J Biochem 130, 589–595 (2001)

Analysis of the Upstream Regulatory Region of the *GTS1* Gene Required for Its Oscillatory Expression

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Received April 19, 2001, accepted August 15, 2001

The protein level of the *GTS1* gene product (Gts1p) fluctuated during the oscillation of energy metabolism in continuous culture of the yeast *Saccharomyces cerevisiae*. Here, we found that the *GTS1* mRNA level oscillated with the same periodicity as the metabolic oscillation, suggesting that the expression of *GTS1* was regulated at the transcriptional level. As the 5′-upstream sequence of *GTS1* contains two short open-reading frames at –310 and –829 bp from the initiation codon, we determined the *GTS1* promoter required for the oscillatory expression. The upstream sequence was truncated into fragments of 183, 355, 1,042, and 1,572 bp, named *GTS1*pr.183 and so on, and their effects on the expression of *lacZ* as a reporter gene and the *GTS1* gene itself were examined. The β-galactosidase activity and Gts1p level oscillated in the continuous cultures when genes were expressed under the control of *GTS1*pr.183 but not *GTS1*pr.355. The disappearance of the metabolic and cell-cycle oscillations in the *GTS1*-deleted mutant was rescued by the transformation with *GTS1*pr.183-*GTS1* but not with *GTS1*pr.355-*GTS1*. However, the stress-resistance oscillations were not found in the cells transformed with *GTS1*pr.183-*GTS1*, differing from the case of *GTS1*pr.1042-*GTS1* reported previously [Wang *et al.* (2001) *FEBS Lett.* 489, 81–86]. Thus, we suggest that the 183-bp upstream sequence of *GTS1* is basically required for the metabolic oscillation, while the 1,042-bp upstream sequence is required for oscillations of stress resistance.

Key words: biological rhythm, budding yeast, *GTS1* gene, oscillatory expression, promoter.

In the yeast *Saccharomyces cerevisiae*, sustained oscillations of energy-metabolism cycling between the respiro-fermentative and respiratory phases with a period of about 4 h have been observed in continuous cultures (*1–5*). These oscillations arise spontaneously under aerobic conditions in a manner dependent on high cell density and are detectable as a periodic change in O_2 consumption, CO_2 production and glucose and ethanol concentrations (*1–5*). Recently, we reported that cellular responses to various stresses such as heat, oxidative agents, and cytotoxic chemicals oscillated in coupling with the ultradian oscillations of metabolism (*6*) and that the Gts1p level oscillated with the same periodicity as the energy metabolism in continuous cultures (*7*). The inactivation of *GTS1* severely affected the metabolic oscillation, resulting in the disappearance of biological rhythms, which was substantially rescued by transformation with *GTS1* (*7*). In those experiments, we used a 1.0-kbp upstream sequence as promoter of *GTS1*, based on the fact that most yeast genes contain *cis*-elements in the 1.0-kbp upstream sequence; and thus the precise region of the promoter remained to be clarified.

In the present study, we found that the expression of *GTS1* was regulated at the transcriptional level, as the *GTS1* mRNA level oscillated during energy-metabolism. As there are two unidentified open reading frames (ORFs) in the 1.0-kbp upstream sequence of *GTS1*, we investigated the regulatory upstream sequence required for the oscillatory expression of *GTS1*. For this purpose, recombinant plasmids carrying *lacZ* as a reporter (*8–10*) or *GTS1* under the control of different lengths of the *GTS1*-upstream sequence were constructed and their effects on the appearance of the biological rhythms in continuous cultures were examined. We found that, basically, the 183-bp upstream sequence is required for the oscillatory expression of *GTS1*, which can induce energy-metabolism oscillation coupled with cell-cycle division in continuous cultures (In this paper, "coupling" means a state where multiple oscillators fluctuate with the same periodicity with and without the same phase.)

MATERIALS AND METHODS

Yeast and Culture Media—The haploid strains W303 (*MAT*a *ade2-1 his3-11 trp1-1 leu2-3 ura3-1 can1-100*) and S288C (*MATα mal gal2 SUC2*) of the yeast *S. cerevisiae* were used for the batch and continuous cultures, respectively. In batch cultures, W303 cells were cultured at 30°C in a synthetic medium containing 2% glucose or other carbon sources as defined elsewhere (*3*). Cell samples in the

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Abbreviations: ORF, open reading frame; A_{550}, absorbance at 550 nm; DO, dissolved oxygen; PCR, polymerase chain reaction

exponentially growing and stationary (post-diauxic) phases were harvested when the absorbance at 550 nm (A_{550}) of the culture reached 0.8–1.0 and 2.0–2.2, respectively. In experiments of carbon source-shift, the cells cultured in the synthetic medium containing 2% glucose were harvested in the stationary phase and resuspended in the same volume of synthetic medium containing glucose. Cells were cultured for 2 h at 30°C, and cell samples for β-galactosidase assay were collected. Cells in the rest of the culture were harvested, washed and resuspended in medium containing ethanol. After incubation for 2 h at 30°C, cell samples for β-galactosidase assay were collected. In continuous cultures, S288C cells were cultured using a bench-top fermenter, MDL-6C (Marubishi Bioengineering, Tokyo), with a constant volume of 500 ml (*6, 7*). The culture was aerated with a flow of 1 liter/min and stirred at an agitation speed of 420 rpm. Continuous cultures were started 2 h after the consumption of ethanol from the medium of batch culture and continued at a dilution rate of 0.1 h^{-1} with the same medium containing 1% glucose at pH 5.5. The periodic change in respiratory-fermentative metabolism was monitored by measuring the level of dissolved oxygen (DO) with an oxygen electrode. Stress conditions with heat, H_2O_2 and cadmium, and determination of the survival rate of the treated cells were described previously (*6*).

