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NOTE

The *Schizosaccharomyces pombe* Gene Encoding γ-Glutamyl Transpeptidase I Is Regulated by Non-fermentable Carbon Sources and Nitrogen Starvation

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In our previous study, the first structural gene (*GGTI*) encoding γ-glutamyl transpeptidase was cloned and characterized from the fission yeast *Schizosaccharomyces pombe*, and its transcription, using the GGTI-*lacZ* fusion gene, containing the 1,085 bp upstream region from the translational initiation point, was found to be enhanced by sodium nitroprusside and L-buthionine-(S,R)-sulfoximine (BSO). In the present work, regulation of the *GGTI* gene was further elucidated. Non-fermentable carbon sources, such as acetate and ethanol, markedly enhanced the synthesis of β-galactosidase from the GGTI-*lacZ* fusion gene. However, its induction by non-fermentable carbon sources appeared to be independent of the presence of the Pap1 protein. Nitrogen starvation also gave rise to induction of *GGTI* gene expression in a Pap1-independent manner. The three additional fusion plasmids, carrying 754, 421 and 156 bp regions, were constructed. The sequence responsible for the induction by non-fermentable carbon sources and nitrogen starvation was identified to exist within a -421 bp region of the *GGTI* gene. Taken together, the *S. pombe GGTI* gene is regulated by non-fermentable carbon sources and nitrogen starvation.

Key words: *Schizosaccharomyces pombe*, γ-glutamyl transpeptidase, non-fermentable carbon sources, regulation, GGTI-*lacZ* fusion gene, Pap1, ethanol, acetate, nitrogen starvation

γ-Glutamyl transpeptidase (GGT; EC 2.3.2.2), a membrane-bound enzyme ubiquitously distributed in living organisms, catalyzes the degradation of GSH (L-γ-glutamyl-L-cysteinylglycine) by cleavage of the γ-glutamyl bond, allowing the supply of extracellular cysteine for intracellular synthesis of GSH. It plays numerous physiological roles, such as protection against oxidative stress, detoxification, transport and enzymic catalysis (Penninckx and Elskens, 1993). GGT is a key enzyme implicated in the homeostasis of intracellular reduced GSH; and hence, in the regulation of the cellular redox state. GGT cleavage of GSH, and the subsequent recapture of cysteine, allows living cells to maintain low levels of cellular reactive oxygen species (ROS), and thereby avoid apoptosis induced by oxidative stress (Karp *et al.*, 2001). When GGT-deficient mice were exposed to hyperoxia, their survival was significantly decreased from that of the control due to the accelerated formation of vascular pulmonary edema, widespread oxidant stress in the epithelium, diffuse depletion of GSH and severe bronchiolar cellular injury, indicating a critical role for GGT in lung glutathione homeostasis and antioxidant defense in normoxia and hyperoxia (Jean *et al.*, 2002). In tumors and cell lines with elevated levels of GGT activity, inhibition of this enzyme led to decreases in the cysteine levels (Ruoso and Hedley, 2004). GGT is important in the utilization of extracellular glutathione as a sulfur source in *B. subtilis* (Minami *et al.*, 2004).

In the yeast *S. cerevisiae*, GGT on the vacuolar membrane plays a role in the vacuolar transport and metabolism of the GSH stored in vacuoles, serving as an alternative nitrogen source during nitrogen starvation (Mehdi and Penninckx, 1997; Mehdi *et al.*, 2001). The expression of the *S. cerevisiae GGT* gene is highly induced in cells growing on a poor nitrogen source, such as urea, through the GATA zinc-finger transcription factors Nil1 and Gln3, with Gzf3, another GATA zinc-finger

