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Effects of Four Oxidants, Menadione, 1-Chloro-2,4-Dinitrobenzene, Hydrogen Peroxide and Cumene Hydroperoxide, on Fission Yeast *Schizosaccharmoyces pombe*

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Several chemical agents have been used to exert oxidative stress in the study of stress response, but differences in the effects of different reagents have received little attention. To elucidate whether such differences exist, the response of *Schizosaccharomyces pombe* to menadione (MD), 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide and cumene hydroperoxide (CHP), which are frequently used to exert oxidative stress, was investigated. Sensitivity to these reagents differed among mutants deficient in genes involved in oxidative stress resistance. *N*-Acetylcysteine restored resistance to MD, CHP and hydrogen peroxide but did not change sensitivity to CDNB. The induction kinetics of genes induced by oxidative stress differed for each reagent. MD, CDNB and hydrogen peroxide caused a transient induction of genes, but the peak times of induction differed among the reagents. CHP gave quite different kinetics in that the induction continued for up to 2 h. The *ctt1*$^{+}$ gene was not induced by CHP. GSH rapidly decreased in the cells treated with high concentrations of these reagents, but at a low concentration only CDNB decreased GSH. These results indicated that *S. pombe* responded differently to the oxidative stress exerted by these different reagents.

Key words: gene induction, GSH depletion, mutants, oxidant sensitivity, *Schizosaccharomyces pombe*.

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; Cu,Zn-SOD, superoxide dismutase with cupper and zinc as cofactors; MD, menadione; NAC, *N*-acetylcysteine; ROS, reactive oxygen species.

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Aerobic organisms use oxygen to generate energy through the complete oxidation of food molecules but the incomplete reduction of oxygen generates highly active molecules called reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, and hydroxyl radical. ROS readily oxidize cellular components and severely damage cells. Unless this damage is repaired properly, it causes such diseases as cancer, diabetes, atherosclerosis, and premature aging (*1–4*). Cells protect themselves from the toxic effects of ROS by various mechanisms. Cells have enzymes and low-molecular-weight scavenging compounds that degrade ROS, repair or degrade damaged molecules, and exclude pro-oxidant molecules from the intracellular environment (*5–9*). The expression of many genes implicated in these processes is induced by oxidative stress (*10–13*).

The defensive machinery for oxidative insults by ROS has been studied extensively. Superoxide dismutase with copper and zinc as cofactors (Cu,Zn-SOD) is a ubiquitous enzyme that catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen. It is located mainly in the cytosol but also in the intermembrane space of mitochondria and in other organelles (*14–18*). The importance of this enzyme for oxidative stress resistance has been shown by reports that mutants of microorganisms deficient in the enzyme are hypersensitive to oxidative stress (*19–24*). Metazoa lacking activity of this enzyme also show some deficiency (*25*, *26*). GSH, which is the most abundant thiol-containing low-molecular-weight substance in the cell, is another molecule which effectively eliminates ROS. GSH is synthesized from its constituent amino acids by two consecutive reactions catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. The elimination of ROS occurs through the oxidation of GSH to GSSG and subsequent regeneration by glutathione reductase. GSH is also used to cope with oxidative stress by conjugating with oxidants, which is catalyzed by glutathione S-transferase, for detoxification. An important role for GSH in cellular resistance to oxidative stress has been verified by many studies (*5*, *27–28*). Catalase and glutathione peroxidase are the enzymes that decompose hydrogen peroxide (*5*). Superoxide dismutase with manganese as a cofactor (*29*, *30*) and thioredoxin (*6*, *31*) also work to protect cells from ROS.

The fission yeast *Schizosaccharomyces pombe* is phylogenically distant from the budding yeast *Saccharomyces cerevisiae* and is often used for the study of cell biology or molecular biology. As in other organisms, oxidative stress activates transcription factors of *S. pombe* to induce the expression of ROS defensive genes. One pathway to activate a transcription factor by oxidative stress is the main stress response pathway of *S. pombe* in which Spc1/Sty1 MAP kinase activates the transcription factor Atf1 (*32*, *33*).