Construction of Recombinant Plasmids Containing lacZ as a Reporter Gene under the Control of GTS1 Upstream Sequences of Different Lengths—The *Sph*I–*Sal*I fragment containing about a 1.6-kbp upstream sequence of *GTS1* was amplified by polymerase chain reaction (PCR) using synthetic primers, 5′-CCCGAAGCATGCTGTGCTCAC-3′ and 5′-AGAACTCGTCGACCTCATTTTTGAC-3′ for 5′- and 3′-primers, respectively, directed on the recombinant plasmid pGTS1 (*11*) (Fig. 1). The 183-bp *Bam*HI–*Sal*I (named *GTS1*pr.183) and 355-bp *Eco*RI–*Sal*I (*GTS1*pr.355) fragments from the PCR product were inserted into the multicloning site of YEP358 vector (*10*) in frame of *lacZ* encoding the *Escherichia coli*–derived β-galactosidase. The recombinant plasmids carrying *lacZ* downstream of *GTS1*pr.183 and *GTS1*pr.355 were named p*GTS1*pr.183-*lacZ* and p*GTS1*pr.355-*lacZ* (Fig. 1), respectively Similarly, the 1,042-bp *Hin*dIII–*Sal*I (*GTS1*pr.1042) and 1,572-bp *Sph*I–*Sal*I (*GTS1*pr.1572) fragments from the PCR products were inserted into YEP357 (*10*) to obtain recombinant plasmids p*GTS1*pr.1042-*lacZ* and p*GTS1*pr.1572-*lacZ* (Fig. 1), respectively. The recombinant plasmids were transformed into the wild-type W303 cells as described previously (*6, 7*).

Construction of Centromere-Based Plasmids Containing GTS1 under the Control of GTS1 Upstream Sequences of Different Lengths—To construct a recombinant plasmid carrying *GTS1* downstream of *GTS1*pr.183, named pACpr.-183-*GTS1*, a PCR product synthesized using 5′-GAAATTCTCGAGAGTTGAAGAAAGTTAGTG-3′ and 5′-GTGCTACTAGTGGGTTTTCCTTACATTACC-3′ as 5′- and 3′-primers, respectively, directed on pGTS1 (*11*) was used. The 1,646-bp *Bam*HI-*Sma*I fragment of the PCR product was blunt-ended and inserted into the centromere-based plasmid pAUR112 (Takara, Tokyo) at the *Sma*I site. pACpr.-355-*GTS1* containing *GTS1*pr.355 was constructed by inserting the 1,866-bp *Eco*RI–*Spe*I fragment of pGTS1 into pAUR112 at the *Sma*I site after blunt-ending. pACpr.1042-*GTS1* was constructed as described in a previous report (*7*), where it was called pAC*GTS1*(NS). pACpr.1572-*GTS1* was obtained by inserting a 3,084-bp *Sph*I–*Spe*I fragment into pAUR112 at the *Sma*I site after blunt-ending. These recombinant plasmids were introduced into the *GTS1*-deleted mutant (*gts1Δ*) of S288C as described previously (*7*).

Determination of β-Galactosidase Activity—Cell samples in batch cultures were harvested from 1 ml of culture medium, and those in the continuous cultures showing DO oscillation were collected from 0.1 ml of effluent from the bioreactor at 30-min intervals. The cells were washed and resuspended in 0.2 ml of Z buffer containing 0.2% sodium lauroyl-sarcosinate and 50 mM β-mercaptoethanol (*12*). The cell suspension was freeze-thawed and incubated for 30 min at 30°C after adding 0.4 ml of Z buffer. The reaction was started by adding 0.15 ml of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside solution in Z buffer, allowed to proceed for 1 h at 30°C, then stopped by adding 0.4 ml of 1.5 M Na_2CO_3. The resulting mixture was centrifuged at 4,000 ×*g* for 10 min. The supernatant was removed and the enzyme activity was determined by measuring A_{420} (*12*). The enzyme activity was corrected for cell density by dividing the A_{420} by the A_{550} of the original culture medium.

Northern and Western Blot Analyses—mRNA levels of *GTS1* and *lacZ* were determined by Northern blot analysis using the 0.82-kbp *Hin*dIII–*Cla*I fragment of YEP358 (*10*) and the 1.24-kbp *Eco*RI–*Kpn*I fragment of pGTS1 (*11*) as probes, respectively. mRNA levels of putative ORFs were determined using oligonucleotides as probes, which were PCR-amplified using primers, 5′-AGACATACCATTGTTGAATG-3′ and 5′-GGAACAGTATTATTCTTCTC-3′ for YGL182C, and 5′-GTTAAACCTCCGTAAATTCC-3′ and 5′-CGTGATTTAGAAATTGCTAG-3′ for YGL183C. For detection of *lacZ* mRNA, the *Hin*dIII–*Cla*I fragment of YEP358 was used as probe. Probes were labeled with a DIG-labeling system (Roche Diagnostics, Mannheim, Germany) and fluorescence was quantified with a fluorescence-imaging analyzer (Fuji Film, LAS1000, Tokyo). The mRNA levels were normalized with the level of actin mRNA as a control.

