Gts1p Activates SNF1-dependent Derepression of HSP104 and TPS1 in the Stationary Phase of Yeast Growth*

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We previously reported that the GTS1 product, Gts1p, plays an important role in the regulation of heat tolerance of yeast under glucose-limited conditions in either batch or continuous culture. Here we show that heat tolerance was decreased in GTS1-deleted and increased in GTS1-overexpressing cells under glucose-depressed conditions during the batch culture and that the disruption of SNF1, a transcriptional activator of glucose-repressible genes, diminished this effect of GTS1. Intracellular levels of Hsp104 and trehalose, which were reportedly required for the acquisition of heat tolerance in the stationary phase of cell growth, were affected in both GTS1 mutants roughly in proportion to the gene dosage of GTS1, whereas those of other Hsps were less affected. The mRNA levels of genes for Hsp104 and trehalose-6-phosphate synthase 1 changed as a function of GTS1 gene dosage. The Q-rich domain of Gts1p fused with the DNA-binding domain of LexA activated the transcription of the reporter gene LacZ, and Gts1p lacking the Q-rich domain lost the activation activity of HSP104 and TPS1. Furthermore, Gts1p bound to subunits of Snf1 kinase, whereas it did not bind to DNA. Therefore, we suggested that GTS1 increases heat tolerance by mainly activating Snf1 kinase-dependent derepression of HSP104 and TPS1 in the stationary phase of yeast growth.

We reported that the GTS1 gene shows pleiotropic effects on yeast in batch cultures, including the effect on heat tolerance as a function of gene dosage (1); overexpression of GTS1 increases and deletion of GTS1 decreases the heat tolerance of yeast in the stationary phase of growth, whereas no such effects were found in exponentially growing cells. Independently, Bossier et al. (2) isolated GTS1 from cDNA library-transformed yeast which re-grew after lethal heat shock and found that overexpression of GTS1 results in an unchanged growth rate at 37 °C compared with 28 °C. On the other hand, we reported that GTS1 is involved in regulating ultradian oscillations of energy metabolism in continuous cultures under aerobic and glucose-limited conditions and in the coupling of oscillations of cellular responses to various stress conditions, including heat with the energy metabolism oscillation (3–5). These results suggested that the gene product Gts1p plays an important role in the regulation of heat and other stress responses under glucose-limited or -depleted conditions in either batch or continuous culture.

Gts1p contains a zinc finger motif similar to that of GATA-transcription factors (7) in the amino-terminal region and a glutamine-rich strand in the carboxyl-terminal region, and thus Gts1p has been conventionally classified as a transcription factor in a yeast genome data base (8). The zinc finger is now shown to be similar in structure to that contained in GTPase-activating proteins of ADP-ribosylation factors, which are considered to play a role in protein interaction rather than DNA binding (9, 10). Recently, we reported that the zinc finger is involved in the binding to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase and that disruption of the zinc finger causes loss of interaction between the proteins and affects the maintenance of ultradian oscillations of energy metabolism in yeast (11). However, the DNA binding activity of the zinc finger has not been tested. On the other hand, although Q-rich sequences have been known as one of the protein sequences common in transcriptional activators in higher organisms and known to work as an activator domain in yeast (12, 13), the function of the Q-rich domain in Gts1p remains to be elucidated.

In yeast, heat shock proteins (Hsps) and trehalose are known to participate in the acquisition of heat tolerance (for review see Refs. 14 and 15). Trehalose is a disaccharide consisting of two glucose molecules bound by the first carbons and known to confer heat tolerance to cells by stabilizing their membrane (15). During the batch culture of yeast, trehalose begins to accumulate at the diauxic shift (after overnight culture) when glucose levels decline and continues until the end of the post-diauxic phase (after several days culture) (16). On the other hand, some Hsps like Hsp70 and Hsp90, which comprise large protein families, are expressed in exponentially growing cells and increase in terms of their protein levels in stationary phase cells, although their specific roles are not well known (17). In contrast, the expression of Hsp104, although it is strongly heat-inducible in exponentially growing cells (18), begins right after the diauxic shift and continues to increase in the stationary phase (19). The expression of Hsp104 in stationary phase cells is crucial for the high tolerance to heat and other stresses (19, 20). Further, Elliott et al. (21) reported that a mutant unable to acquire heat tolerance in the stationary phase contains two mutations, one at the TPS2 locus encoding trehalose-6-phosphate phosphatase, another regulatory enzyme to trehalose synthesis along with TPS1, and the other at the HSP104 locus. They concluded that both trehalose and Hsp104 synergistically contribute to the acquisition of heat tolerance in the stationary phase cells, because a double mu-

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1 The abbreviations used are: Hsps, heat shock proteins; GFP, green fluorescent protein; GST, glutathione S-transferase; DAPI, 4',6'-diamidino-2-phenylindole; SC, synthetic medium; ORF, open reading frame.