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protein, acting as a negative regulator of nitrogen-source control in the expression of GGT (Springael and Penninckx, 2003). The GGT activity in *Candida albicans* is influenced by the carbon and nitrogen sources, and its increased activity is responsible for the rapid decrease of the intracellular GSH in *C. albicans* during yeast-to-mycelium conversion (Gunasekaran *et al.*, 1995; Manavathu *et al.*, 1996). In adult humans, the serum GGT activity was increased by ethanol (Nakajima *et al.*, 1994), and also induced GGT activity in the C2 rat hepatoma cell line (Barouki *et al.*, 1989). Meningococcal GGT is processed into two subunits in *Neisseria meningitidis,* at the conserved amino acid, threonine 427, with the majority of meningococcal GGT associated with inner membrane facing to the cytoplasmic side (Takahashi and Watanabe, 2004). The first structural gene encoding GGT, later known as *GGTI*, was previously cloned from the fission yeast *Schizosaccharomyces pombe*, and its transcription was induced by NO-generating sodium nitroprusside and GSH-depleting L-buthionine-(S,R)-sulfoximine (BSO) in a Pap1-dependent manner (Park *et al.*, 2004). In the present study, the regulation of the *GGTI* gene from *S. pombe,* by non-fermentable carbon sources and nitrogen starvation, is reported.

The original fusion plasmid carries the GGTI-*lacZ* fusion gene, containing the 1,085 bp upstream from the translational initiation point of the *GGTI* gene (Park *et al.*, 2004). The *E. coli* strain, MV1184 [*ara Δ(lac-proAB) rpsL thi* (*Φ80 lacZAM15*) Δ(*srl-recA*)*306::Tn10*(*tet*^{r})], and *S. pombe,* KP1 (*h*$^+$ *leu1-32 ura4-294*), were typically used as hosts for the transformation. The yeast cells were grown in minimal medium (Kim *et al.*, 2004), and the 2-day-grown culture was diluted 500-fold for inoculation. The yeast cells were grown at 30°C, with shaking, and the cell growth was monitored by the absorbance at 600 nm. The β-galactosidase activity in the extracts was measured by a spectrophotometric method, using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate (Guarente, 1983). The protein content of the extracts was measured by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.

Growth of the budding yeast on non-fermentable carbon sources, such as lactate, ethanol or acetate, requires the coordinated expressions of several genes, which are dispensable in the presence of substrates easily utilized by glycolysis (e.g. glucose or fructose) (Hiesinger *et al.*, 2001). Especially, growth with a non-fermentable carbon source requires the coordinated transcriptional activation of gluconeogenic structural genes by an upstream activation site (UAS) element, designated CSRE (carbon source-responsive element). An *S. pombe* culture, containing the fusion plasmid pGT98, was grown in supplemented minimal medium, and split at the early exponential phase. A switch was then made from 2% glucose to 2% potassium acetate or 2% ethanol as the sole carbon source. The β-galactosidase activity was measured at 3, 6 and 9 h after the switch. Initially, the switch to a non-fermentable carbon source gave rise to an arrest in the growth of the treated cultures (Fig. 1A, Fig. 2A). A switch to 2% potassium acetate significantly increased the synthesis of β-galactosidase from the GGTI-*lacZ* fusion gene (Fig. 1B). The yeast cells grown in 2% potassium acetate appeared to contain 2.19-, 3.70- and 2.51-fold higher β-galactosidase activities at 3, 6 and 9 h after the switch, respectively, compared with that in the control culture (Fig. 1B). Ethanol (2%), the other non-fermentable carbon source used in this study, gave a similar induction pattern (Fig. 2B), together with growth arrest (Fig. 2A). Taken together, non-fermentable carbon sources are able to up-regulate the expression of the *GGTI* gene in *S. pombe* cells. However, the relation between growth arrest and induction by