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Activation of another transcription factor Pap1 by oxidative stress is regulated by nuclear localization of the factor (*10*) with the modification of cysteine residues in the protein (*34*, *35*). Prr1 is a transcription factor involved in the response to oxidative stress (*36*) with homology to the transcription factor Skn7p of *S. cerevisiae*, but the regulatory mechanism of this factor is not well understood.

Many chemical agents are used to exert oxidative stress on cells. Since oxidative stress is a state in which the intracellular environment becomes oxidized, differences in agents exerting the stress have not attracted much attention. We examined the difference in the response of *S. pombe* to several reagents that exert oxidative stress on the cell. We report here that menadione (MD), 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide and cumene hydroperoxide (CHP), which are commonly used to exert oxidative stress on cells, had different effects on *S. pombe*. Care should be taken when interpreting the results obtained from an experiment where a specific oxidant is used.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—The *S. pombe* strains used in this study are listed in Table 1. The construction of mutants was as follows. MN475, which is a mutant deficient in *gsh1* (the gene that encodes γ-glutamylcysteine synthetase) and has the same genetic background as wild-type JY741, was constructed by crossing MN55 (*38*) with JY746 and then JY741 and selected based on hypersensitivity to cadmium. The GSH content of the mutant is less than 1/40 that of JY741. Deletion mutants of *atf1* (encodes a transcription factor involved in general stress response), *ctt1* (encodes catalase) and *pap1* (encodes a transcription factor involved in oxidative stress response) were constructed by transfecting DNA fragments containing deletions of genes (deletion between two *Hin*dIII sites for *atf1*, two *Msc*I sites for *ctt1*, and two *Bgl*II sites for *pap1*) into corresponding strains in which these genes disrupted by $ura4^+$ gene [CN803 (*40*) for *atf1*, CN513 (*39*) for *ctt1* and TP108-3C (*41*) for *pap1*], and 5-fluoroorotic acid-resistant clones were selected. The deletion of genes was confirmed by PCR using primers flanking the deletion sites. Primers used were AGCCGT-TATTACTTCTGTCTTG and AACGGAGGGAAAGTC-AAAGGGA for *atf1*, TGCGACTGAAAAGTCTAGCT and AAGCTTCCTAAGATTACAAA for *ctt1*, and TGTTAATTA-ATTGATAATTATG and TGCCTTTGGTCGACGAATAT for *pap1*. Cells were grown in YE (0.5% yeast extract, 3% glucose) with vigorous shaking at 30°C for most experiments. EMM (*42*) supplemented with the necessary nutrients at 50 μg/ml was used for selecting auxotrophic strains in the construction of mutants. ME (3% malt extract) was used to induce mating and sporulation for genetic crossing. For solid media, 1.5% agar was added.

Table 1. **Strains used in this work.**

Strain	Genotype	Reference or source
JY741	h^-, *ade6-M216*, *ura4-D18*, *leu1*	Laboratory stock
JY746	h^+, *ade6-M210*, *ura4-D18*, *leu1*	Laboratory stock
MN415	h^-, *ade6-M216*, *ura4-D18*, *leu1*, Δ*sod1*	*37*
MN55	h^-, *leu1*, *gsh1*	*38*
MN475	h^-, *ade6-M216*, *ura4-D18*, *leu1*, *gsh1*	This study
CN513	h^-, *ade6-M216*, *ura4-D18*, *leu1*, *ctt1*::*ura4*	*39*
MN407	h^-, *ade6-M216*, *ura4-D18*, *leu1*, Δ*ctt1*	This study
CN803	h^-, *ade6-M216*, *ura4-D18*, *leu1*, *atf1*::*ura4*	*40*
MN508	h^-, *ade6-M216*, *ura4-D18*, *leu1*, Δ*atf1*	This study
TP108-3C	h^-, *ura4*, *leu1*, *pap1*::*ura4*	*41*
MN602	h^-, *ura4*, *leu1*, Δ*pap1*	This study
DP1	h^-, *ade6-M216*, *ura4-D18*, *leu1*, *prr1*::*ura4*	*36*

