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Transcriptional Analysis and Pap1-Dependence of the Unique Gene Encoding Thioredoxin Reductase from the Fission Yeast

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The unique gene encoding thioredoxin reductase (TrxR) was previously cloned and characterized from the fission yeast *Schizosaccharomyces pombe*, and its expression was induced by oxidative stress. To elucidate the regulatory mechanism of the *S. pombe* TrxR gene, three fusion plasmids were generated using polymerase chain reaction: pYUTR20, pYUTR30, and pYUTR40. Plasmid pYUTR20 has an upstream region of 891 base pairs, pYUTR30 has 499 in this region, and pYUTR40 has an 186 bp upstream region. Negatively acting sequence is located between −1,526 ~ −891 bp upstream of the gene. The upstream sequence, responsible for the induction of TrxR by menadione (MD), is situated on the −499 ~ −186 bp region, which is also required for TrxR induction by mercuric chloride. The same region also appeared to be required for Pap1-mediated transcriptional regulation of the TrxR gene, which contains the two plausible Pap1 binding sites, TTACGAAT and TTACGCGA. Consistently, basal and inducible expression of the TrxR gene was markedly lower in the Pap1-negative TP108-3C cells than in wild-type yeast cells. In summary, up-regulation of the *S. pombe* TrxR gene is mediated by Pap1 via the transcriptional motif(s) located on the −499 ~ −186 bp region.

Keywords: fission yeast, Pap1, regulation, *Schiaosaccharomyces pombe*, thioredoxin reductase

The dimeric enzyme thioredoxin reductase (TrxR; EC1.6.4.5) is a member of a family of pyridine nucleotide-disulfide oxidoreductases that includes the closely related enzymes lipoamide dehydrogenase, glutathione reductase, trypanothione reductase, and mercuric ion reductase (Williams, 1992). TrxR was originally identified as the catalyst for NADPH-dependent reduction of the active site disulfide in oxidized thioredoxin (Trx-S_2) to give a dithiol in reduced thioredoxin (Trx-$(SH)_2$). Since then, TrxR has been demonstrated to reduce not only Trxs, but also several low molecular weight substrates: 5,5'-dithiolbis (2-nitrobenzoic acid) (DTNB), lipoic acid, lipid hydroperoxides, the cytotoxic peptide NK-lysin, vitamin K, selenodiglutathione, alloxan, dehydroascorbic acid, and the tumor-suppressor protein p53 (Arner *et al.*, 1996; Williams *et al.*, 2000). TrxR is also implicated in defense against oxidative stress, regulation of apoptosis, redox regulation of cell signaling, and control of cell growth and proliferation (Yoshitake *et al.*, 1994; Ejima *et al.*, 1999). For example, TrxR is a signaling factor in the regulation of AP-1 activity via a cysteine motif located in the protein. Yeast cells lacking TrxR have a diminished capacity for detoxifying oxidants and/or to repairing oxidative damage (Carmel-Harel *et al.*, 2001). A recent report has shown that a novel TrxR inhibitor induces growth inhibition and apoptosis in different cancer cells. This suggests some relationship between TrxR inactivation and apoptosis or inhibition of proliferation (Zhao *et al.*, 2005).

The fungal pathogen *Crytococcus neoformans* encodes the low-molecular-weight isoform of TrxR, which is essential for viability but shares little homology with that of its mammalian host (Missall and Lodge, 2005). TrxR is not essential in the gram-positive bacterium *Lactococcus lactis,* but its inactivation triggers induction of several mechanisms acting at the membrane and metabolic levels (Vido *et al.*, 2005). TrxR of the respiratory yeast *Kluyveromyces lactis* is induced by oxidative stress (Tarrío *et al.*, 2004). *Staphylococcus aureus* TrxR is essential for growth and is upregulated following exposure to oxidative and disulfide stress, resulting in increased disulfide bond formation (Uziel *et al.*, 2004). Cadmium-induced TrxR ex-

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pression in vascular endothelial cells is mediated by the activation of NF-E2-related factor-2 (Nrf2) via binding to antioxidative responsive element (ARE) in the promoter (Sakurai *et al.*, 2005).

