, Austin, TX
- 6 Singer, M. A. and Lindquist, S. (1998) Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. *Trends Biotechnol.* **16**, 460–468
- 7 Kuchin, S., Treich, I. and Carlson, M. (2000) A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 7916–7920
- 8 Akiyama, S. and Tsurugi, K. (2003) The *GTS1* gene product facilitates the self-organization of the energy-metabolism oscillation in the continuous culture of the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **228**, 105–110
- 9 Porro, D., Martegani, E., Ranzi, B. M. and Alberghina, L. (1988) Oscillations in continuous cultures of budding yeast: a segregated parameter analysis. *Biotechnol. Bioeng.* **32**, 411–417
- 10 Wang, J., Liu, W., Uno, T., Tonozuka, H., Mitsui, K. and Tsurugi, K. (2000) Cellular stress responses oscillate in synchronization with the ultradian oscillation of energy metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **189**, 9–13
- 11 Tonozuka, H., Wang, J., Mitsui, K., Saito, T., Hamada, Y. and Tsurugi, K. (2001) Analysis of the upstream regulatory region of the *GTS1* gene required for its oscillatory expression. *J. Biochem. (Tokyo)* **130**, 589–595
- 12 Uno, T., Wang, J., Mitsui, K., Umetani, K., Tamura, K. and Tsurugi, K. (2002) Ultradian rhythm of trehalose levels coupled to heat resistance in continuous cultures of the yeast *Saccharomyces cerevisiae*. *Chronobiol. Int.* **19**, 361–375
- 13 Thevelein, J. M. and Hohmann, S. (1995) Trehalose synthase: guard to the gate of glycolysis in yeast? *Trends Biochem. Sci.* **20**, 3–10
- 14 Francois, J. and Parrou, J. L. (2001) Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **25**, 125–145
- 15 Hardie, D. G. and Hawley, S. A. (2001) AMP-activated protein kinase: the energy charge hypothesis revisited. *BioEssays* **23**, 1112–1119
- 16 Cortassa, S. and Aon, M. A. (1998) The onset of fermentative metabolism in continuous cultures depends on the catabolite repression properties of *Saccharomyces cerevisiae*. *Enzyme Microbial Technol.* **22**, 705–712
- 17 Rouwenhorst, R. J., Van der Baan, A. A., Scheffers, W. A. and Van Dijken, J. P. (1991) Production and localization of β -fructose in asynchronous and synchronous chemostat cultures of yeasts. *Appl. Environ. Microbiol.* **57**, 557–562
- 18 Gancedo, J. M. (1992) Carbon catabolite repression in yeast. *Eur. J. Biochem.* **206**, 297–313
- 19 Ronne, H. (1995) Glucose repression in fungi. *Trends Genet.* **11**, 12–17
- 20 Carlson, M. (1999) Glucose repression in yeast. *Curr. Opin. Microbiol.* **2**, 202–207
- 21 Yaguchi, S., Mitsui, K. and Tsurugi, K. (2000) Phosphorylation of the *GTS1* gene product of the yeast *Saccharomyces cerevisiae* and its effect on heat tolerance and flocculation. *FEMS Microbiol. Lett.* **187**, 179–184
- 22 Parulekar, S. J., Semones, G. B., Rolf, M. J., Lievense, J. C. and Lim, H. C. (1986) Induction and elimination of oscillations in continuous cultures of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **28**, 700–710
- 23 Martegani, E., Porro, D., Ranzi, B. M. and Alberghina, L. (1990) Involvement of a cell size control mechanism in the induction and maintenance of oscillations in continuous cultures of budding yeast. *Biotechnol. Bioeng.* **36**, 453–459
- 24 Goldstein, A. and Lampen, J. O. (1975) β -D-Fructofuranoside fructohydrolase from yeast. *Methods Enzymol.* **42**, 504–511
- 25 Wilson, W. A., Hawley, S. A. and Hardie, D. G. (1996) Glucose repression/derepression in budding yeast: *SNF1* protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.* **6**, 1426–1434
- 26 McCartney, R. R. and Schmidt, M. C. (2001) Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *J. Biol. Chem.* **276**, 36460–36466
- 27 Teusink, B., Walsh, M. C., van Dam, K. and Westerhoff, H. V. (1998) The danger of metabolic pathways with turbo design. *Trends Biochem. Sci.* **23**, 162–169
- 28 Woods, A., Munday, M. R., Scott, J., Yang, X., Carlson, M. and Carling, D. (1995) Yeast *SNF1* is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase *in vivo*. *J. Biol. Chem.* **269**, 19509–19515
- 29 Klevecz, R. R., Bolen, J., Forrest, G. and Murray, D. B. (2004) A genomewide oscillation in transcription gates DNA replication and cell cycle. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 1200–1205
- 30 Satroudinov, A. D., Kuriyama, H. and Kobayashi, H. (1992) Oscillatory metabolism of *Saccharomyces cerevisiae* in continuous culture. *FEMS Microbiol. Lett.* **77**, 261–267
- 31 Keulers, M., Satroudinov, A. D., Suzuki, T. and Kuriyama, H. (1996) Synchronization affector of autonomous short-period sustained oscillation of *Saccharomyces cerevisiae*. *Yeast* **12**, 673–682
- 32 Sohn, H. Y., Murray, D. B. and Kuriyama, H. (2000) Ultradian oscillation of *Saccharomyces cerevisiae* during aerobic continuous culture: hydrogen sulphide mediates population synchrony. *Yeast* **16**, 1185–1190
- 33 Liu, W., Wang, J., Mitsui, K., Shen, H. and Tsurugi, K. (2002) Interaction of the *GTS1* gene product with glyceraldehyde-3-phosphate dehydrogenase 1 required for the maintenance of the metabolic oscillations of the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **269**, 3560–3569
- 34 Adams, C. A., Kuriyama, H., Lloyd, D. and Murray, D. B. (2003) The Gts1 protein stabilizes the autonomous oscillator in yeast. *Yeast* **20**, 463–470

Received 8 June 2004/14 June 2004; accepted 1 July 2004

Published as BJ Immediate Publication 1 July 2004, DOI 10.1042/BJ20040967