Assay for Sensitivity to Oxidants—For the assay of sensitivity on plates, cells grown to the mid-logarithmic phase in YE were diluted sequentially and spotted onto plates containing reagents at the appropriate concentration. For the assay of antioxidative action, *N*-acetylcysteine (NAC) was added to the plates at a concentration of 400 μM. Plates were incubated at 30°C for 4 days. For the assay of survival in liquid culture, reagents were added to the culture at the mid-logarithmic phase (2×10^7 cells/ml) in 5 ml of YE. After 30 min of incubation at 30°C, cells were appropriately diluted with sterile water and spread onto YE plates. Plates were incubated at 30°C for 4 days. The number of colonies that appeared on the plate was counted to estimate the number of viable cells in the culture. To score growth at a low concentration of reagents, reagents were added to the culture at the mid-logarithmic phase in 5 ml of YE. Aliquots were appropriately diluted and spread onto YE plates at the time indicated. Plates were incubated at 30°C for 4 days. The number of colonies that appeared on the plate was counted to estimate the number of viable cells in culture.

Hybridization Analysis of RNA—Cells grown to the mid logarithmic phase in 40 ml of YE were treated with reagents for a specific period. Total cellular RNA was isolated by the method of Elder *et al*. (*43*). Aliquots of 40 μg of total cellular RNA were electrophoresed in 0.8% agarose gel containing formaldehyde. RNA was transferred to nitrocellulose membranes and hybridized with ^{32}P-labeled probe (*44*) prepared by the random priming method *45*. A probe for the *leu1* gene (*46*) of *S. pombe* was used as an internal control for hybridization. A BAS 1800 image analyzer was used to analyze the results of hybridization.

Determination of GSH Content—Cells grown to the mid logarithmic phase in 25 ml of YE were treated with reagents for an appropriate period and harvested by centrifugation. Harvested cells were washed with ice-cold 50 mM Na-phosphate buffer pH 7.0 and frozen at –80°C. Frozen cell pellets were mechanically disrupted with a Cool Mill (Tokken, Japan) and extracted with 200 μl of ice-cold 50 mM Na-phosphate buffer, pH 7.0. Unbroken cells were removed by centrifugation at 2,000 × *g* for 1 min. Protein content in the cell extract was determined by the method of Lowry *et al*. (*47*) using bovine serum albumin as a standard. To estimate cellular GSH content, cell extract was deproteinized with 5% trifluoroacetic acid and separated by ODS80 Tm (Tosoh, Japan) reverse-phase HPLC as described previously (*48*). The thiol content of the fraction in which GSH was eluted was determined by the method of Ellman (*49*) to estimate GSH content.

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RESULTS

Sensitivity of S. pombe *Mutants to the Reagents Tested on Plates*—Spotting cells on a plate containing a reagent is a convenient way to test the sensitivity of the cell to the reagent. Four reagents, MD, CDNB, hydrogen peroxide and CHP, were used to test the sensitivity of *S. pombe* mutants defective in systems to destroy ROS. MD is a quinone that produces superoxide radical by redox cycling in the cell. CDNB exerts oxidative stress by depleting intracellular GSH. Hydrogen peroxide, a ROS, oxidizes cellular components and is converted to highly active hydroxyl radical by the Harber-Weiss reaction in the presence of transition metals. CHP is an organic hydroperoxide that initiates radical reaction in the presence of transition metals. *S. pombe* MN415, MN475 and MN407, which have a mutation in the *sod1*, *gsh1*, and *ctt1* genes, respectively, and JY741 (wild type) were sequentially diluted and spotted onto plates containing these reagents at the concentration indicated (Fig. 1). MN415 (*sod1* mutant, deficient in Cu,Zn-SOD) was more sensitive to all reagents tested than JY741. MN475 (*gsh1* mutant, deficient in GSH biosynthesis) was more sensitive to the reagents tested except for CHP than JY741 but more resistant to all reagents tested than MN415. MN407 (*ctt1* mutant, deficient in catalase) was very sensitive to hydrogen peroxide and slightly sensitive to MD. Concentrations of the reagents used here were the highest at which JY741 could grow at the highest dilution and the lowest at which sensitive mutants showed growth inhibition. Twice this concentration of the reagents completely inhibited the growth of JY741 on the plate even without dilution and sensitive mutants grew well at half of this concentration of the reagents in many cases (data not shown). When the anti-oxidant NAC was included in the plate containing reagents, resistance to MD and CHP recovered dramatically. Resistance to hydrogen peroxide recovered slightly but sensitivity to CDNB did not change. The weak recovery of resistance to hydrogen peroxide might be caused by the high concentration of hydrogen peroxide used (2 mM) compared to the concentration of NAC used (400 μM). Lower concentration of NAC (100 μM) was enough to restore resistance to MD and CHP (data not shown). Mutants defective in transcription factors involved in the response to oxidative stress were examined for their sensitivity to the reagents on the plate. Mutants used were strains defective in the *atf1*, *pap1* or *prr1* gene. As shown in Fig. 2, these strains each had a different spectrum of sensitivity. DP1 (*prr1* mutant) was sensitive to CDNB, CHP and hydrogen peroxide and slightly sensitive to MD. MN602 (*pap1* mutant) was sensitive to all reagents used. MN508 (*atf1* mutant) was sensitive to MD and hydrogen peroxide but was not as sensitive as MN602 to MD or as DP1 or MN602 to hydrogen peroxide. These results revealed that different gene products were implicated in resistance to these reagents and that these reagents acted differently on *S. pombe* cells in spite of their action as oxidants.