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tant for TPS1 and HSP104 showed severe heat sensitivity in the stationary phase (21).

In this report, we investigated the mechanism by which Gts1p modulates the acquisition of heat tolerance in yeast during the stationary phase of cell growth. We found that heat tolerance was affected depending on the GTS1 gene dosage under derepressed conditions and that Gts1p acts as a transcriptional activator for HSP104 and TPS1 depending on Snf1 kinase. We further showed that the carboxy-terminal Q-rich domain of Gts1p is essential for the transcriptional activation, whereas the protein lacks DNA binding activity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—All of the mutants and transformants used in this report were derived from Saccharomyces cerevisiae W303-1A (MATa SUC2 ade2 can1 his3 trp1 lac2 ura3). Cells were cultured at 30 °C in a synthetic (SC) medium containing 2% or 0.2% glucose and required amino acids (22). The glucose-repressed cells were collected after culture for 8 h on 2% glucose. To collect the glucose-repressed cells in most experiments, cells pre-cultured overnight on glucose and required amino acids (22). The glucose-repressed cells were diluted 50-fold with the synthetic medium containing 0.2% glucose and cultured for 18 h.

Production of Δts1 and the Mutants for GTS1 Dosage—An S. cerevisiae Δts1-disrupted mutant, Δts1 (S. cerevisiae Δts1::URA3), was obtained by transforming W303-1A with the recombinant plasmid pUCGTS1-2, which was derived from pUCGTS1 (24) after deleting the BamHI-KpnI fragment spanning nucleotide positions −426 to +883, with respect to the A of the initiation codon of GTS1, from the plasmid. The GTS1-overexpressing mutant, named TmpGTS1, was obtained as described previously (25). To produce a GTS1-overexpressing transformant in the culture on galactose, the PCR-amplified Sall-HindIII fragment carrying the GTS1 ORF was inserted into the cognate sites of the multicopy vector YEps1 downstream of the GAL10 promoter. The resultant recombinant plasmid was inserted with the BamHI-SpeI fragment from pPER119 containing the GTS1 terminator at the BamHI-BclI sites. The plasmid named pGAL-GTS1 was transformed into Δts1.

Construction of Plasmids to Produce HSP104- and TPS1-deleted Mutants—To produce the HSP104-deleted mutant, named hsp104Δ, the PCR-amplified Smal-KpnI fragment containing nucleotides −385 to +580 with respect to the first residue A of HSP104 was inserted into the cognate sites of pBluescript SK(+), and HSP104 in the recombinant plasmid was disrupted by replacing the 436-bp Apal-EcoRI fragment with the Apal-L-NcoI fragment carrying HIS3 from pRS413 (Stratagene, Kirkland, WA). To obtain the TPS1-deleted mutant, named ipsΔ, the PCR-amplified SmaI-KpnI fragment containing nucleotides −500 to +1485 with respect to the first residue A of TPS1 was inserted into the cognate sites of pBluescript SK(+), and TPS1 was disrupted by replacing the 1146-bp NcoI-NcoI fragment with the NcoI-PvuII fragment carrying TRPI from pGBD9 (Clontech).

Construction of Recombinant Plasmid Carrying GTS1 Fused to DNA-binding Domain of LexA—To construct plexA-GTS1, for expression of Gts1p fused to the DNA-binding domain of LexA, the PCR-amplified EcoRI-SalI fragment directed on pPER119 (24) was cloned into the same sites of pSH2-1 (26), which was a gift from Dr. M. Carlson (Columbia University). To construct plexA-Q, for expression of the Q-rich domain of Gts1p (the sequence from nucleotide positions 946 to 1154 with respect to the A of the initiation codon of GTS1), which was fused to the DNA-binding domain of LexA, the EcoRI-NcoI fragment of plexA-GTS1 was deleted. The plasmid pJK1621 carrying lacZ as a reporter gene (27) was a gift from Dr. M. Carlson (Columbia University).

