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NOTE

Transcriptional Regulation of the Gene Encoding γ-Glutamylcysteine Synthetase from the Fission Yeast *Schizosaccharomyces pombe*

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Transcriptional regulation of the *Schizosaccharomyces pombe* γ-glutamylcysteine synthetase (GCS) gene was examined using the two GCS-*lacZ* fusion plasmids pUGCS101 and pUGCS102, which harbor 607 bp and 447 bp upstream regions, respectively. The negatively-acting sequence was located in the -607 ~ -447 bp upstream region of the GCS gene. The upstream sequence responsible for induction by menadione (MD) and L-buthionine-(S, R)-sulfoximine (BSO) resides in the -607 ~ -447 bp region, whereas the sequence which codes for nitric oxide induction is located within the -447 bp region, measured from the translational initiation point. Carbon source-dependent regulation of the GCS gene appeared to be dependent on the nucleotide sequence within -447 bp region. The transcription factor Pap1 is involved in the induction of the GCS gene by MD and BSO, but not by nitric oxide. Induction of the GCS gene occurring due to low glucose concentration does not depend on the presence of Pap1. These data imply that induction by MD and BSO may be mediated by the Pap1 binding site, probably located in the -607 ~ -447 region, and also that the nitric oxide-mediated regulation of the *S. pombe* GCS gene may share a similar mechanism with its carbon-dependent induction.

Key words: γ-glutamylcysteine synthetase, glutathione, Pap1, *Schizosaccharomyces pombe*, transcriptional regulation

The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is the predominant cellular non-protein thiol. It is synthesized in millimolar concentrations by most living cells. GSH exhibits multiple physiological functions, such as detoxification of various cytotoxic compounds, acting as a cofactor for several enzymes, protection of proteins' SH groups, and transportation of amino acids via the γ-glutamyl cycle (Inoue and Kimura, 1995; Sugiyama *et al.*, 2000b). In addition, GSH, one of the most important antioxidants, directly reduces HO· to H_2O, and serves as an electron donor for glutathione peroxidase, which in turn catalyzes the reduction of H_2O_2 and lipid hydroperoxide to H_2O and the corresponding alcohol (Michiels *et al.*, 1994; Russel *et al.*, 1994; Sugiyama *et al.*, 2000b). GSH synthesis involves two sequential, ATP-dependent enzymatic reactions, catalyzed by γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS). GCS catalyzing the formation of γ-glutamylcysteine from L-glutamate and L-cysteine is the rate-limiting step in *de novo* GSH biosynthesis, and regulated by feed-back competitive inhibition by GSH and the availability of L-cysteine (DeLeve *et al.*, 1991; Huang *et al.*, 2000). The eukaryotic GCS holoenzyme is normally composed of catalytic (heavy, Mr ~73,000) and regulatory (light, Mr ~30,000) subunits, which are encoded by different genes, and dissociate under reducing conditions (Yan and Meister, 1990). However, GCS from the fission yeast *Schizosaccharomyces pombe* is known to possess only a single polypeptide chain (Coblenz and Wolf, 1995; Mutoh *et al.*, 1995).

Regulation of GCS is of considerable importance, as GCS plays a principal role in modulating GSH homeostasis and, consequently, the capacity of the cell to withstand the deleterious effects of oxidative stress. Expression of the budding yeast *Saccharomyces cerevisiae* GCS gene is regulated by hydrogen peroxide, and also by heavy metals, such as mercury and cadmium (Westwater *et al.*, 2000). It is also increased by heat-shock stress in a Yap1p-dependent fashion, and consequently

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intracellular GSH content increases under such condition (Sugiyama *et al.*, 2000a). Heat shock-induced GSH synthesis protects mitochondrial DNA from potentially cytotoxic oxidative damage (Sugiyama *et al.*, 2000b; Lee *et al.*, 2001). A rapid downshift in the growth temperature of *S. cerevisiae* also induces transcription of the GCS gene, in a Yap1p-independent manner (Zhang *et al.*, 2003). In a previous study, expression of the *S. pombe* GCS gene was shown to be induced by metals, oxidative and nitrosative stresses, and glutathione-depleting agents (Kim *et al.*, 2004). In this work, we further analyzed the regulatory mechanisms of the *S. pombe* GCS gene, at the transcriptional level.