Sensitivity of S. pombe *Cells to the Reagents in Liquid Culture*—The rate of survival of *S. pombe* at higher concentrations of oxidants in liquid culture was examined. The assay in which survival after treatment with reagents for 30 min was scored showed that 500 μM MD killed more than 90% of cells, whereas 4 mM CDNB was required to kill

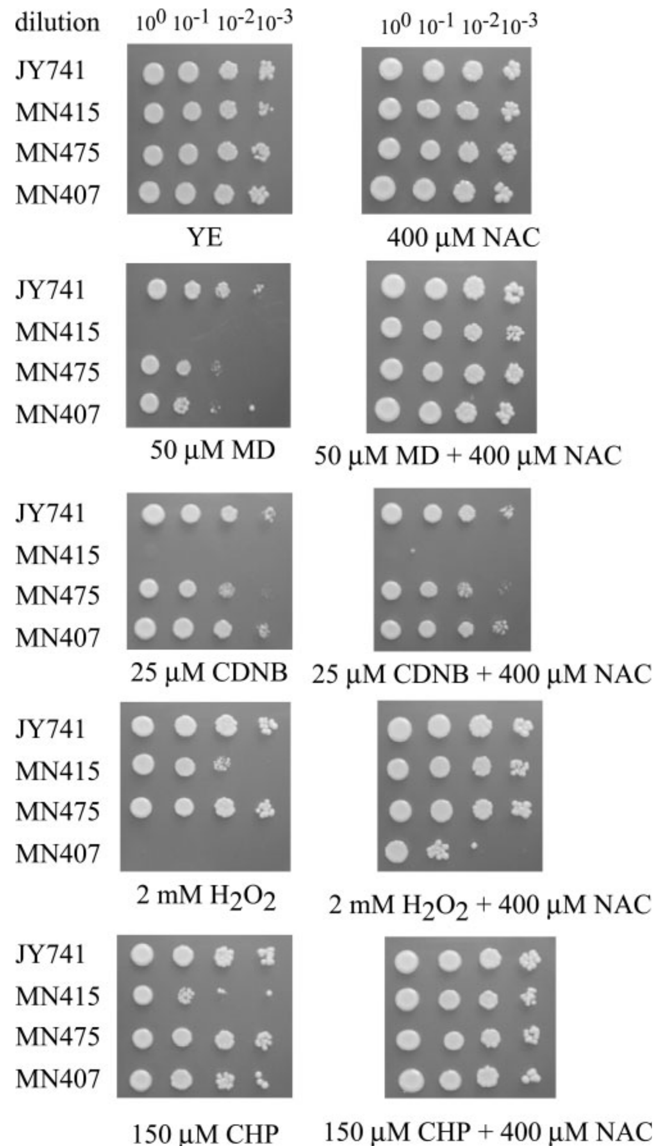

Fig. 1. **Sensitivity of *S. pombe* mutants deficient in ROS resistant genes to CDNB, MD, hydrogen peroxide and CHP on plates and recovery of resistance on addition of NAC.** JY741 (wild type), MN415 (*sod1*), MN475 (*gsh1*), and MN407 (*ctt1*) were serially diluted 10-fold, and 2.5 μl aliquots were spotted onto YE plates containing MD (50 μM), CDNB (25 μM), hydrogen peroxide (2 mM) or CHP (150 μM) with or without NAC (400 μM). Plates were incubated at 30°C for 4 days.