Construction of a Plasmid Carrying GTS1 Lacking the Q-rich Domain—To obtain a mutant expressing Gts1p lacking the carboxy-terminal sequence, which contains the Q-rich sequence from amino acid positions 316 to 396, named Gts1p-ΔQ, the Sall-PvuII fragment from pGBT9 (Clontech, Palo Alto, CA), carrying TRPI followed by the ADH1 terminator, was inserted into the NcoI site of plexA-GTS1 (24). The recombinant plasmids were integrated into the GTS1 locus of W303-1A.

Construction of Plasmids Carrying Gts1p-Fused to GFP—A fusion gene encoding GTS1 and GFP in-frame under the control of the promoter of TDH3 was integrated into the GTS1 locus. For this purpose, a HindIII-EcoI fragment carrying a gene encoding green fluorescent protein (GFP) from pEGFP-N1 (Clontech) was inserted into the cognate sites of pBluescript SK(+), and the HindIII-SacI fragment from the resulted plasmid was inserted into the cognate sites of pXY223 (R&D Systems, Minneapolis, MN) generating pXY223-GFP. Then, a PCR-amplified EcoRI-HindIII fragment carrying the GTS1 ORF was inserted into the cognate sites of pXY223-GFP obtaining pXY223-GTS1-GFP. To construct the integration plasmid, pHIS1-GTS1-GFP, the BamHI-BamHI fragment carrying the 5′-half of GTS1 and TDH3 promoter from pGTS1, which had been constructed by inserting the EcoRI-HindIII fragment of the PCR-amplified GTS1 ORF into the cognate sites of pKT10 (28), was inserted into the BamHI site of pUCGTS1-2, and then the EcoRV-SacI fragment from pXY223-GTS1-GFP, carrying the 3′-half of GTS1 and GFP in-frame, was inserted into
were digested with either subunit of Snf1 kinase tagged with myc13 at the carboxyl terminus.

The expressed His6 and Gts1p-His6 in yeast were purified with EcoRI and the SNF1 upstream sequence of the resulted plasmid. Then, PCR-amplified ORFs of EcoRI inserted into the RI-I sites of pUC and the cognate sites of the resulted plasmid. Finally, the BamHI-SacI fragment from pUCAGTGS1-2 carrying the 1.7-kbp downstream sequence of GTS1 was inserted into the EcoRI and cloned into the cognate sites of the resulted plasmid. Finally, the BamHI-SacI fragment from pUCAGTGS1-2 carrying the 1.7-kbp downstream sequence of GTS1 was inserted into the EcoRI-SacI fragment from the recombinant plasmid was inserted into the EcoRI-SacI sites of pYX222 (R&D Systems), generating pYX222myc. Then, PCR-amplified ORFs of SNF1, SNF4, and GAL83 were digested with SacI and cloned into the EcoRI-SacI sites of pYX222myc, generating recombinant plasmids carrying genes encoding either subunit of Snf1 kinase tagged with myc13 at the carboxyl terminus.

Determination of Heat Tolerance—Heat tolerance of yeast cells was determined as described previously (25).

Enzyme Assays—For determination of invertase activity, cells cultured for varying time periods in SC medium containing 2% or 0.2% glucose were harvested, washed with 10 mM sodium azide four or five times, weighed, and frozen at −80°C until use for invertase assays (31). Invertase activity is expressed as micromoles of glucose released per minute per 1 g of cells (wet weight). β-Galactosidase was assayed after permeabilization of the cells and expressed in Miller units as described previously (32).

Determination of the Levels of Hsp and Trehalose—Proteins were isolated from cell lysates with Isogen (Nippon Gene, Tokyo, Japan), and Hsp levels were analyzed by one-dimensional SDS-PAGE, and the intracellular trehalose level was determined as described previously (33).

Northern and Western Blot Analyses—Total RNA was isolated with Isogen (Nippon Gene), and 20 μg of RNA was electrophoresed on an agarose gel after denaturation with glyoxal and dimethyl sulfoxide. Hybridization and probe labeling were performed with the PCR digitonin labeling mix (Roche Applied Science) (5). Western blotting of Gts1p was performed as described previously (24). Northern and Western blots were visualized with a lumino-image analyzer (RAS-1000, Fuji film, Tokyo, Japan). RNA was stained with CYBR green I (Takara, Tokyo, Japan).