Materials and Methods

Chemicals

Restriction enzymes (*Bam*HI, *Hin*dIII), T4 DNA ligase, and RNase A were obtained from Roche Molecular Biochemicals (Germany). Seakem LE agarose was from Bioproducts (USA). Two PCR primers (primer 1, 5'-tctgagtttacactgtcaagcttcatgtcgtta-3' and primer 2, 5'-attctggccaatctaaaggatccccgagta-3') and *Ex Taq* DNA polymerase were ordered from the TaKaRa Shuzo Co. (Japan). Unless stated otherwise, all biochemical reagents used in this study were purchased from Sigma Chemical Co. (USA).

Strain and growth condition

The *S. pombe* strains KP1 (*h⁺ leu1-32 ura4-294*) (Kim *et al.*, 2004) and TP108-3C (*h⁻ leu1-32 ura4-D418 pap1::ura4⁺*) were used in this study. The yeast cells were grown in minimal medium. The medium (per 1 L) contained KH phthalate (3 g), Na_2HPO_4 (1.8 g), NH_4Cl (5 g), D-glucose (20 g), 1,000×vitamin mixture (nicotinic acid, 81.2 μM; inositol, 55.5 μM; biotin, 40.8 μM; pantothenic acid, 4.2 μM) (1 ml), 10,000X minerals (H_3BO_3 8.1 μM; $MnSO_4$ 2.37 μM; $ZnSO_4 \cdot 7H_2O$, 1.39 μM; $FeCl_3 \cdot 6H_2O$, 0.74 μM; $MoO_4 \cdot 2H_2O$, 0.25 μM; KI, 0.6 μM; $CuSO_4 \cdot 5H_2O$, 0.16 μM; citric acid, 4.76 μM) (0.1 ml), 50X salts ($MgCl_2$, 5.2 mM; $CaCl_2 \cdot 2H_2O$, 0.1 mM; KCl, 13.4 mM; Na_2SO_4, 0.28 mM) (20 ml), and L-leucine (250 mg) (Kim *et al.*, 2004). The culture, after 2 days of growth, was diluted 500-fold for inoculation. The yeast cells were grown at 30°C with shaking. The cell growth was monitored by absorbance at the wavelength of 600 nm.

Cell harvesting and disruption

Cell harvesting and disruption were performed as previously described (Kim *et al.*, 2004). Supernatant, obtained after centrifugation, was used as a crude extract for enzyme assays.

PCR

The two synthetic primers, primer 1 and primer 2, were used for PCR amplification by *Ex Taq* DNA polymerase. PCR was performed as described in the users sheet offered by the TaKaRa Shuzo Co. (Japan). The conditions used in this study were as follows: denaturation at 94°C for 2 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min.

β-Galactosidase assay

β-Galactosidase activity in the extracts was measured at 25°C by the spectrophotometric method, using ONPG as a substrate (Guarente, 1983). Its specific activity was represented as A_{420}/min/mg protein.

γ-Glutamylcysteine synthetase assay

γ-Glutamylcysteine synthetase (GCS) activity was determined as previously described (Seelig and Meister, 1985; Lee *et al.*, 2003). Enzyme activity was determined at 37°C in reaction mixtures of 1.0 ml containing 0.1 M TrisHCl buffer (pH 8.2), 150 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-γ-aminobutyrate, 20 mM $MgCl_2$, 2 mM EDTA, 0.2 mM NADH, 17 μg of pyruvate kinase and 17 μg of lactate dehydrogenase. The reaction was initiated by the addition of extract. The absorbance was monitored at 340 nm.

Protein determination

Protein concentrations in the extracts were determined according to the procedure described by Bradford, using BSA as a standard (Bradford, 1976).