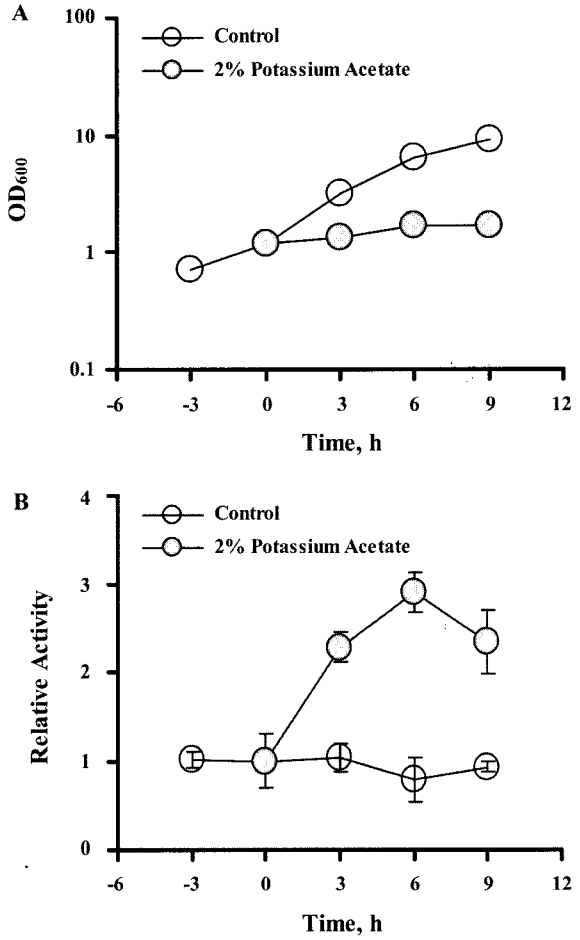

Fig. 1. Effect of potassium acetate, as the sole carbon source, on the β-galactosidase synthesis from the GGTI-*lacZ* fusion gene in wild-type *S. pombe* cells. The *S. pombe* cells harboring the fusion plasmid, pGT98, were grown in minimal medium, and split at the early exponential phase. (A) Growth curves. (B) β-Galactosidase activity. The β-galactosidase activity was determined at 37°C by the spectrophotometric assay using ONPG as a substrate, with the specific activity expressed in ΔA_{420}/min/mg protein. The relative activity was calculated by considering the specific activity of the untreated culture as 1.

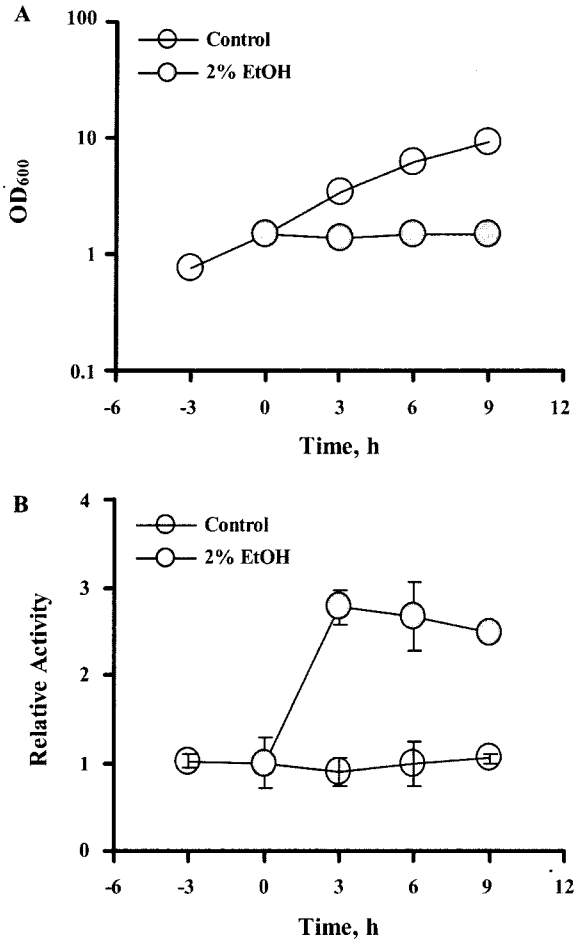

Fig. 2. Effect of ethanol, as the sole carbon source, on the β-galactosidase synthesis from the GGTI-*lacZ* fusion gene in wild-type *S. pombe* cells. The *S. pombe* cells harboring the fusion plasmid, pGT98, were grown in minimal medium, and split at the early exponential phase. (A) Growth curves. (B) β-Galactosidase activity. The β-galactosidase activity was determined at 37°C by the spectrophotometric assay using ONPG as a substrate, with the specific activity expressed in ΔA_{420}/min/mg protein. The relative activity was calculated by considering the specific activity of the untreated culture as 1.