Gel Shift Assay—For overexpression of Gts1p tagged with six molecules of histidine (Gts1p-His6) under the control of the TPI promoter in yeast, the HindIII-StyI fragment of pET22b+ (Novagen, Darmstadt, Germany) was inserted into the HindIII-HindIII fragment of pYX222 (R&D Systems) obtaining a recombinant plasmid encoding His6 as a control. The resulted plasmid was inserted with the EcoRI-HindIII fragment of the PCR-amplified GTS1 ORF into the cognate sites to express Gts1p-His6. The expressed His6 and Gtslp-His6 in yeast were purified with nickel-nitrioltriacetic acid-agarose (Qiagen, Hilden, Germany) according to the manufacturer’s product manual.

As DNA probes, the upstream sequences of HSP104 from −271 to −1 and from −380 to −192 with respect to the A residue of the initiation codon of HSP104 were amplified by PCR and labeled with the digitonin nick.
oligonucleotide 3′-end-labeling kit (Roche Applied Science). The coding sequence of GTS1 from 6 to 162 was PCR-amplified and used as a nonspecific competitor. Gel-shift assay was performed according to Conlan et al. (34), and signals were detected as in the Northern blotting.

Microscopic Observation—The nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI) after fixing with 4% paraformaldehyde for 30 min. Fluorescence of GFP and DAPI was visualized using an Olympus BX-50 microscopy.

RESULTS

Effect of Different Gene Dosages of GTS1 on the Acquisition of Heat Tolerance in the Stationary Phase—When cells were cultured for 18 h on a synthetic medium containing 2% glucose as a sole carbon source, the heat tolerance of the GTS1-overexpressing mutant (TMpGTS1) was increased while that of the GTS1-deleted mutant (gts1Δ) was unchanged compared with that of the wild-type cells (Fig. 1A). Although the heat tolerance of gts1Δ had not changed on the second day of the culture (data not shown), it was decreased on the third day, whereas in other cells it remained constant (Fig. 1A). Thus, the gene dosage effect of GTS1 on the acquisition of heat tolerance took 3 days to appear when cells were cultured on 2% glucose. Because we speculated that the slow acquisition of heat tolerance was caused by a delayed consumption of glucose, we determined the activity of invertase encoded by SUC2, a representative glucose-repressible gene (35), during the culture. The time course of the invertase activity showed a slight rise after the first day of culture and a very slow increase until the third day (Fig. 1B), indicating that the culture took a long time to reach a sufficient level of invertase activity.

TPS1 between gts1Δ and snf1Δ. The mRNA levels were determined by densitometry of the three independent Northern blots, and ratios of every mRNA level in gts1Δ to those in snf1Δ were calculated. The ratio of ACT1 was normalized to 1.0. Statistical analysis of t-test (n = 3) indicates: *, no significant difference (p > 0.1); **, significant differences at p < 0.001 compared with the value of ACT1 as a control.
degree of derepression due to the high glucose concentration (2%) in the medium. So, we cultured cells on 0.2% glucose and found that they showed strong invertase activity after 18 h of culture (Fig. 1B) accompanying changes of heat tolerance depending on the gene dosage of GTS1 (Fig. 1A). Therefore, we suggested that the gene dose effect of GTS1 on heat tolerance occurred under the derepressed conditions. Hereafter, we employed these culture conditions (18 h of culture on 0.2% glucose) to obtain derepressed cells.

Then, we examined whether the effect of Gts1p on heat tolerance under the derepressed conditions is mediated by SNF1, a catalytic subunit of Snf1 kinase, which is known as a gene and metabolic activator under derepressed conditions (Fig. 2A). The heat tolerance of either the wild-type or GTS1-overexpressing cells derived from the SNF1-deleted mutant snf1Δ was decreased down to the level of gts1Δ, whereas that of the double mutant gts1Δ snf1Δ was unchanged. Under the derepressed conditions (8 h culture on 2% glucose), on the other hand, the heat tolerance was not significantly decreased in either snf1Δ or gts1Δ compared with that in the wild-type, although it was very low (Fig. 2B). Thus, the results suggested that the effect of Gts1p on the heat tolerance was caused in combination with Snf1 kinase under the derepressed conditions.