Results and Discussion

Construction of the additional fusion plasmid pUGCS102

To conveniently monitor the expression of the *S. pombe* GCS gene, the GCS gene was first fused into the promoterless β-galactosidase gene of the shuttle vector YEp367R (Myers *et al.*, 1986). The resultant fusion plasmid pUGCS101, which harbors the 607 bp upstream region of the *S. pombe* GCS gene, was used in our previous study (Kim *et al.*, 2004). In this work, one additional fusion plasmid, which contained the shorter upstream region, was constructed. The 447 bp upstream region of the *S. pombe* GCS gene was amplified from the fusion plasmid pUGCS101 by PCR, using primers 1 and 2. The amplified DNA fragment was electro-eluted from 1% agarose gel, and digested with *Bam*HI and *Hin*dIII. The digested DNA fragment was ligated into the vector YEp367R, and digested with *Bam*HI and *Hin*dIII. After transformation into the *E. coli* strain MV1184 ((*ara* Δ(*lac-proAB*) *rpsL thi* (φ80 *lacZ*ΔM15) Δ(*srl-recA*)306::*Tn* 10(*tet*ʳ)) (Ishii *et al.*, 1995), the desired clone was confirmed by restriction mapping and nucleotide sequencing and named pUGCS102 (Fig. 1). The fusion plasmid pUGCS102 contains a 447 bp upstream sequence and the region encoding the N-terminal 7 amino acids of *S. pombe* GCS.

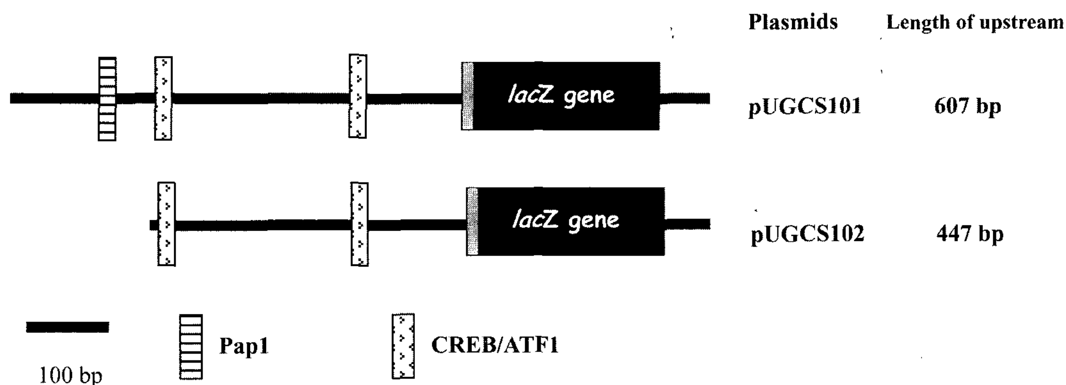

Fig. 1. Construction of the additional GCS-*lacZ* fusion plasmid, harboring the 447 bp upstream region of the *S. pombe* γ-glutamylcysteine synthetase (GCS) gene. The fusion plasmid pUGCS102 was constructed by PCR from the previously constructed pUGCS101 (Kim *et al.*, 2004). The length of the upstream region is expressed as the distance from the translational initiation point of the GCS gene.

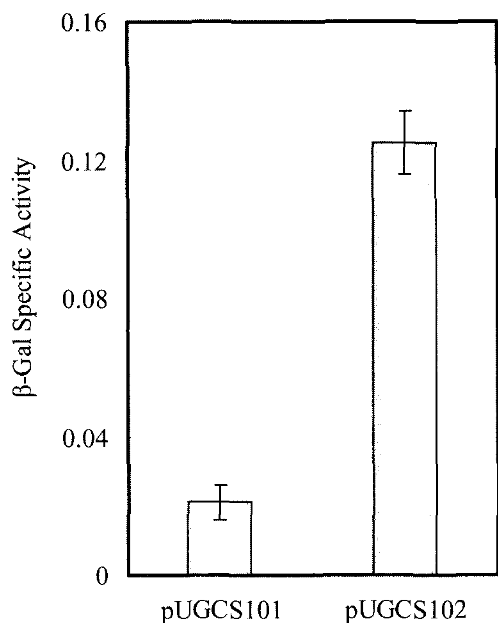

Fig. 2. β-Galactosidase synthesis from the two GCS-*lacZ* fusion plasmids pUGCS101 and pUGCS102. The yeast cells harboring the fusion plasmids, pUGCS101 or pUGCS102, were harvested at the mid-exponential phase. β-Galactosidase activity was measured, as described in Materials and Methods.