Western blotting of Gts1p and actin was performed and the relative Gts1p to actin level was determined as described previously (*7*).

RESULTS AND DISCUSSION

Regulation of GTS1 Expression at the Transcriptional Level—We previously found that the Gts1p level oscillated with the same periodicity as energy-metabolism, peaking at the respiratory phase (*7*). Here, to ascertain whether the expression of *GTS1* is regulated at the transcriptional level, we determined the mRNA level by Northern blot analysis (Fig. 1). The *GTS1* mRNA level fluctuated with a broad peak at the respiro-fermentative phase, which is the opposite of the Gts1p fluctuation (*7*), suggesting that *GTS1* expression was regulated in an oscillatory manner at the transcriptional level.

In the upstream sequence of *GTS1*, there are two putative ORFs of 324 bp (YGL182C) and of 525 bp (YGL183C), located at –310 and –829 bp upstream from the first residue A of the initiation codon (+1) of *GTS1*, respectively, prior to the next gene, *STR3* (YGL184C), which encodes the yeast homologue of cystathionine-β-lyase (Fig. 2A). As *cis*-regulatory elements in most genes of yeast exist in 1.0-kbp upstream sequences, it should be clarified whether the two ORFs comprise a regulatory region of *GTS1*. To search

for the regulatory region required for the oscillatory expression of *GTS1*, we constructed four recombinant plasmids carrying *lacZ* as reporter under the control of *GTS1* upstream sequences of different lengths (Fig. 2B). The upstream sequences of the recombinant plasmids, p*GTS1*-pr.183-*lacZ* and p*GTS1*pr.355-*lacZ*, do not contain any full-length ORFs, although the latter contains the 48-bp 3′-side sequence of YGL182C. p*GTS1*pr.1042-*lacZ* contains YGL-182C and the 206-bp 3′-half of YGL182C, and p*GTS1*pr.-1572-*lacZ* contains the full lengths of YGL182C and YGL183C. It should be added that a main transcription-initiation site of *GTS1* was the T residue at –30, determined by a primer-extension method (data not shown), and so a putative TATA box for RNA polymerase II is predicted to lie at –60.

Effect of Carbon Sources on the Expression of lacZ Reporter Gene in Batch Cultures—To test the *GTS1* expression level under the control of upstream sequences of different lengths, β-galactosidase activity of the transformants was measured in batch cultures using glucose or ethanol as a carbon source (Fig. 3). The cells transformed with p*GTS1*-pr.183-*lacZ* showed markedly higher transcriptional activity than those transformed with other recombinant plasmids irrespective of carbon source or growth phase (Fig. 3). Similar results were obtained when fermentable carbons like galactose and trehalose were used in the place of glucose and non-fermentable glycerol was used in place of ethanol (data not shown). It should be noted that in the stationary phase, the cells transformed with p*GTS1*pr.183-*lacZ* exhibited 1.6-times higher activity when cultured on ethanol than on glucose (Fig. 3B). The result suggested that under glucose-limited conditions, the transcription of *GTS1* was more responsive to ethanol than to glucose. In agreement with this, the transcription activity of p*GTS1*pr.183-*lacZ* in the stationary phase cells was increased by 20% after the shift from glucose to ethanol (Fig. 4). These results may reflect the fact that in the continuous culture, where glucose is limited, the transcription of *GTS1* is increased in

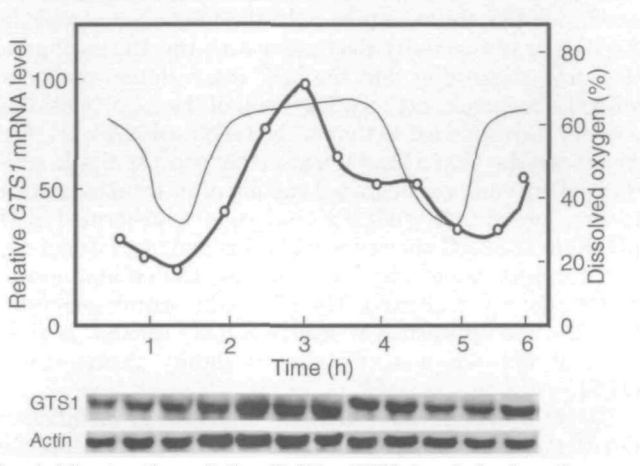

Fig 1 **Fluctuation of the *GTS1* mRNA level during the energy-metabolism oscillation in the continuous culture of the wild-type S288C.** Cell samples were harvested on the second day of the dissolved oxygen (DO) oscillation (solid line) Relative *GTS1* mRNA to actin mRNA level (open circles) was determined with the fluorescence-imaging analyzer after Northern blotting, and the *GTS1* mRNA level is presented as a percentage of the highest value