In response to various stresses, yeast cells induce transcription of genes required for detoxification of stressful agents. The two transcription factors, Pap1 and Atf1, mediate the induction of stress-related genes in the fission yeast *Schizosaccharomyces pombe* (Nguyen *et al.*, 2000). The transcriptional factor Pap1, an *S. pombe* bZIP protein homologous to mammalian AP1, plays a crucial role in defense against oxidative stress and a variety of cytotoxic agents (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). This *S. pombe* homologue of mammalian stress-activated protein kinases (SAPKs) activates Atf1 through the Wis1-Spc1-Atf1 signal pathway (Degols and Russell, 1997; Nguyen *et al.*, 2000). Atf1 is necessary for the induction of the *S. pombe* catalase gene by osmotic stress, UV irradiation, and heat shock. The induction of this gene by menadione treatment requires Pap1 to produce superoxide anion (Nakagawa *et al.*, 2000). The fission yeast *S. pombe* is widely used to study fundamental processes like the cell cycle or gene expression. In the preceding work, the unique *S. pombe* gene encoding TrxR was shown to be up-regulated by oxidative stress (Hong *et al.*, 2004). In this study, transcriptional regulation of the *S. pombe* TrxR gene was further analyzed using various TrxR-*lacZ* fusion genes.

Materials and Methods

Chemicals

Ampicillin, bovine serum albumin (BSA), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), L-leucine, uracil, adenine, glucose, menadione, mercuric chloride, and Bradford reagent were purchased from Sigma Chemical Co. (USA). Restriction enzymes (*Hin*dIII and *Bam*HI), T4 DNA ligase, RNase A, and *Ex Taq* polymerase were obtained from TaKaRa Shuzo Co. (Japan). Yeast extract and agar were obtained from Amersham Life Science (USA). PCR primers were from TaKaRa Shuzo Co. (Japan). All other chemicals used were of the highest grade commercially available.

Strains and growth conditions

E. coli strain MV1184 was used for the construction of fusion plasmids, and *S. pombe* KP1 (h^+ *leu1-32 ura4-294*) and TP108-3C (h^- *leu1-32 ura4D18 pap1::ura4*$^+$) were used for regulation studies. The yeast cells were grown in a liter of minimal medium containing 3 g KH phthalate, 1.8 g Na_2HPO_4, 5 g NH_4Cl, 20 g D-glucose, 1 ml 1,000 × vitamin mixture, 0.1 ml 10,000 × minerals, 20 ml 50 × salts, and 250 mg L-leucine (Kim *et al.*, 2004d). Salt stock solutions (50 ×) contained 5.2 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$, 13.4 mM KCl, and 0.28 mM Na_2SO_4. Mineral stock solutions (10,000 ×) contained 8.1 μM H_3BO_3, 2.37 μM $MnSO_4$, 1.39 μM $ZnSO_4 \cdot 7H_2O$, 0.74 μM $FeCl_3 \cdot 6H_2O$, 0.25 μM $MoO_4 \cdot 2H_2O$, 0.6 μM KI, 0.16 μM $CuSO_4 \cdot 5H_2O$, and 4.76 μM citric acid. Vitamin stock solutions (1,000 ×) contained 81.2 μM nicotinic acid, 55.5 μM inositol, 40.8 μM biotin, and 4.2 μM pantothenic acid. The grown for 2-day culture was diluted 500-fold for inoculation. The yeast cells were shaken at 30°C, and cell growth was monitored by absorbance at 600 nm.

Plasmid and nucleotide sequencing

E. coli-yeast shuttle vector YEp367R (Myers *et al.*, 1986) was used for the construction of the three additional fusion plasmids. Nucleotide sequencing was performed with an automatic DNA sequencer from Bionex, Inc.