Presence of the negatively-acting sequence

The two YEp367R derivatives were individually introduced into wild-type *S. pombe* strain KP1, and the cells harboring each plasmid were grown in minimal medium, and then harvested at the mid-exponential phase. β-Galactosidase activity and protein content were then measured in the cell extracts. Synthesis of β-galactosidase from the fusion plasmid pUGCS102 was about 6 times higher than that observed from the fusion plasmid pUGCS101 (Fig. 2). This finding indicates that deletion of the upstream -607 ~ -447 bp region significantly increases the expression of β-galactosidase. In other words, it points to the presence of a negatively-acting sequence in this -607 ~ -447 bp region, although its action mechanism remains to be explained.

Upstream regions for transcriptional induction

Quinone compounds were previously found to induce GSH synthesis in various cell lines (Shi *et al.*, 1994). Menadione (2-methyl-1,4-naphthoquionone), a quinone which imposes oxidative stress by generating superoxide radicals via redox cycling, was shown to induce the synthesis of β-galactosidase from the fusion plasmid pUGCS101 (Kim *et al.*, 2004). This result was reconfirmed in the present study (Fig. 3A). The synthesis of β-galactosidase from the fusion plasmid pUGCS102 was not induced by menadione (Fig. 3B). Similar results were obtained with L-buthionine-(S, R)-sulfoximine (BSO), a specific transition-state inhibitor of GCS, which is used to deplete GSH in higher eukaryotic cells. BSO (50 μM) was able to induce the synthesis of β-galactosidase from the fusion plasmid pUGCS101, although in relatively low quantities. However, BSO was not found to induce the synthesis of β-galactosidase from the fusion plasmid pUGCS102 (Fig. 3). Taken together, these data imply that the upstream sequence responsible for menadione and BSO induction is located somewhere in the -607 ~ -447 region (Fig. 1).

Synthesis of β-galactosidase from the fusion plasmid pUGCS101 was previously shown to be enhanced by treatment with NO-generating sodium nitroprusside (SN, 1.5 mM) (Kim *et al.*, 2004). SN (3 mM) was able to enhance the synthesis of β-galactosidase from the fusion plasmid pUGCS101, and exhibited a similar effect on the synthesis of β-galatosidase from the fusion plasmid pUGCS102 (Fig. 3). This indicates that the upstream sequence responsible for SN-mediated induction is located within the 447 bp region, measured from the translational initiation point. Clearly, the induction of the GCS gene by nitric oxide occurs in completely different transcriptional mechanism with that by menadione and BSO. Additionally, GCS mRNA levels were previously found to be markedly augmented by treatment with SN or