Intracellular Levels of Hsps and Trehalose under the Derepressed Conditions—In yeast, heat shock proteins and trehalose are known to participate in the acquisition of heat toler-

Fig. 6. The time course of the mRNA level of TPS1 during the culture on 0.2% glucose. A, patterns of Northern blots of TPS1 mRNA from the wild-type, TmpGTS1, and gts1Δ during the incubation for 5, 10, and 18 h in the medium containing 0.2% glucose. These are representative patterns of two independent experiments. B, the TPS1 mRNA level determined by densitometry of the Northern blots in A.

Fig. 7. The function of the Q-rich domain of Gts1p for transcriptional activation. A, transcriptional activation of LexA as a reporter by Gts1p (LexA-Gts1p) and its Q-rich domain (LexA-Q) fused to the DNA-binding domain of LexA. LexA (vector alone), LexA-Gts1p, and LexA-Q were expressed in the wild-type cell transformed with a recombinant plasmid carrying LexA as a reporter gene under the control of the LexA operator, and the transformed cells under the repressed (left) and derepressed conditions (right) were collected. β-Galactosidase activity was expressed in Miller units (32). Statistical analysis of t-test (n = 3) indicates: *, no significant difference (p > 0.1); ** and ***, significant differences at p < 0.001 and p < 0.0005, respectively. B, the protein level of the Q-rich domain-deleted Gts1p (Gts1p-Q) determined by Western blotting. C, the effect of Gts1p-Q on the activation of HSP104 and TPS1 under the derepressed conditions. The mRNA levels of HSP104 and TPS1 in the wild-type (WT), Gts1p-Q expressing, and gts1Δ cells were determined by Northern blotting. In the mutant cell, GTS1-ΔQ was introduced into the GTS1 locus.
near the baseline, whereas Hsp90 and Hsp70 levels returned half the value in the wild-type cells. On the other hand, the trehalose level was most increased in the GTS1-overexpressing cells among the three, but it was also increased about 2.5-fold in gts1Δ (Fig. 3C), which is inconsistent with the levels of heat tolerance (Fig. 1A). Therefore, because the trehalose levels between gts1Δ and the wild-type were reversed, the levels of heat tolerance among the cells under the derepressed conditions can be attributed, if not totally, to the differences in the Hsps, especially Hsp104, and to a lesser extent, trehalose levels.

**Effect of Gts1p on Transcription of Genes of Hsps and Trehalose Synthesis in the Glucose-derepressed Cell**—To investigate whether Gts1p regulates the expression of the Hsps and trehalose at the transcription level under derepressed conditions, the mRNA levels of HSP104 and TPS1 were determined by Northern blotting. We also determined the mRNA levels of HSP26 and HSP12, instead of HSP70s and HSP90s, because they consist of single molecules and are known to be induced in the stationary phase like HSP104 (38, 39). In this experiment, we conventionally used the rRNA levels stained with CYBR green for the quantitative control of RNA amounts applied to SDS-PAGE in Northern blot analysis, because the mRNA levels of ACT1, PGK1, TBPI, and ADH1, which are usually used for this purpose, were all affected in the GTS1 dosage transformatants, especially in TmpGTS1. The mRNA levels of Hsps 104, 26, 12, and Tps1p were increased in the derepressed cells compared with the repressed cells in a GTS1 gene dosage-dependent manner (Fig. 4). The effect of GTS1 on the mRNA levels disappeared in snf1Δ suggesting a dependence on Snf1 kinase. It should be pointed out that the mRNA levels of Hsps 26 and 12 were much higher in gts1Δ than in snf1Δ, whereas those of Hsp104 and Tps1p in gts1Δ were as low as in snf1Δ (Fig. 4B). To verify the differential effect of GTS1 on the SNF1-dependent expression of the four genes, we compared the mRNA levels in gts1Δ with those in snf1Δ using ACT1 for the quantitative control instead of rRNA (Fig. 4C). The result suggested that Gts1p is partially involved in the SNF1-dependent activation of HSP26 and HSP12, whereas it is fully involved in that of HSP104 and TPS1.