The appropriate number of yeast cells was harvested by centrifugation. They were resuspended in 20 mM Tris buffer (pH 8.0) containing 2 mM EDTA (buffer A) and disrupted using a glass bead beater. After centrifugation, the supernatant was used as a crude extract for enzyme assay and protein determination.

Enzyme assay

β-Galactosidase activity in extracts was measured at 25°C by spectrophotometric methods using ONPG as a substrate (Guarente, 1983). Its specific activity was represented as ΔA_{420}/min/mg protein. Protein content in extracts was determined according to the procedure of Bradford using BSA as a standard (Bradford, 1976).

Polymerase chain reaction

Appropriate synthetic primers, *Ex Taq* polymerase, and a PTC-150 Minicycler (MJ Research, USA) were used for PCR amplication. PCR was performed according to the manufacturer's instructions. The typical PCR conditions used in this work were 94°C (1 min), 55°C (1 min), and 72°C (1min) for 30 cycles. The amplified DNA fragments contained *Hin*dIII and *Bam*HI restriction sites derived from the synthetic primers.

General recombinant DNA techniques

Other recombinant DNA techniques used in this study were performed according to the book 'Molecular Cloning: A laboratory manual' (Sambrook *et al.*, 1989). The *S. pombe* cells were transformed as previously described (Bröker, 1993).

Results and Discussion

Sequential deletion of the upstream sequence

In previous studies, the 1,526- bp upstream region of the TrxR gene was fused into the promoterless β-galactosidase gene of the shuttle vector YEp367R (Myers *et al.*, 1986) to generate the fusion plasmid pYUTR10 (Hong *et al.*, 2004). Using the fusion plasmid pYUTR10 and RT-PCR, expression of the *S. pombe* TrxR gene was up-regulated by superoxide-generating menadione and mercuric chloride on the transcriptional level (Hong *et al.*, 2004). To understand the upstream sequence and transcriptional mechanism responsible for up-regulation, the upstream sequence in the fusion plasmid pYUTR10 was serially shortened by PCR and the amplified fragments ligated back into YEp367R. The resultant fusion plasmids pYUTR20, pYUTR30, and pYUTR40 were confirmed by restriction mapping and nucleotide sequencing after transformation into *E. coli* strain MV1184. Plasmid pYUTR20 contained 891 base pairs, pYUTR30 had 499, and pYUTR40 had 186 bp upstream of the translation initiation site (Fig. 1).

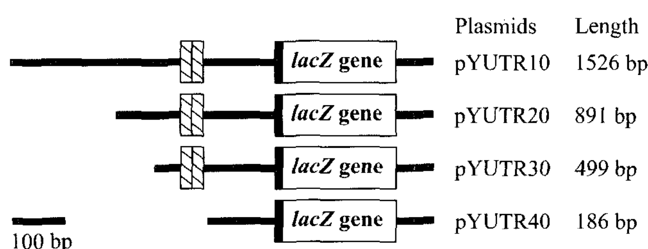

Fig. 1. Schematic representation of fusion plasmids constructed from *E. coli*-yeast shuttle vector YEp367R carrying *leu2*$^+$ as a selective marker (Meyers *et al.*, 1986). The shaded boxes indicate plausible Pap1 binding sites.

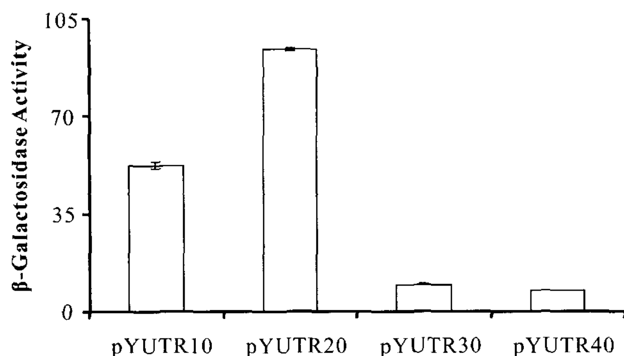

Fig. 2. Synthesis of β-galactosidase from the four fusion plasmids, pYUTR10, pYUTR20, pYUTR30, and pYUTR40, in wild-type *S. pombe* strain KP1. The yeast cells were harvested at the mid-exponential phase from the individual cultures, and the extracts were prepared as described in 'Materials and Methods'. The specific activities of β-galactosidase are expressed as ΔA_{420}/min/mg protein.