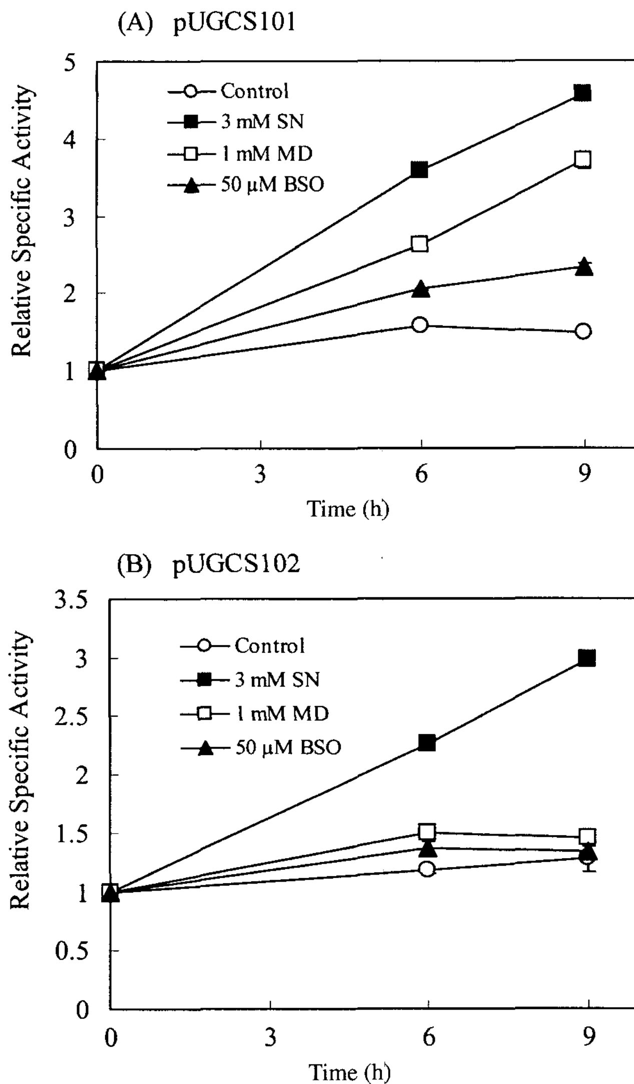

Fig. 3. Effect of sodium nitroprusside (SN), menadione (MD) and L-buthionine-(S, R)-sulfoximine (BSO) on the β-galactosidase synthesis from the two GCS-*lacZ* fusion plasmids pUGCS101 (A) and pUGCS102 (B). The *S. pombe* cells harboring the fusion plasmid pUGCS101 or pUGCS102 were grown in minimal medium, and split at the early exponential phase. The relative specific activity of β-galactosidase at the split point was considered to be 1.0.

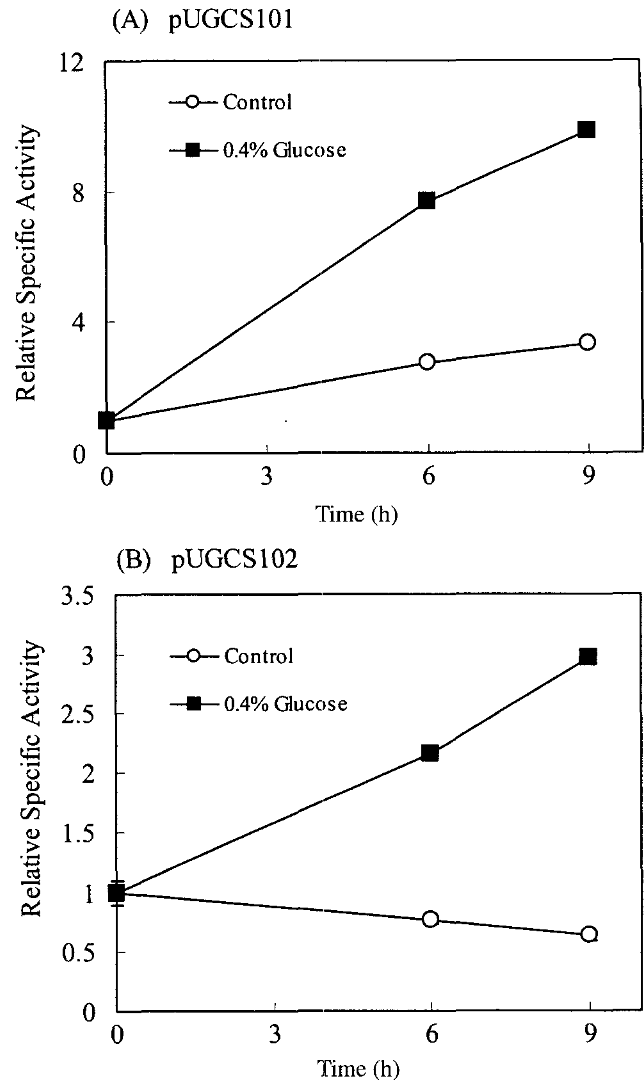

Fig. 4. Effect of low concentration of glucose as a sole carbon source on the β-galactosidase synthesis from the two GCS-*lacZ* fusion plasmids pUGCS101 (A) and pUGCS102 (B). The *S. pombe* cells harboring individual fusion plasmids were grown in minimal medium, and split at the early exponential phase. The relative specific activity of β-galactosidase at the corresponding split point was considered to be 1.0.