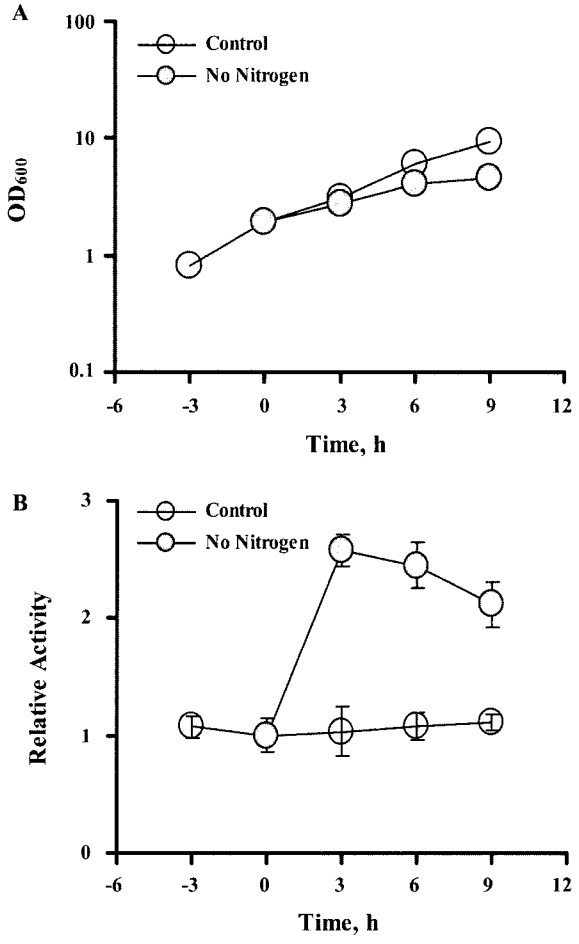

Fig. 3. Effect of nitrogen starvation on the β-galactosidase synthesis from the GGTI-*lacZ* fusion gene in wild-type *S. pombe* cells. The *S. pombe* cells harboring the fusion plasmid, pGT98, were grown in minimal medium, and split at the early exponential phase. (A) Growth curves. (B) β-Galactosidase activity. The β-galactosidase activity was determined at 37°C by the spectrophotometric assay using ONPG as a substrate, with the specific activity expressed in ΔA_{420}/min/mg protein. The relative activity was calculated by considering the specific activity of the untreated culture as 1.

non-fermentable carbon sources still remains unclear.

Adaptation of cells to nutritional stress is paralleled by alterations in the expression of the genes responsible, which may be associated with selective degradation of superfluous proteins. Yeast can use GSH as an endogenous sulfur source, and GSH stored in the yeast vacuoles can serve as an alternative nitrogen source during nitrogen starvation (Mehdi *et al.*, 2001). In *Saccharomyces cerevisiae*, expression of the *CIS2* gene encoding GGT, the main GSH-degrading enzyme, is highest in cells growing on a poor nitrogen source such as urea (Springael and Penninckx, 2003). During growth on a preferred nitrogen source, like $NH4^+$, *CIS2* expression is repressed through a mechanism involving the Gln3-binding protein, Ure2/GdhCR (Springael and Penninckx, 2003). When ammonium-grown *S. pombe* KP1 cells, harboring the fusion plasmid pGT98, were transferred to a minimal medium lacking a nitrogen source, the synthesis of β-galactosidase from the GGTI-*lacZ* fusion gene was significantly enhanced (Fig. 3B). Three hours after exposure to nitrogen-starved conditions, the β-galactosidase activity in fission yeast cells increased 2.27-fold (Fig. 3B). Unlike the fission yeast cells under glucose-limited conditions, the growth of the nitrogen-starved yeast cells was not arrested, although it was slightly delayed (Fig. 3A). In brief, the expression of the *S. pombe GGTI* gene is induced under nitrogen starvation.