Negatively acting sequence(s)

The four fusion plasmids were individually introduced into wild-type *S. pombe* KP1 cells. The yeast cells harboring fusion plasmids were grown in minimal medium and harvested at the mid-exponential phase. β-Galactosidase activity and protein content were measured in the cellular extracts. Synthesis of β-galactosidase from plasmid pYUTR20 was about 1.8-fold higher than that from plasmid pYUTR10 (Fig. 2). This finding suggests that deletion of the upstream −1,526 ~ −891 bp region enhances expression of the TrxR gene. Similar effects were observed in Pap1-negative TP108-3C cells, though the increase was smaller (data not shown). These results indicate the presence of negatively acting sequence(s) in the −1,526 ~ −891 bp region. However, further deletion in the upstream sequence, shown with plasmids pYUTR30 and pYUTR40 (Fig. 2), markedly decreased expression of the TrxR gene. It may be possible that positively acting sequence(s) are located between −891 and −499 bp, although this region is not essential for the expression of the TrxR gene.

Upstream sequence(s) responsible for induction

Synthesis of β-galactosidase from the original fusion plasmid pYUTR10 was found to be induced by menadione and mercury (Hong *et al.*, 2004). This study was designed for the elucidation of upstream sequence(s) and transcriptional mechanisms responsible for the induction of the TrxR gene. The wild-type *S. pombe* KP1 cells harboring individual fusion plasmid were grown in minimal medium, treated with 2.5 mM MD, and harvested 3, 6 and 9 hours after treatments. Growth of the four cultures harboring the individual fusion plasmid, as estimated from changes in absorbance, was not delayed or arrested (data not shown). As seen in previous findings (Hong *et al.*, 2004), synthesis of β-galactosidase from the plasmid pYUTR10 was notably enhanced, increasing up to about 4.3-fold 9 hours after treatment (Fig. 3A). Synthesis of β-galactosidase from the plasmids pYUTR20 and pYUTR30, which contain shorter upstream sequences, was also significantly induced by 2.5 mM MD (Fig. 3B, Fig. 3C). The induction by 2.5 mM MD of β-galactosidase from the plasmid pYUTR20 was relatively low (Fig. 3B). However, absolute β-galactosidase activities in yeast cells harboring plasmid pYUTR20 were higher than those in cells harboring plasmid pYUTR10 after treatment with 2.5 mM MD (data not shown). This was a result of enhanced levels of basal β-galactosidase activity in yeast cells harboring the plasmid pYUTR20 due to the absence of the inhibitory sequence located between −1,526 and −891 bp. The preexisting enhanced level of β-galactosidase might resist subsequent induction of the TrxR-*lacZ* fusion gene by MD, which is