menadione (Kim *et al.*, 2004).

Carbon source-dependent induction

Yeast cells growing in the presence of glucose or related rapidly-fermented sugars differ strongly in a variety of physiological properties, as opposed to cells grown in the absence of glucose (Thevelein *et al.*, 2000). In part, these differences appear to be due to the protein kinase A (PKA), and related signal transduction pathways. When the fission yeast cells were forced to switch to low concentration of glucose or sucrose as a sole carbon source, after the disruption of normal glucose concentration by centrifugation, β-galactosidase synthesis from the fusion plasmid pUGCS101 increased significantly (Kim *et al.*, 2004). When 0.4% glucose, or 2.0% sucrose, was selected as the sole carbon source, the GCS activity of the *S. pombe* cells increased (data not shown). This finding supports the notion that regulation of the *S. pombe* GCS gene proceeds in a carbon source-dependent manner. To ascertain the upstream sequence responsible for induction due to low concentration of glucose, the fusion plasmid pUGCS102 was used in this study. As shown in Fig. 4, the induction of β-galactosidase synthesis from both fusion plasmids (pUGCS101 and pUGCS102) was observed to be very similar. This indicates that the

upstream sequence of the GCS gene which is responsible for induction due to low glucose concentration is located within -447 bp region.

Pap1-dependence

In response to various stresses, yeast cells induce transcription of genes required for the detoxification of stressful agents. The two transcription factors, Pap1 and Atf1, have been known to be responsible for the induction of some stress-related genes in the fission yeast *S. pombe*, which is widely used to study fundamental processes such as the cell cycle or gene expression (Nguyen *et al.*, 2000). Pap1, an *S. pombe* basic leucine zipper (bZIP) protein which is homologous to mammalian AP1, was shown to play an important role in the response to both oxidative stress and a variety of cytotoxic agents, and also in the binding of DNA containing the consensus sequence (Toone *et al.*, 1998; Fujii *et al.*, 2000). Atf1, a bZIP transcription factor with homology to mammalian ATF / CREB, is activated by Spc1 / Sty1 mitogen activated protein kinase (MAPK), the *S. pombe* homologue of mammalian stress-activated protein kinase (SAPKs), through the Wis1-Spc1-Atf1 signal pathway (Degols and Russell, 1997; Nguyen *et al.*, 2000). Atf1 was previously shown to be necessary for the induction of the *S. pombe* catalase gene by osmotic stress, UV irradiation or heat shock, whereas its induction by menadione, which produces the superoxide anion, was determined to require Pap1 (Nakagawa *et al.*, 2000). These results may imply that Pap1 and Atf1 individually mount responses against stressful agents, albeit in different ways. To ascertain examine whether Pap1 is involved in the induction of the GCS gene by SN, menadione, BSO or low glucose concentration, the Pap1-negative *S. pombe* strain TP108-3C was used. SN (3 mM) induced β-galactosidase synthesis from the fusion plasmid pUGCS101 in TP108-3C, in the same quantities as when applied to the wild-type strain (Fig. 5). However, induction due to 1 mM menadione or 50 μM BSO was unobserved in the TP108-3C strain (Fig. 5). These data suggest that the transcription factor Pap1 is involved in induction of the GCS gene by menadione and BSO, but not by SN. The plausible Pap1 binding site might be located in the -607 ~ -447 bp upstream region of the GCS gene (Fig. 1). Induction of the GCS gene by nitrosative stress was concluded to be Pap1-independent.