To further determine the effect of GTS1 on the expression of HSP104 and TPS1, we examined whether deletion of the genes abrogates the increased heat tolerance associated with GTS1 overexpression. In this experiment, cells were cultured on galactose as a carbon source (40), because the growth rate of tps1Δ was severely inhibited in the culture on glucose. When the wild-type cells were grown on galactose for 24 h, the heat tolerance was increased about 2-fold compared with that of the glucose-repressed cells (compare the second lane in Fig. 5 with the second lane in Fig. 1), and the heat tolerance was further increased about 2-fold by overexpression of Gts1p (Fig. 5). The heat tolerance was decreased in tps1Δ, hsp104Δ, and tps1Δ hsp104Δ in this order compared with the wild-type, and the effect of GTS1 overexpression on the heat tolerance was decreased in the mutants in proportion to the strength of heat tolerance (Fig. 5). In the double mutant tps1Δ hsp104Δ, the heat tolerance was only a little increased by overexpression of GTS1 suggesting that the two genes are predominantly involved in the acquisition of heat tolerance in derepressed cells. It should be added that a similar result was obtained concerning HSP104 using hsp104Δ cultured on glucose into the derepressed conditions (data not shown).

Recently, Grably et al. (41) reported that transcriptional control of HSP104 is achieved exclusively or cooperatively through activation of heat shock elements and stress response elements. So, we examined the effect of GTS1 gene dosage on the transcription of HSP104 and other genes tested above in response to heat shock. The heat shock responses of the genes were not affected by the different gene dosage of GTS1 (data not shown) suggesting that the transcriptional activation of the genes by GTS1 under the derepressed conditions was not caused through heat shock factors and stress response elements.

**Change of the TPS1 mRNA Level during the Culture on Glucose**—We determined the time course of TPS1 mRNA expression during the culture on 0.2% glucose, because we found that the mRNA levels of TPS1 in the derepressed cells changed as a function of GTS1 dosage (Fig. 4B), which was inconsistent with the distribution of trehalose levels (Fig. 3C). The result revealed that the level in gts1Δ was most increased among the three in the repressed cells (cultured for 5 h), whereas it was most decreased under the derepressed conditions (Fig. 6). The same result was obtained in an experiment comparing the TPS1 mRNA levels between the repressed (cultured for 6 h) and derepressed (cultured for 72 h) cells on 2% glucose (data not shown). These results account, at least in part because the Tps1p level has not determined, for the fact that gts1Δ contained more trehalose than the wild-type cell after 18 h of culture on 0.2% glucose (Fig. 3C).

**Transcriptional Activation of the Q-rich Domain of Gts1p**—Because it was shown that Gts1p activates the transcription of genes, we examined the ability of the Q-rich domain to activate the expression of a reporter gene when it is forced to bind to DNA. We constructed recombinant genes encoding GTS1 and the Q-rich domain, which are fused in-frame after the DNA-binding domain of LexA and named LexA-GTS1 and LexA-Q, respectively. They were transformed together with a recombinant plasmid carrying LacZ as a reporter under the control of
Fig. 9. DNA binding activity of Gts1p determined by gel-shift assays. A, Gts1p-His$_6$ and His$_6$ as a control were incubated with digitonin-labeled DNA fragments spanning from −271 to −1 (Probe 1) and from −380 to −192 (Probe 2) of HSP104 as probes. Complex formation was tested by visualizing with a lumino-image analyzer after gel electrophoresis. B, labeled probes were incubated with Gts1p-His$_6$, in the presence of cold specific and nonspecific probes as competitive inhibitors added at a concentration 50 times higher than labeled probes and antibody against Gts1p. Arrows and arrowheads in A and B indicate the position of probes 1 and 2, respectively. *, position of a nonspecific signal.

Fig. 10. The binding activities of Gts1p for Snf1 kinase subunits Snf1p, Snf4p, and Gal83p. A myc$_3$-tagged subunit of Snf1 kinase was expressed in the wild-type, and complex formation was tested by immunoprecipitation with anti-Gts1p antibody followed by immunoblotting using anti-myc tag antibody. Arrowheads in the upper panel indicate the positions of the respective myc$_3$-tagged subunits of Snf1 kinase. The arrow in the lower panel indicates the position of Gts1p.

The LexA operator and CYC1 promoter (Fig. 7A). LexA-Q activated the transcription of the reporter more than LexA-Gts1p in both the glucose-repressed and derepressed cells, whereas LexA-Gts1p activated the transcription more in the derepressed than repressed cells (Fig. 7A). These results suggested that the Q-rich domain of Gts1p functions as a transcriptional activation domain and that some other region of Gts1p is required for the activation under the derepressed conditions.

To examine whether the Q-rich domain of Gts1p is required for the activation of HSP104 and TPS1, we introduced GTS1ΔQ lacking the sequence encoding the Q-rich domain into the GTS1 locus and examined the effect on expression (Fig. 7, B and C). Although the expression level of Gts1p-ΔQ was about half that of Gts1p in the wild-type (Fig. 7B), the expression of both HSP104 and TPS1 was suppressed as low as gts1Δ (Fig. 7C) suggesting that the Q-rich domain is required for the activation of the genes in vivo.