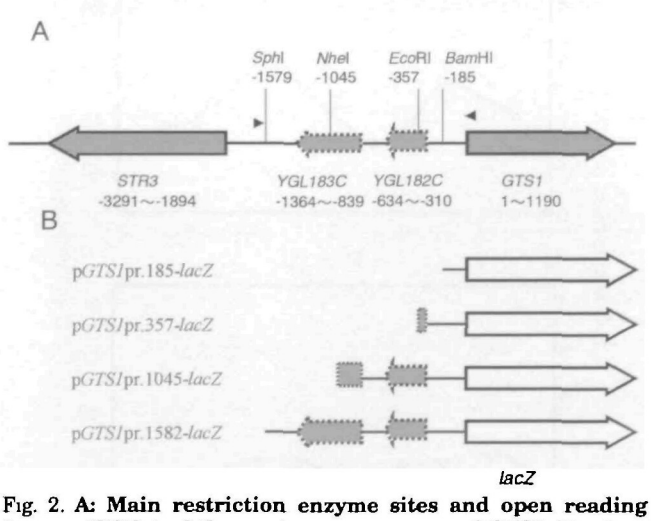

Fig. 2. **A: Main restriction enzyme sites and open reading frames (ORFs) of the upstream sequence of *GTS1*.** Numbers indicate nucleotide positions from the first nucleotide A of the initiation codon of *GTS1* Arrowheads indicate approximate positions of primers used for amplification of the *SphI–SalI* fragment containing about a 1.6-kbp upstream sequence of *GTS1* Arrows indicate approximate positions and transcriptional directions of identified genes (solid line) and putative ORFs (broken line). **B: Schemes of gene constructs inserted into the recombinant plasmids containing *lacZ* as a reporter gene under the control of different lengths of *GTS1* upstream sequence.**

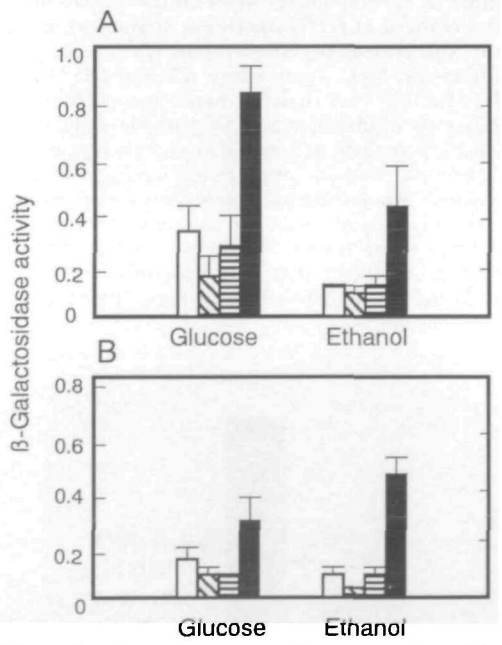

Fig 3 **Effect of carbon sources on the expression of *lacZ* under the control of *GTS1* upstream sequences of different lengths in the exponentially-growing (A) and stationary-phase cells (B).** W303 cells transformed with p*GTS1*pr.183-*lacZ* (closed bars), p*GTS1*pr 355-*lacZ* (lateral-stripped bars), p*GTS1*pr-1042-*lacZ* (hatched bars), and p*GTS1*pr 1572-*lacZ* (open bars) were incubated at 30°C in synthetic medium containing glucose or ethanol at 2%. Cell samples (1 ml) were harvested when the A_{650} of the culture reached 0 8–1 0 (exponentially growing phase) or 2.0–2 2 (stationary phase) β-Galactosidase activity was measured and corrected by the A_{650} of the culture as described in "MATERIALS AND METHODS." Mean values and standard deviations were calculated from three independent experiments.

the respiro-fermentative phase (Fig. 1) during which ethanol accumulates (*3*).

Expression of the Putative ORFs in the Upstream of GTS1—The results using the *lacZ* reporter (Fig. 3) raised the possibility that the transcriptional products or proteins from putative ORFs affected the reporter gene expression under the control of the 183-bp sequence having the oscillatory transcription activity. Alternatively, the region further upstream than the 183-bp sequence may contain inhibitory elements which are required for the oscillatory transcription. To examine the former possibility, the lysate from the cells transformed with p*GTS1*pr.1572-*lacZ* (Fig. 5) carrying both ORFs in full length was applied to Northern blot analysis to detect their products. The products of the putative ORFs were not accumulated in detectable levels, while *lacZ* mRNA was clearly detected. Together with the finding that there are no obvious TATA boxes in putative promoter regions of the two putative ORFs, these results suggest that the ORFs are not expressed *in vivo*.

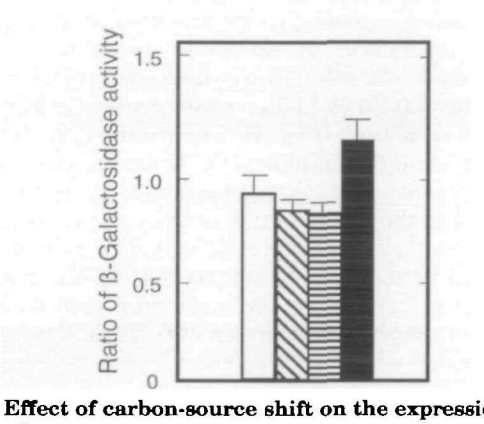

Fig 4 **Effect of carbon-source shift on the expression of *lacZ* under the control of *GTS1*-upstream sequences of different lengths in the stationary-phase cells.** W303 cells transformed with pGTS1pr183-*lacZ* (open bars), pGTS1pr355-*lacZ* (crossed bars), pGTS1pr1042-*lacZ* (hatched bars), and pGTS1pr.1572-*lacZ* (closed bars) were incubated at 30°C in synthetic medium containing glucose at 2% and harvested in the stationary phase. Cells were then incubated in synthetic medium containing glucose and then ethanol, each for 2 h. β-Galactosidase activity was measured before and after the carbon-source shift and corrected by the A_{550} of the culture. The ratios of the enzyme activity were calculated by dividing the activity on ethanol by that on glucose in every transformant. Mean values and standard deviations were calculated from three independent experiments.