a comparable number of cells (Fig. 3). This result reversed the result of the spotting assay, where CDNB worked at a lower concentration than MD. CHP worked in liquid culture at a concentration two to three times higher than that at which CDNB worked, but CHP worked at a six times higher concentration than CDNB in spotting assay. Hydrogen peroxide required a much higher concentration than other reagents to kill *S. pombe* cells in liquid culture, as was found in the assay on the plate shown in Fig. 1. To test the effects of low concentrations of the reagents in liquid culture, the growth curve of JY741 with a low concentration of the reagents was examined (Fig. 4). MD, CDNB, and hydrogen peroxide stopped the growth of cells but did not

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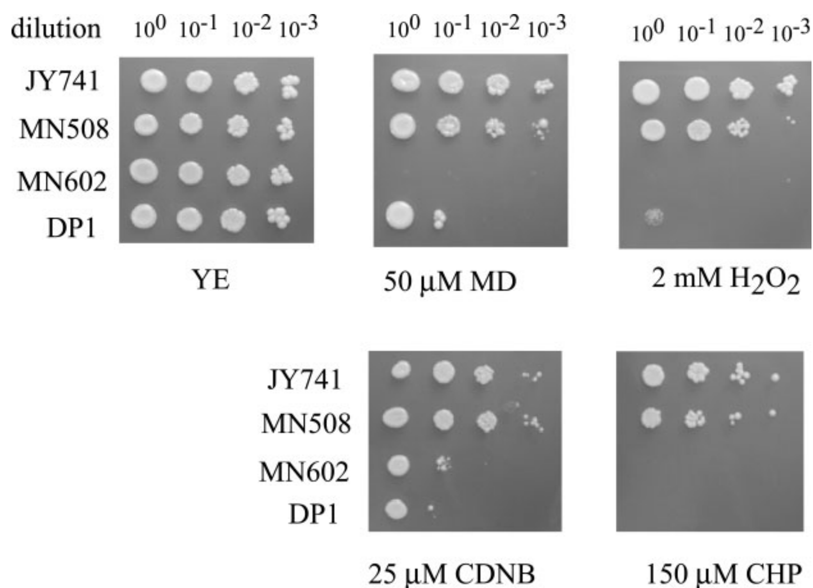

Fig. 2. **Sensitivity of *S. pombe* mutants deficient in genes of transcription factors involved in oxidative stress resistance to CDNB, MD, hydrogen peroxide and CHP on plates.** JY741 (wild type), MN508 (*atf1*) MN602 (*pap1*) and DP1 (*prr1*) were serially diluted 10-fold and 2.5 μl aliquots were spotted onto YE plates containing MD (50 μM), CDNB (25 μM), hydrogen peroxide (2 mM) or CHP (150 μM). Plates were incubated at 30°C for 4 days.