The yeast cells are known to induce the transcription of genes required for detoxification of stressful agents (Lee *et al.*, 2003). The two transcription factors, Pap1 and Atf1, are believed to be responsible for the induction of stress-related genes in the fission yeast *S. pombe* (Nguyen *et al.*,

Table 1. Effects of non-fermentable carbon sources and nitrogen starvation on the synthesis of β-galactosidase from the GGTI-*lacZ* fusion gene in Pap1-negative *S. pombe*, strain TP108-3C

Nutritional Agents	Induction Fold[a]		
	3 h	6 h	9 h
2% Acetate	4.43 ± 0.11	3.51 ± 0.39	3.30 ± 0.08
2% Ethanol	3.07 ± 0.22	2.69 ± 0.39	2.38 ± 0.08
No Nitrogen	3.99 ± 0.11	2.46 ± 0.09	1.45 ± 0.07

The *S. pombe* TP108-3C cells harboring the fusion plasmid pGT98 were grown in minimal medium, and split at the early exponential phase. At 3, 6 and 9 h after the treatments, the β-galactosidase activity was determined at 37°C by the spectrophotometric assay using ONPG as a substrate.

[a]Induction fold in the β-galactosidase activity was calculated by considering the activity of the untreated culture as 1.

Table 2. Induction of β-galactosidase synthesis by the addition of acetate and ethanol, and nitrogen starvation by four different GGTI-*lacZ* fusion genes in *S. pombe* cells

Plasmids	Induction Fold[a]		
	Acetate	Ethanol	No Nitrogen
pGT98	2.07 ± 0.12	2.85 ± 0.18	2.49 ± 0.42
pGT754	2.33 ± 0.17	3.95 ± 0.89	3.20 ± 0.88
pGT421	1.97 ± 0.17	1.39 ± 0.13	2.57 ± 0.36
pGT156	1.09 ± 0.13	0.69 ± 0.21	1.03 ± 0.15

[a]Induction fold indicates β-galactosidase activity in *S. pombe* cells after treatment with 2% potassium acetate, 2% ethanol or nitrogen starvation compared to that in untreated *S. pombe* cells cultured in minimal glucose medium.

2000). The transcription factor Pap1, an *S. pombe* bZIP protein homologous to mammalian AP1, plays an important role in responses to both oxidative stress and a variety of cytotoxic agents, and binds DNA containing the consensus sequence, TTACGTAA (Toone *et al.*, 1998; Fujii *et al.*, 2000). In one plausible Pap1 binding site, TATCGTAA, 6 out of the 8 nucleotides, identical to the consensus sequence, in located in the -246 ~ -238 bp upstream region of the *S. pombe GGTI* gene. To find whether Pap1 was involved in the induction of the *S. pombe GGTI* gene by non-fermentable carbon sources and nitrogen starvation, the Pap1-negative *S. pombe* strain, TP108-3C, was used. The TP108-3C cells harboring the fusion plasmid pGT98 were exposed to non-fermentable carbon sources and nitrogen starvation, and the β-galactosidase activities in the treated cells measured. The basal β-galactosidase synthesis from the GGTI-*lacZ* fusion plasmid dropped to 0.61-fold at mid-exponential phase in the TP108-3C cells compared to that in wild-type KP1 cells (data not shown). However, the synthesis of β-galactosidase from the GGTI-*lacZ* fusion gene was normally induced in Pap1-negative cells by the non-fermentable carbon sources and nitrogen starvation (Table 1). These results suggest that the *S. pombe GGTI* gene is regulated by non-fermentable carbon sources and nitrogen starvation, in a Pap1-independent manner.