As shown in Fig. 6, β-galactosidase synthesis from the fusion plasmid pUGCS102 could be induced in the Pap1-negative TP108-3C by lowering the concentration of glucose. Augmentation of β-galactosidase synthesis in TP108-3C was observed to be similar to that occurring in the wild-type strain (Fig. 4A). These results indicate that the induction of the GCS gene due to low glucose concentration is independent of the presence of Pap1. The consensus sequence for the binding site of the Pap1 protein was previously determined to be TTACGTAA (Fujii *et al.*, 2000). The plausible binding site for TTACGAAA is found in the -485 ~ -495 bp region, upstream of the *S. pombe* GCS gene (Fig. 1). It could be responsible for the regulation of the *S. pombe* GCS gene by the Pap1 protein. Within the -447 bp region, the plausible binding sites for stress-related transcription factor CREB / ATF1 were identified (Fig. 1). However, their roles in the regulation of the GCS gene remain unknown.

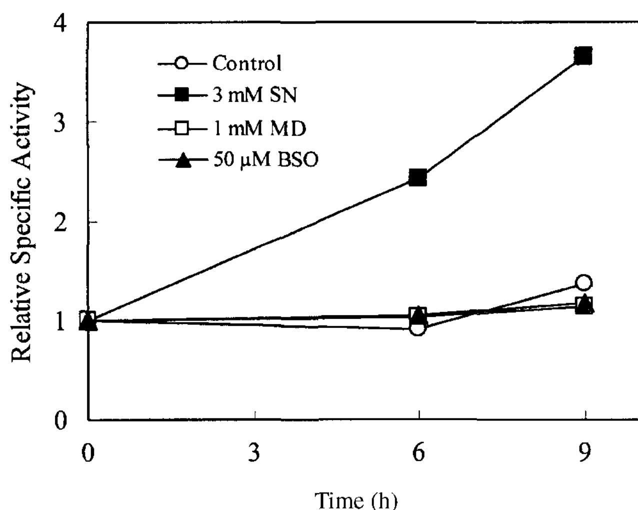

Fig. 5. Effect of sodium nitroprusside (SN), menadione (MD) and L-buthionine-(S, R,) sulfoximine BSO on the synthesis of β-galactosidase from the fusion plasmid pUGCS101 in the Pap1-negative mutant *S. pombe* strain TP108-3C. The *S. pombe* culture, grown in minimal medium, was split at the early exponential phase. The relative specific activity of β-galactosidase at the split point was considered to be 1.0.

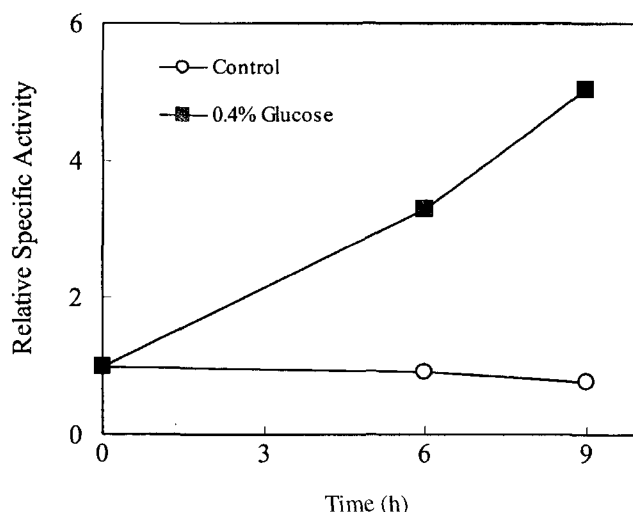

Fig. 6. Effect of low concentration of glucose as a sole carbon source on the β-galactosidase synthesis from the fusion plasmid pUGCS101 in the Pap1-negative mutant *S. pombe* strain TP108-3C. The *S. pombe* culture, grown in minimal medium, was split at the early exponential phase. The relative specific activity of β-galactosidase at the split point was considered to be 1.0.

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