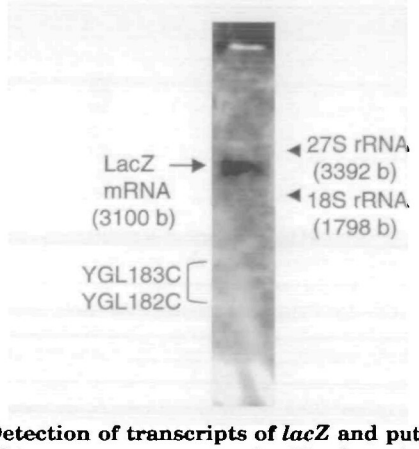

Fig 5. **Detection of transcripts of *lacZ* and putative ORFs in the *GTS1* upstream sequence by Northern blot analysis in the wild-type cell (W303) transformed with p*GTS1*pr.1572-*lacZ* in a multi-copy plasmid.** "[" indicates approximate position of transcripts of the putative ORFs, YGL183C and YGL182C, calculated from the migration rates of rRNAs.

Oscillatory Expression of lacZ in Continuous Culture under the Control of GTS1-Upstream Sequences of Varying Lengths—To examine whether the 183-bp upstream sequence having the highest transcription activity is enough for the oscillatory expression of *GTS1*, wild-type S288C cells transformed with p*GTS1*pr.183-*lacZ* were subjected to continuous culture. The transformant showed a stable DO oscillation like the wild-type cells (data not shown) and the β-galactosidase activity fluctuated with the DO oscillation (Fig. 6A), suggesting that the *lacZ* transcription oscillated with the metabolic activity. The peak of the β-galactosidase activity corresponded to that of the *GTS1* mRNA level (Fig. 1) but not the Gts1p level (*7*), probably because the *E. coli*–derived enzyme was degraded rapidly after translation as a foreign protein. In contrast, the cells transformed with p*GTS1*pr.355-*lacZ* showed low enzyme activity without significant fluctuation (Fig 6B), while the DO oscillation was stable (data not shown). Therefore, the result suggested that the 183-bp upstream sequence (*GTS1*pr.183) is sufficient, if not complete, for the oscillatory expression of *GTS1*.

Effect of GTS1 Expression on the Energy-Metabolism Oscillation in the GTS1-Deleted Mutant (gts1Δ)—As the *lacZ* reporter gene was expressed in an oscillatory manner under the control of *GTS1*pr.183, we examined whether the

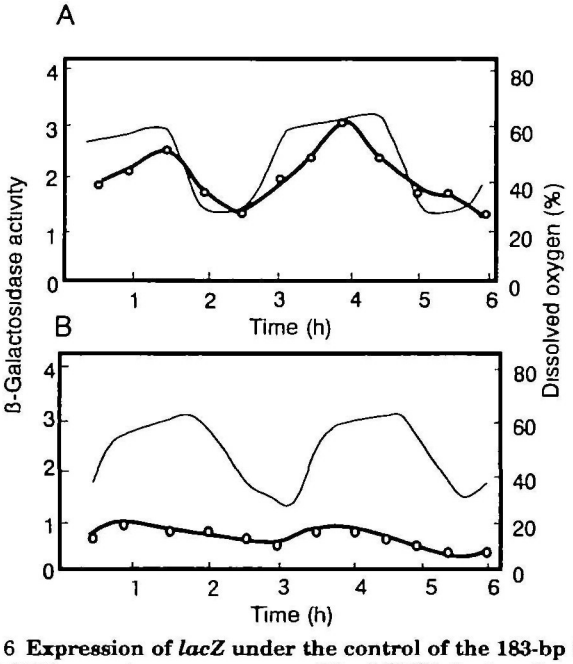

Fig. 6 **Expression of *lacZ* under the control of the 183-bp (A) and 355-bp upstream sequences (B) of *GTS1* during the energy-metabolism oscillation in the continuous culture.** The wild-type S288C cells transformed with p*GTS1*pr183-*lacZ* (A) and with p*GTS1*pr355-*lacZ* (B) were incubated at 30°C in the continuous culture. Cell samples were collected on the second day of the DO oscillation (solid line) and β-galactosidase activity (open circles) was measured.

*GTS1*pr.183-controlled expression of *GTS1* can generate metabolic oscillation in *gts1Δ* by transforming the cells with the centromere-based recombinant plasmid, pACpr183-*GTS1*. The DO oscillation appeared and continued for about 4 days in the transformant (Fig. 7A), although the amplitude of the waves of the DO oscillation was shallow compared with that of the wild-type cells (7) (Table I). On the other hand, *gts1Δ* transformed with pACpr.355-*GTS1* showed irregular oscillations which ceased within a few days (Fig. 7B). Thus, the result suggested that the 183-bp upstream sequence is basically required for oscillatory expression of *GTS1*, although the DO oscillation was shallow compared with that of the wild-type cells (Table I). The result also supported the notion that the oscillatory expression of Gts1p is required for the stable oscillation of the energy-metabolism pathway (7).