Subcellular Localization of Gts1p—Although Gts1p without the Q-rich domain lost the ability to activate target genes, it is possible that deletion of the Q-rich domain changed its subcellular localization. So, to test this possibility, the localization of Gts1p was examined by integrating a recombinant gene encoding GFP-fused Gts1p in the GTS1 locus. The transformant expressing the GFP-fused Gts1p showed almost the same heat tolerance as the wild-type under the derepressed condition, suggesting that the fusion protein was functional (data not shown). The fusion protein was predominantly localized in nuclei irrespective of the presence of the Q-rich domain in the derepressed cells (Fig. 8A). This result supported our notion that the Q-rich domain is required for the transcriptional activation. The protein level (Fig. 8B) and intracellular distribution of Gts1p (data not shown) did not differ between the repressed and derepressed cells, suggesting that the function of Gts1p as a transcriptional activator under the derepressed conditions is not regulated by its intracellular location and transcription.

DNA Binding Activity of Gts1p—Because Gts1p possesses a zinc finger motif in the amino-terminal portion, we examined whether Gts1p directly binds to DNA by the gel-shift assay. Gts1p tagged with His$_6$ at the carboxyl terminus was overexpressed in yeast, and the purified protein was named Gts1p-His$_6$. DNA fragments of the upstream sequence of HPS104 ranging from −271 to −1 (probe 1) and from −380 to −192 (probe 2), with respect to the A of the initiation codon of HPS104, were used as probes, because Grably et al. (41) reported that a 334-bp fragment upstream of the first coding AUG is sufficient and essential for maximal basal activity. We tested the gel-shift assay under various binding conditions and found that neither probe formed any complexes with Gts1p-His$_6$ except for a faint signal that was also found for His$_6$ as a control (Fig. 9A). This proved to be a nonspecific signal, because it was not competitively reduced by addition of either specific or nonspecific probe and because the signal did not show a suprer-
shift after addition of antibody against Gts1p (Fig. 9B). It should be noted that no specific complexes were detected between the probes and proteins of cell lysates from Gts1p-overexpressing cells in the place of purified Gts1p (data not shown). Furthermore, because the zinc finger of Gts1p is similar to that of GATA family transcription factors (7), the binding activity was tested using synthetic oligonucleotides containing binding motifs such as GATA, GATT, and GAAT (42), but no complexes were detected by gel-shift assay between Gts1p and these probes (data not shown). These results suggested that Gts1p does not have DNA binding activity. It should be mentioned that the cells overexpressing the zinc finger-disrupted Gts1p encoded by GTS1(C53Y) (11), inserted in a multicopy vector, still showed heat tolerance that was as high as that of TmpGTS1 (data not shown), suggesting that the zinc finger is not involved in the activation of the gene.

Interaction of Gts1p with Subunits of Snf1 Kinase—Snf1 kinase is a heterotrimeric protein composed of Snf1p (α), Snf4p (γ), and any molecule of the three β isoforms, Gal83p, Sip1p, and Sip2p, which are involved in targeting the kinase into different subcellular components (43). Because Gal83p directs Snf1 kinase to the nucleus in response to glucose depletion, we examined the binding activity with Gts1p of Gal83p in addition to Snf1 kinase. An myc13-tagged subunit of Snf1 kinase was expressed in the wild-type, and complex formation between them was tested by immunoprecipitation with anti-Gts1p antibody followed by immunoblotting (Fig. 10). Apparently, all subunits of Snf1 kinase were detected showing similar band densities in the immunoprecipitate. It should be added that a similar result was obtained in an experiment using glutathione S-transferase (GST)-tagged Gts1p and myc13-tagged subunits of Snf1 kinase coexpressed in gts1Δ; GST-tagged Gts1p was detected in the immunoprecipitates with anti-myc antibody, and, conversely, myc13-tagged subunits of Snf1 kinase were detected in the affinity precipitate with a glutathione-Sepharose beads, although the band densities were different among the subunits (data not shown). Thus, it is likely that Gts1p activates target genes associating with Snf1 kinase rather than directly binding to DNA.