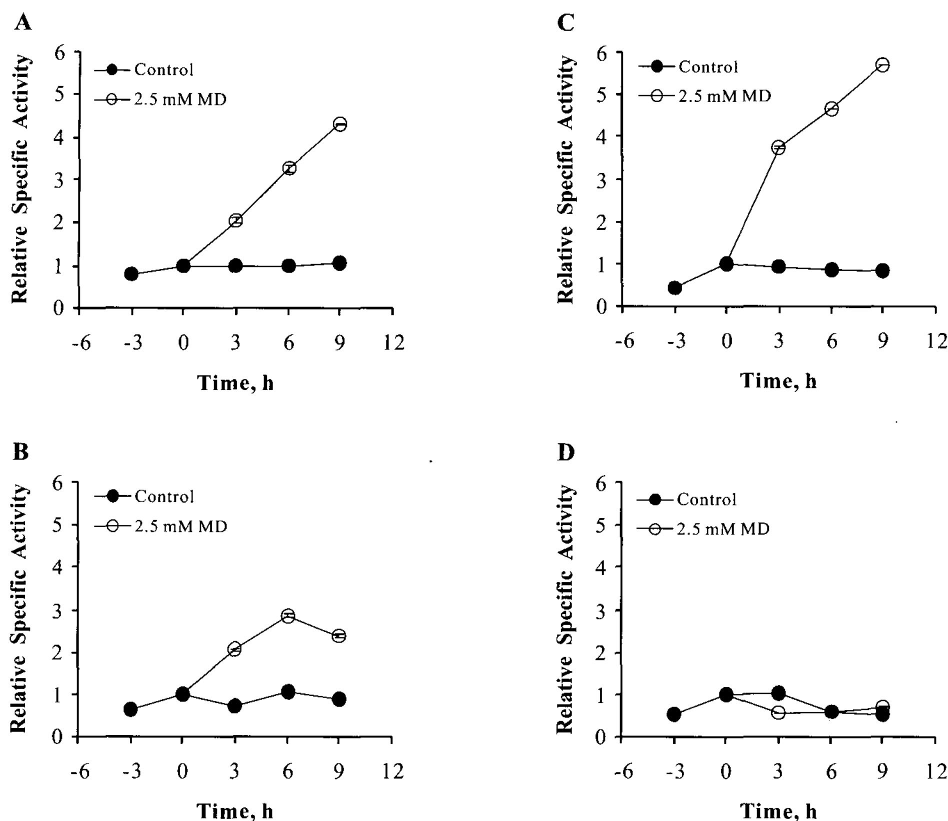

Fig. 3. Effect of menadione (MD, 2.5 mM) on the synthesis of β-galactosidase from the fusion plasmids pYUTR10 A, pYUTR20 B, pYUTR30 C, and pYUTR40 D in *S. pombe* wild-type KP1 cells. The *S. pombe* cells harboring the appropriate plasmid were grown in minimal medium, and split at the early exponential phase. Solid circle (-•-) indicates the untreated cells, and open circle (-o-) indicates cells treated with 2.5 mM MD. The β-galactosidase activity was determined at 25°C by spectrophotometric assay using ONPG as a substrate. β-Galactosidase activities are expressed relative to the value (1) in the yeast culture at the split point.

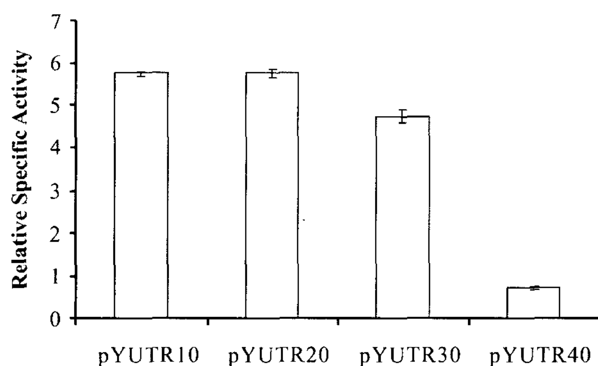

Fig. 4. Effect of 5 mM MD on the synthesis of β-galactosidase from the four fusion plasmids in *S. pombe* wild-type KP1 cells. The *S. pombe* cells harboring individual fusion plasmid were grown in minimal medium, split at the early exponential phase, treated with 5 mM MD, and harvested 6 h after the treatments. β-Galactosidase activities are expressed as relative values against those of the corresponding untreated cells.