The Western blots showed that the fluctuation of Gts1p level in *gts1Δ* transformed with pACpr.183-*GTS1* was coupled with the DO oscillation (Fig. 8) The maximal (peak) level of Gts1p was about 50% higher and the minimal (trough) level was three times higher than those of the wild-type cells and cells transformed with pACpr.1042-*GTS1* (7) (Fig. 8 and Table II). Thus, the amplitude of the Gts1p level was significantly restricted on a high basal level in *gts1Δ* transformed with pACpr.183-*GTS1*. The result is in agreement with the finding that the transformant with p*GTS1*pr.183-*lacZ* showed markedly higher transcriptional activity of the reporter gene than the others (Fig. 3). It should be pointed out that the amplitude of the Gts1p level oscillated in parallel with the amplitudes and duration of the DO oscillation among different strains (Table II).

Coupling of Various Biological Rhythms with the Metabolic Oscillation Regulated by pACpr.183-GTS1—In a previous report, we suggested that the rhythmic expression of Gts1p is more important than the protein level for the maintenance of biological rhythms, as the constitutive expression of *GTS1* under the control of the *TPI* promoter resulted in the disappearance of biological rhythms (7). As the amplitude of Gts1p fluctuation was restricted in the cells transformed with pACpr.183-*GTS1* (Fig. 8), we examined whether various biological rhythms are coupled with the metabolic oscillation. The metabolic oscillation regu-

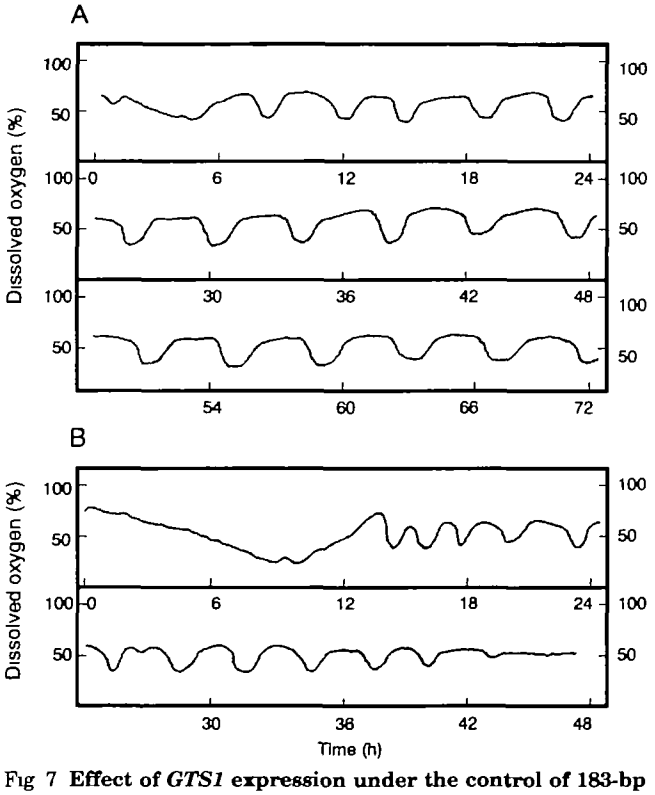

Fig 7 **Effect of *GTS1* expression under the control of 183-bp (A) and 355-bp upstream sequences (B) of *GTS1* on the DO oscillation in *gts1Δ*.** *gts1Δ* (S288C) was transformed with pACpr.-183-*GTS1* (A) or pACpr355-*GTS1* (B) and subjected to continuous culture. The continuous culture was started at time zero

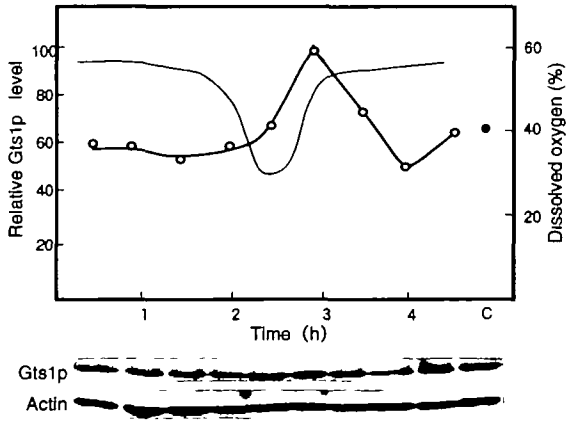

Fig 8 **Fluctuation of the Gts1p level in *gts1Δ* transformed with a recombinant plasmid carrying *GTS1* under the control of its own 183-bp upstream sequence.** The Gts1p level was determined by Western blot analysis in *gts1Δ* transformed with pACpr 183-*GTS1* on the second day of the DO oscillation (Fig 7A) Relative protein level of Gts1p to actin (open circles) was determined with a fluorescence-imaging analyzer after Western blotting, and the Gts1p level is presented as a percentage of the highest value. The last column (C) indicates the relative Gts1p level at its peak during the DO oscillation in the wild-type strain as a control (closed circle)

TABLE I. **Wavelengths and amplitudes of the DO oscillations in the wild-type and *gts1Δ* transformed with p*GTS1*pr.183-*GTS1* or p*GTS1*pr.355-*GTS1*.** Numbers in parentheses indicate % control.