DISCUSSION

In this report, we showed that Gts1p induced the acquisition of heat tolerance of yeast in the stationary phase by activating the transcription of HSP104 and TPS1 genes, depending on the presence of Snf1 kinase. Also, we reported for the first time that Snf1 kinase is required for the acquisition of heat tolerance under the derepressed conditions by activating the genes. As far as we tested in this report, other glucose-repressible genes like HSP26 and HSP12 were also activated by Gts1p, but the activation occupied only a part of the total increase by Snf1 kinase under the derepressed conditions. A similar result was obtained in the activation of SUC2, because the invertase activity in gts1Δ, which was decreased compared with that in the wild-type, was much higher than that in snf1Δ (data not shown). In addition, we showed that the expression of the Hsp70 and Hsp90 protein families was partially activated by Gts1p in a gene dosage-dependent manner, although it remains possible that particular members of the protein families are activated by Gts1p specifically under the derepressed conditions, because we did not separately analyze all members in the assay using gel electrophoresis. Together, although the derepression of the glucose-repressible genes was absolutely dependent on Snf1 kinase, only some of them such as HSP104 and TPS1 are derepressed by Gts1p in combination with Snf1 kinase.

Snf1 kinase is a yeast homologue of mammalian AMP-activated protein kinase that is known as a metabolic sensor of the eukaryotic cell, which regulates various metabolic reactions depending on the energy state of the cell (44). AMP-activated protein kinase acts as a metabolic regulator both acutely by phosphorylation of metabolic enzymes and chronically by effects on gene expression (45). Although Gts1p is a phosphorylated protein (25), it is unlikely that Gts1p is a phosphorylation target of Snf1 kinase, because it does not have any sequence motifs for the kinase. Rather, because Gts1p was shown to bind to Snf1 kinase by our affinity precipitation analysis, we suggest that Gts1p functions as one of transcriptional modulators for Snf1 kinase, which then functions in the RNA polymerase II holoenzyme complex (46). Alternatively, because Snf1 kinase has been recognized as a component of a histone kinase complex that works in concert with the histone acetyltransferase Gcn5 (47), it is possible that Gts1p regulates transcription by controlling histone modification via interaction with Snf1 kinase. We have started experiments to address these questions.

We showed in this report that the Q-rich domain of Gts1p activates the transcription of some genes in agreement with previous reports (12, 13). However, we found that Gts1p lacking the Q-domain was still able to associate with Snf1 kinase subunits by immunoprecipitation analysis (data not shown). The Q-rich domain of the transcriptional activator Sp1 in higher organisms has been reported to associate with the general transcription factor TFIIID, which contains the TATA-binding factor and several TATA-binding factor-associated factors (48–50). Thus, it is possible that the Q-rich domain is involved in the binding to some basic transcription factors rather than to Snf1 kinase. So, at present, it is unclear how Gts1p binds to Snf1 kinase.

Previously, we found that GTS1 is involved in the coupling of ultradian oscillations of heat tolerance with the energy metabolism oscillation, which is a periodic change between respiratory and respiro-fermentative phases (4). Recently, we reported that heat tolerance oscillation is induced by a fluctuation in the trehalose level and not by an oscillatory expression of Hsps (33). The increase in the trehalose level began at the start of the respiro-fermentative phase, and the decrease began after the elevation of the cAMP level. Thus, we hypothesized that the synthesis of trehalose parallels the activation of the glycolytic pathway sharing glucose 6-phosphate as an initiating substrate and trehalose is degraded by neutral trehalase activated by cAMP in coupling with the metabolic oscillation in the continuous culture of yeast (33). The results shown in this report raised the possibility that the expression of TPS1/TPS2 is regulated at the transcription level depending on the intracellular energy state, which periodically changes during the metabolic oscillation. In fact, a preliminary experiment showed that the mRNA level of TPS1 fluctuated in concert with the metabolic oscillation and was affected by the disruption of GTS1. Thus, it is possible that GTS1 acts as a transcription regulator during the metabolic oscillation in the continuous culture. In addition, because Gts1p has been shown to interact with many proteins, including metabolic enzymes and protein kinases, besides transcription factors by a genome-wide analysis employing the two-hybrid system (51, 52), it is possible that Gts1p, like Snf1 kinase, regulates metabolic enzymes in addition to transcription factors. Further studies must be done to answer these questions.

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Gts1p Activates SNF1-dependent Derepression of HSP104 and TPS1 in the Stationary Phase of Yeast Growth
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