of unknown reason. Synthesis of β-galactosidase in the yeast cells harboring plasmid pYUTR40, the shortest construct, was not induced by 2.5 mM MD (Fig. 3D). Similar experiments were repeated with the concentration of MD increased to 5 mM. As with 2.5 mM MD, synthesis of β-galactosidase in yeast cells harboring the plasmid pYUTR10, pYUTR20, or pYUTR30 was significantly enhanced by 5 mM MD. However, it was not changed in yeast cells harboring the plasmid pYUTR40 after the same treatment (Fig. 4). Six hours after treatment with 5 mM MD, β-galactosidase activity went up more than 5-fold in yeast cells harboring plasmids pYUTR10, pYUTR20, and pYUTR30 (Fig. 4). Since mercuric chloride has been shown to induce the TrxR gene, it was also used on yeast cells with the four fusion plasmids. Mercuric chloride (1 μM) was able to induce expression of the TrxR-*lacZ* fusion gene contained in plasmids pYUTR10, pYUTR20, or pYUTR30, but not in plasmid pYUTR40 (Fig. 5A). This induction by mercuric chloride appears to be parallel with that by MD, suggesting that mercuric chloride and MD cause an induction of the TrxR-*lacZ* gene via an identical mechanism. The results indirectly confirm that mercuric chloride also

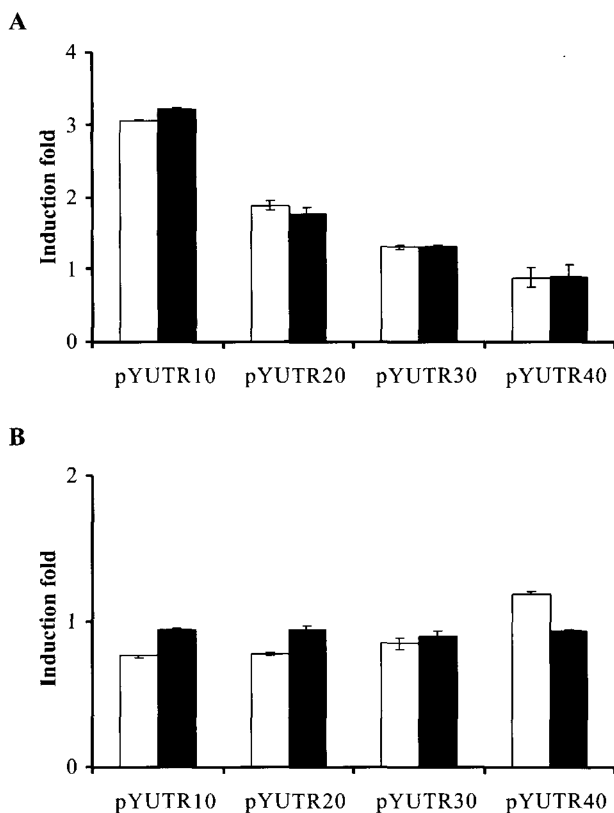

Fig. 5. Effect of mercury on the synthesis of β-galactosidase from the four fusion plasmids in *S. pombe* wild-type KP1 (A) and Pap1-negative TP108-3C (B) cells. The *S. pombe* cells harboring individual fusion plasmid were grown in minimal medium, split at the early exponential phase, treated with 1 μM mercuric chloride, and harvested 3 hours (open box) and 9 hours (solid box) after treatment. β-Galactosidase activities are expressed as relative values against those of the corresponding untreated cells.

generates oxidative stress in the fission yeast. Collectively, the sequence responsible for induction by MD and mercury is located between −499 and −186 bp upstream of the TrxR gene.