Strains	No of waves[b] (waves/run)	Wavelength[c] (h/wave)	Amplitude[d] (%DO)
Wild type	34 (n = 5)[a]	3 90 ± 0 34 (100)	40 4 ± 2.21 (100)
gts1Δ transformed with			
p*GTS1*pr.183-*GTS1*	22 (n = 3)[a]	3.92 ± 0 33 (101)	21 8 ± 3.70 (54)
p*GTS1*pr.355-*GTS1*	10 (n = 3)[a]	2 53 ± 0.61 (65)	15 1 ± 4 16 (37)

[a]The number of experiments used for statistical analysis. [b]Average of the number of waves in a run of oscillations. [c]Average and standard deviation of the wavelength determined with all experimental values for each strain. [d]Average and standard deviation of the amplitude of all waves for each strain estimated by subtracting the DO concentration (%) of the trough from that of the peak of the next wave

TABLE II. **Comparison of the Gts1p-level fluctuation with the DO and stress-resistance oscillations in the wild-type and *gts1Δ* transformed with p*GTS1*pr.183-*GTS1* or p*GTS1*pr.1042-*GTS1*.**

Strains	Gts1p level	DO oscillation		Stress-resistance oscillation
	Peak/Trough[b] = Amplitude	Amplitude	Duration[c]	
Wild type[a]	100/35 = 2.86	40.0	132	+++
gts1Δ transformed with				
pACpr 1042-*GTS1*[a]	95/33 = 3 03	28.4	117	++
pACpr 183-*GTS1*	155/90 = 1 73	21.8	86	–

[a]Parameters for these strains were calculated using the data presented in a previous paper (7) [b]Relative Gts1p to actin level at the peak and trough of the fluctuations. The Gts1p level in each strain was normalized by taking the peak level of the wild-type cells as 100, which was used as a control in each experiment [c]Average duration time (h) of the DO oscillation calculated by multiplying the average number of waves by the average wavelength

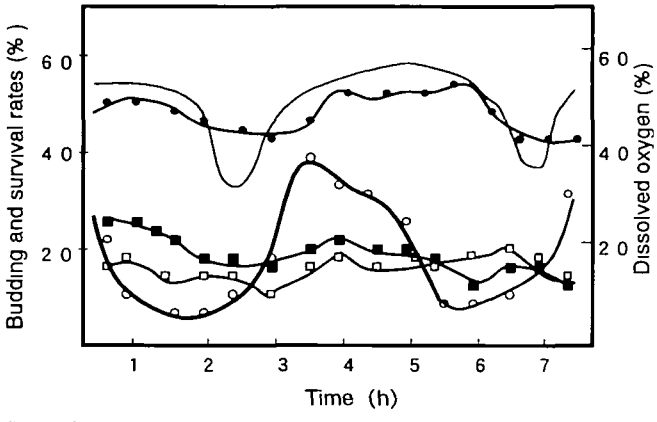

Fig. 9. **Coupling of cell-cycle and stress-resistance oscillations in *gts1Δ* transformed with a recombinant plasmid carrying *GTS1* under the control of its own 183-bp upstream sequence in a centromere-based plasmid.** Budding rate (open circles) and survival rates on treatment with heat (55°C, 3 min) (closed circles), hydrogen peroxide (5 mM) (open squares), and cadmium (10 mM) (closed squares) were determined in *gts1Δ* transformed with pACpr-183-*GTS1* (Fig. 7A) on the second day of the DO oscillation (solid line).

lated by pACpr.183-*GTS1* in *gts1Δ* coupled well with the cell-cycle oscillation but not with stress-resistance oscillations (Fig. 9). As we previously reported (7), *gts1Δ* transformed with pACpr.1042-*GTS1* generated Gts1p oscillations similar to the wild-type cells, which induced coupling, if not completely, of the stress-resistance oscillations with the DO oscillation (7). Thus, the result supported the possibility that the region further upstream than the 183-bp sequence contains some inhibitory elements which are required for the oscillatory expression of *GTS1* capable of inducing the stress-resistance oscillations. It should be added that *gts1Δ* transformed with pACpr.1572-*GTS1* showed a little unstable DO oscillation which ceased within a few days and was followed by irregular and frequent oscillations. Thus, these results suggested that, for the appearance of stress-resistance oscillations, an upstream sequence ranging over 1.0 kbp is required.

A search of the database TFSEARCH ver. 1.3 (Kyoto University, Kyoto) for consensus sequences of known *cis*-elements revealed no obvious matches in the 183-bp upstream sequence of *GTS1*. However, we found a consensus sequence for general carbohydrate regulator 1 (*GCR1*) (*13, 14*) at nucleotide –88 of the *GTS1*-upstream sequence. The *GCR1* protein is a major activator for main glycolytic enzymes like enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphofructokinase and others (*15, 16*), and is activated by glucose (*17*). However, p*GTS1*pr.183-*lacZ* was more activated by non-fermentable carbons than by glucose in the glucose-limited conditions. Thus, it is unlikely that the oscillatory expression was controlled only by *GCR1* even if the *cis*-element participates in the transcription of *GTS1*.