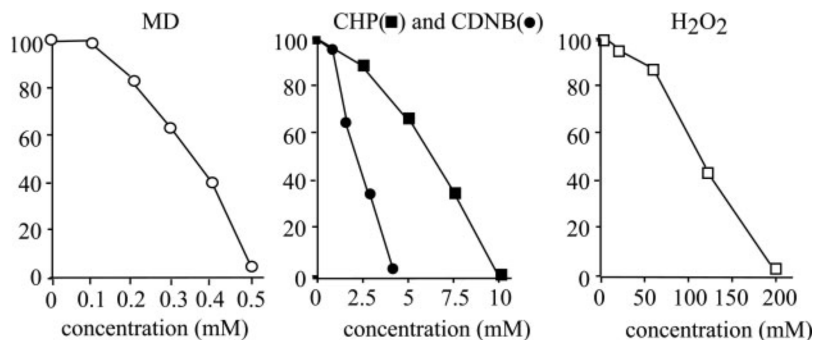

Fig. 3. **Survival assay of JY741 treated with high concentrations of MD (left panel) CDNB (closed circle) and CHP (closed square) (center panel) and hydrogen peroxide (right panel).** MD, CDNB, hydrogen peroxide and CHP were added to JY741 culture grown in YE to mid logarithmic phase at the concentrations indicated. After 30 min incubation at 30°C, cells were diluted appropriately and spread onto YE plates to count viable cells in the culture. Plates were incubated at 30°C for 4 days. The absolute value corresponding to 100% is 2×10^7 cells/ml. The data presented here are results representative of three independent experiments.

decrease their viability. However, when CHP was added to the culture, viability did not decrease in the first 2 h but rapidly decreased thereafter.

Induction of Oxidative Stress Related Genes by Oxidants—S. pombe adapts to oxidative stress by rapidly inducing defensive genes. We examined the induction of *ctt1*$^+$, *gpx1*$^+$, and *apt1*$^+$ after administration of a non-lethal concentration of oxidant (50 μM MD, 50 μM CDNB, 0.1 mM CHP, and 0.3 mM hydrogen peroxide) by hybridization assay (Fig. 5). Expression of *apt1*$^+$ is controlled by Pap1 (*10*, *41*), while expression of *gpx1*$^+$ is controlled by Atf1 (*50*). Expression of *ctt1*$^+$ is controlled both Atf1 and Pap1 (*40*, *51*), and Prr1 is also involved in *ctt1*$^+$ expression (*36*). Although the induction kinetics of *ctt1*$^+$ and *gpx1*$^+$ by MD, CDNB and hydrogen peroxide was that of a typical stress-responsive gene, a difference in the peak of induction was observed. The peak for these genes was 30 min for MD and hydrogen peroxide and 60 min for CDNB. The amount of mRNA of these genes rapidly decreased after the peak. Kinetics of *apt1*$^+$ induction was a little different from that of *ctt1*$^+$ and *gpx1*$^+$ induction. Some delay in the induction of *apt1*$^+$ was observed. The peak of induction by MD and CDNB occurred at 90 min, though hydrogen peroxide showed a peak of induction at 30 min as found in *ctt1*$^+$ and *gpx1*$^+$ genes. In the case of MD and hydrogen peroxide,

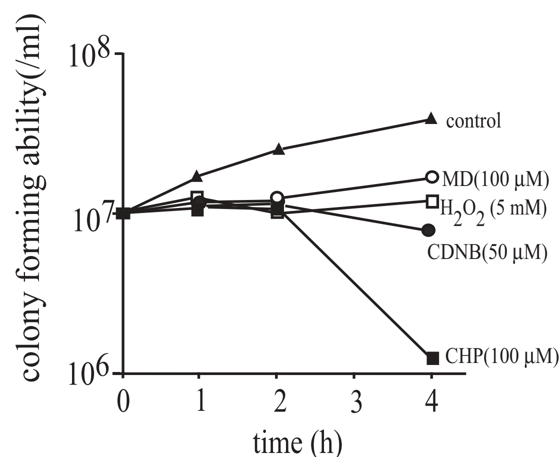

Fig. 4. **Growth curve of JY741 treated with low concentrations of MD, CDNB, hydrogen peroxide and CHP.** MD (100 μM) (open circle), CDNB (50 μM) (closed circle), hydrogen peroxide (5 mM) (open square) and CHP (100 μM) (closed square) were added to cells grown in mid logarithmic phase in YE and incubation was continued. As a control, a culture without reagents (closed triangle) was used. Cells were diluted appropriately and spread onto YE plates to count viable cells in the culture. Plates were incubated at 30°C for 4 days. The data presented here are results representative of three independent experiments.