To identify the upstream region responsible for the induction of the *GGTI* gene by non-fermentable carbon sources and nitrogen starvation, the upstream region was serially deleted from the original fusion plasmid pGT98 by PCR (Fig. 4). Appropriately synthesized primers were used for the PCR amplification by *Ex Taq* DNA polymerase (TaKaRa, Shuzo Co., Japan). The newly constructed fusion plasmids, pGT754, pGT421 and pGT156, carried 754, 421 and 156 bp fragments upstream from the translational initiation point, respectively (Fig. 4). The wild-type *S. pombe* cells harboring the fusion plasmid pGT421 gave rise to markedly higher β-galactosidase activity than the cells harboring pGT98 or pGT754. These results imply the existence of negatively acting sequence (s) in the -754 ~ -421 bp upstream region, although its definite functions remain to be explained. However, the cells

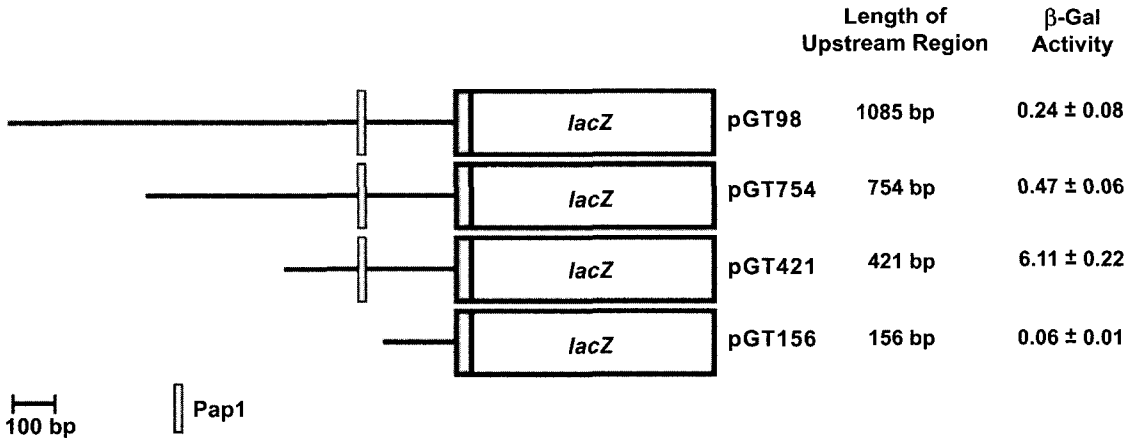

Fig. 4. Construction of three additional fusion plasmids harboring 754, 421 and 156 bp upstream regions from the translational initiation point in the *S. pombe GGTI* gene. The fission yeast cells carrying individual fusion plasmids were grown in minimal glucose medium, and harvested at the mid-exponential phase. The β-galactosidase activity was determined at 37°C by the spectrophotometric assay using ONPG as a substrate, with the specific activity expressed in ΔA_{420}/min/mg protein.

harboring the fusion plasmid pGT156 showed drastically diminished β-galactosidase activity compared with those harboring other fusion plasmids. This might be explained by the assumption that part of the core promoter is deleted in the fusion plasmid pGT156. The wild-type *S. pombe* cells, harboring different fusion plasmids and grown to the early exponential phase, were exposed to non-fermentable carbon sources and nitrogen starvation. As shown in Table 2, the synthesis of β-galactosidase from the fusion plasmids pGT98, pGT754 and pGT421, but not that from the fusion plasmid pGT156, can be induced by non-fermentable carbon sources and nitrogen starvation. These findings suggest that the responsible sequence for the induction by non-fermentable carbon sources and nitrogen starvation is located in the -421 ~ -156 bp upstream region. However, it is not certain whether an identical sequence is responsible for the induction by both a non-fermentable carbon source and nitrogen starvation.

In conclusion, the *S. pombe GGTI* gene is induced under nutritional stress. Its enhanced expression might help fission yeast cells survive under poor nutrient conditions.

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