Why did *GTS1*pr.355 inhibit the oscillatory expression of *GTS1* and *lacZ*, while *GTS1*pr.183 and *GTS1*pr.1042 facilitated it? It is unlikely that the putative ORF YGL182C inhibited the expression, because *GTS1*pr.1042 also contains the full-length ORF and because the transcript was not detected in the transformant with multi-copy recombinant plasmids. Rather, it is possible that there are inhibitory elements in the upstream sequence between position –375 and –183 which are regulated by some stimulatory element(s) in the region further upstream up to –1042 and –355. The results in this report suggest that these yet-unidentified elements are required for the full oscillation of the Gts1p level to induce the coupling of the stress-resistance oscillations with the energy-metabolism oscillation. *GTS1*-pr.1042-355 contains several putative E-boxes (CANNTG), which are known as *cis*-elements for basic-helix-loop-helix (bHLH) transcription activators (*18*), which are involved in the oscillatory expression of the clock gene *period* in *Drosophila* (*19*). Interestingly, of several known bHLH transcription factors in yeast (*20*), the *SGC1* product is recognized as a transcription activator for the genes of main glycolytic enzymes like the *GCR1* product (*21*). However, further experiments should be done to determine *cis*-regulatory elements for basic oscillation of *GTS1* expression within the 183-bp upstream fragment and those in the region further upstream.

REFERENCES

1 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
2 Strässle, C., Sonnleitner, B., and Fiechter, A. (1988) A predictive model for spontaneous synchronization of *Saccharomyces cerevisiae* grown in continuous culture. I. Concept. *J Biotechnol* **7**, 299–318
3 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
4 Chen, C.-I. and McDonald, K.A. (1990) Oscillatory behavior of *Saccharomyces cerevisiae* in continuous culture· II Analysis of cell synchronization and metabolism. *Biotechnol. Bioeng.* **36**,

28–38
5 Münch, T, Sonnleitner, B, and Fiechter, A (1992) The decisive role of the *Saccharomyces cerevisiae* cell cycle behavior for dynamic growth characterization. *J Biotechnol.* **22**, 329–352
6 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
7. 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
8 Rose, M. and Botstein, D. (1983) Construction and use of gene fusion to *lacZ* (beta-galactosidase) that are expressed in yeast *Methods Enzymol* **101**, 167–180
9 Guarente, L (1983) Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast in *Methods in Enzymology* (Wu, R, Grossman, L, and Moldave, K., eds.) Vol 101, pp 181–191, Academic Press, New York
10 Myers, A.M., Tzagoloff, A., Kinney, D M, and Lusty, C J (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusion *Gene* **45**, 299–310
11. Mitsui, K., Yaguchi, S, and Tsurugi, K. (1994) The *GTS1* gene, which contains a Gly-Thr repeat, affects the timing of budding and cell size of the yeast *Saccharomyces cerevisiae Mol Cell Biol.* **14**, 5569–5578
12 Kawabata, K., Mitsui, K., Uno, T, Tamura, K., and Tsurugi, K. (1999) Protein interactions of Gts1p of *Saccharomyces cerevisiae* throughout a region similar to a cytoplasmic portion of some ATP-binding cassette transporters. *Eur J Biochem* **259**, 112–119
13 Baker, H V (1991) *GCR1* of *Saccharomyces cerevisiae* encodes a DNA binding protein whose binding is abolished by mutations in the CTTCC sequence motif. *Proc. Natl Acad. Sci. USA* **88**, 9443–9447
14. Huie, M.A, Scott, E.W, Drazinic, C M, Lopez, M.C., Hornstra, I K., Yang, T P., and Baker, H V (1992) Characterization of the DNA-binding activity of *GCR1* In vivo evidence for two *GCR1*-binding sites in the upstream activating sequence of *TPI* of *Saccharomyces cerevisiae*. *Mol Cell. Biol* **12**, 2690–2700
15 Baker, H V (1986) Glycolytic gene expression in *Saccharomyces cerevisiae* Nucleotide sequence of *GCR1*, null mutants, and evidence for expression. *Mol. Cell Biol* **6**, 3774–3784
16 Holland, M J, Yokoi, T, Holland, J P, Myambo, K., and Innis, M.A. (1987) The *GCR1* gene encodes a positive transcriptional regulator of the enolase and glyceraldehyde-3-phosphate dehydrogenase gene families in *Saccharomyces cerevisiae*. *Mol Cell Biol* **7**, 813–820
17 Kuroda, S, Otaka, S., and Fujisawa, Y (1994) Fermentable and nonfermentable carbon sources sustain constitutive levels of expression of yeast triosephosphate dehydrogenase 3 gene from distinct promoter elements. *J Biol Chem* **269**, 6153–6162
18 Ferre-D'Amare, A.R, Prendergast, G C, Ziff, E B, and Burley, S K. (1993) Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature* **363**, 38–45
19 Hao, H., Allen, D.L, and Hardin, P.E (1997) A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster*. *Mol Cell Biol* **17**, 3687–3693
20 Robinson, K.A. and Lopes, J M. (2000) *Saccharomyces cerevisiae* basic helix-loop-helix proteins regulate diverse biological processes *Nucleic Acids Res.* **28**, 1499–1505
21 Nishi, K., Park, C S, Pepper, A.E, Eichinger, G, Innis, M A., and Holland, M J (1995) The *GCR1* requirement for yeast glycolytic gene expression is suppressed by dominant mutations in the *SGC1* gene, which encodes a novel basic-helix-loop-helix protein *Mol Cell. Biol* **15**, 2646–2653