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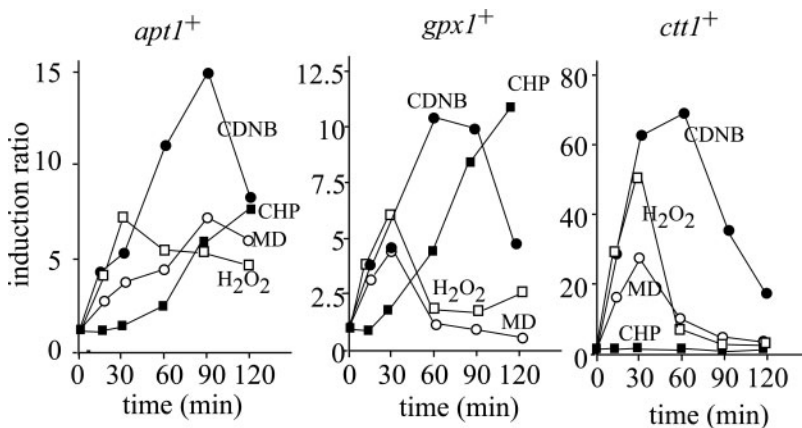

Fig. 5. **Hybridization analysis of induction kinetics of genes by the reagents.** Induction of *apt1*$^+$ (left panel), *gpx1*$^+$ (center panel), and *ctt1*$^+$ (right panel) genes by 50 μM MD (open circle), 50 μM CDNB (closed circle), 300 μM hydrogen peroxide (open square) and 100 μM CHP (closed square) was examined. Total cellular RNA of JY741 was prepared from cells treated with the reagents for the time indicated and analyzed by Northern blot hybridization. The amount of mRNA was estimated by use of a BAS 1800 Image analyzer. The induction rate was calculated from the amount of each mRNA relative to *leu1* mRNA. The data presented here are results representative of three independent experiments.

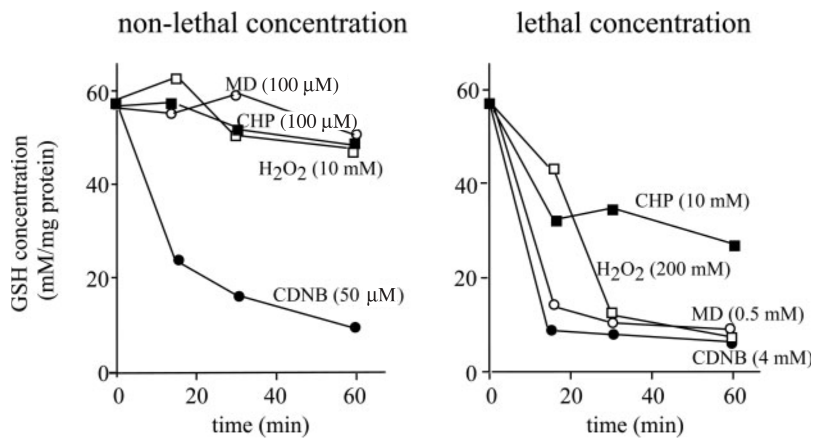

Fig. 6. **Consumption of GSH by oxidant.** Cell extract obtained from JY741 treated with MD (open circle), CDNB (closed circle) hydrogen peroxide (open square) and CHP (closed square) at non-lethal (left panel) and lethal (right panel) concentrations was deproteinized with 5% trifluoracetic acid and separated by reverse phase HPLC. Thiol contents of the fraction in which GSH was eluted were determined to estimate GSH content. Concentrations used were 50 μM (non-lethal) and 4 mM (lethal) CDNB, 100 μM (non-lethal) and 500 μM (lethal) MD, 500 μM (non-lethal) and 10 mM (lethal) CHP, and 10 mM (non-lethal) and 200 mM (lethal) hydrogen peroxide.

the amount of *apt1*$^+$ mRNA did not decrease rapidly. These differences might be caused by a difference in the transcription factors involved in induction of the genes. CHP gave different induction kinetics. Both *apt1*$^+$ and *gpx1*$^+$ mRNA levels began to rise 30 min after administration of CHP and continued to increase up to 2 hours after administration of CHP. Surprisingly, *ctt1*$^+$ was not induced at all by CHP. MN407 (*ctt1* mutant) was resistant against CDNB in the plate assay (Fig. 1), though the *ctt1* gene was found to be highly expressed after CDNB treatment (Fig. 5). This indicated that some genes responsible for resistance to a reagent are not always inducible by the reagent.