Pap1-dependence

Yeasts have been model systems to elucidate cellular stress responses in eukaryotes. One set of genes required for detoxification of ROS has been identified in yeasts (Moradas-Ferreira *et al.*, 1996). There are two known stress signal pathways in the fission yeast *S. pombe*. One is the Spc1-Atf1 pathway in which stress-activated Spc1 induces Atf1, and the other is mediated by Pap1 independently of Spc1 activation (Nguyen *et al.*, 2000). Pap1, required for survival to oxidative stress, shows high homology to mammalian c-Jun and its *S. cerevisiae* homologue Yap1 (Toda *et al.*, 1991). The *S. pombe* cells deleted in $Pap1^{+}$ show high sensitivity to oxidative stress but not to osmotic stress or nutrient deprivation (Toone *et al.*, 1998). Spc1 is activated by high osmolarity, oxidative stress, and heat shock (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Kato *et al.*, 1996). It was previously shown that the induction of the TrxR gene by mercury and MD did not occur according to the Wis1-Spc1-Atf1 signal pathway (Hong *et al.*, 2004). The Pap1-dependence of the TrxR gene was further examined using the four fusion plasmids. Expression of the TrxR-*lacZ* fusion genes was 33 ~ 40% lower in the Pap1-negative TP108-3C cells than in the wild-type KP1 cells (data not shown). This indicates that basal expression of the TrxR gene is partly dependent on Pap1. The induction by 2.5 mM MD of the TrxR-*lacZ* fusion genes contained in the plasmids pYUTR10, pYUTR20, and pYUTR30 was abolished in the TP108-3C cells (data not shown). No induction was observed in the yeast cells harboring the plasmid pYUTR40 either (data not shown). Treatment with 1 μM mercuric chloride gave rise to a similar pattern in the TP108-3C cells (Fig. 5B). These results indirectly confirm that mercury and MD control the TrxR gene in the same way.

TrxR reduces thioredoxin through binding flavin adenine dinucleotide and NADPH. TrxR, which is active in a variety of biological functions, is identified as a potential molecular target in tumor cells for enhancing the cytotoxicity of anticancer agents that induce oxidative stress (Smart *et al.*, 2004). The unique TrxR of the fission yeast *S. pombe* shows 69% homology with TrxR1 and 73% homology with TrxR2 from the budding yeast *S. cerevisiae* (Pedrajas *et al.*, 1999). And TrxR from the fission yeast is 66% homologous to that from the filamentous fungus *Penicillium chrysgenum* (Cohen *et al.*, 1994). In contrast with the existence of two TrxR genes in *S. cerevisiae*, the single TrxR in fission yeast cells may have a wider variety of physiological functions. According to computer analysis using the program Geno3D, the hypothetical three-dimensional structure of the *S. pombe* dimeric TrxR contains 10 α-helices and 24 β-sheets, and βαβ domains that bind FAD (data not shown). It resembles those of *S. cerevisiae* and *P. chrysogenum* (data not shown), which indicates their close relationship in the fungal phylogeny. The precise understanding of the *S. pombe* TrxR would be helpful in elucidating other fungal TrxRs.

The consensus sequence for the binding site of Pap1 was previously determined to be TTACGTAA (Fujii *et al.*, 2000). The two plausible binding sites TTACGAAT and TTACGCGA are found at the −247 ~ −254 and −281 ~ −288 regions upstream of the *S. pombe* TrxR gene. Their location is included in the −499 ~ −186 bp region upstream of the TrxR gene. This is the region responsible for induction by mena-

dione and mercury. They may be responsible for regulation by Pap1 for induction of the *S. pombe* TrxR gene by menadione and mercury. The other plausible Pap1 binding sites were previously located at −1,240 ~ −1,247 and −1,275 ~ −1,282 bp regions upstream of the TrxR gene (Hong *et al.*, 2004). However, subsequent data suggest that they do not act as actual Pap1 binding sites. Other genes in *S. pombe* involved in the response against various kinds of stresses have been identified to be positively regulated by Pap1. These regulated genes encode monothiol glutaredoxins (Kim *et al.*, 2005; Moon *et al.*, 2005), dithiol glutaredoxin (Lim *et al.*, 2003), γ-glutamylcysteine synthetase (Kim *et al.*, 2004d), glutathione synthetase (Kim *et al.*, 2004c), thioredoxin (Cho *et al.*, 2002), and glutathione S-transferase (Lim *et al.*, 2002; Kim *et al.*, 2004a; Kim *et al.*, 2004b). Some genes, like the gene encoding glutathione synthetase (Kim *et al.*, 2003), are also regulated by Atf1. Currently available data on *S. pombe* indicates that Pap1, rather than Atf1, is assumed to be a major transcriptional regulator in the stress response. It is considered to play a crucial role in the protection of the fission yeast cells from a variety of stressful agents through enhancing downstream defense proteins and diminishing cellular damage.

Acknowledgement

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