Consumption of GSH by Oxidant—GSH is a thiol compound present in large amounts in cells and plays an important role in maintaining the reductive environment of the cell. Since GSH is used to detoxify ROS because of its strong reducing power and itself is oxidized to glutathione disulfide, the intracellular GSH concentration was expected to decrease in the cells treated with an oxidant. The consumption of GSH in *S. pombe* treated with the four reagents was examined (Fig. 6). When JY741 was treated with the reagents at the concentration at which the survival rate of cells at 30 min is less than 10%, the intracellular concentration of GSH decreased rapidly. CDNB, MD and hydrogen peroxide reduced the cellular GSH content to less than 10% of the original level but CHP did not reduce the cellular GSH content as much. About 40% of the original level of GSH was detected in cells treated with a high concentration of CHP. At lower non-lethal concentrations of reagents, only treatment with CDNB markedly decreased the cellular GSH content. MD, CHP and hydrogen peroxide did not decrease cellular GSH level significantly at non-lethal concentrations. Since CDNB is a good substrate for glutathione S-transferases of *S. pombe* (*52*), the reduction of GSH at low concentrations of CDNB was probably caused by the conjugation of GSH with CDNB catalyzed by this enzyme.

DISCUSSION

MD, CDNB, hydrogen peroxide and CHP, which are frequently used to induce oxidative stress in the study of cellular stress response, were examined for their effects on *S. pombe*. MN415 (*sod1* mutant) and MN475 (*gsh1* mutant) were more sensitive to these reagents than JY741 (wild type) and the resistance was restored by the antioxidant NAC, though resistance to CDNB was not restored. This indicated that they worked as oxidants as expected. However, their effects on *S. pombe* differed. Among the four reagents, CHP was most different. The induction kinetics of genes by CHP was very different from the typical induction kinetics of genes induced by oxidative stress. Expression of the *ctt1*$^+$ gene was not induced by CHP in spite of the extensive induction of the gene by other reagents tested. *apt1*$^+$ and *gpx1*$^+$ showed some delay in their induction and a high level of expression for up to 2 h after the administration of CHP. In contrast the induction of these genes by other reagents peaked at

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30 to 90 min post-administration. GSH consumption at a high concentration of CHP was also different from other reagents. GSH content was not reduced much in *S. pombe* treated with a high concentration of CHP, in contrast to *S. pombe* treated with other reagents at high concentrations.

NAC restored resistance to MD and CHP but did not change sensitivity to CDNB. The restoration of MD resistance occurred in MN475 (*gsh1* mutant) as well as the wild type. This indicated that NAC, which is used frequently as a precursor of GSH, did not need to be converted to GSH to cope with oxidants, and a high concentration of thiol in the cell was enough to protect *S. pombe* against MD toxicity. The reason why NAC did not restore resistance to CDNB is not clear. One possibility is that 400 μM NAC might not give enough thiol to restore oxidant resistance in *S. pombe*, since the cellular GSH level decreased at the low concentration of CDNB used in the spot assay.

Hydrogen peroxide required a much higher concentration than other reagents to act on JY741 both on plates and in the liquid culture. A catalase-deficient mutant showed sensitivity to hydrogen peroxide at a micromolar concentration in liquid culture (*32*), which is a similar concentration to other oxidants in liquid culture (Fig. 3). This indicated that efficient decomposition of hydrogen peroxide by catalase makes *S. pombe* cells resistant to higher concentrations of hydrogen peroxide. Rapid decomposition of hydrogen peroxide in the wild-type cell (*53*) could be the cause of the rapid decline in induction of the $apt1^+$ gene shown in Fig. 5.

Mutants defective in transcription factors involved in oxidative stress resistance were differently resistant to the reagents. This means different genes were involved in resistance to the reagents. Resistance to MD was mainly determined by the gene whose transcription is controlled by Pap1, and the gene whose transcription was controlled by Atf1 and Prr1 is also involved in MD resistance. Resistance to CDNB was determined by the gene whose transcription is controlled by Prr1 and Pap1. All mutants of one of the three transcription factors were sensitive to hydrogen peroxide. This is consistent with the expression of catalase, an important enzyme for hydrogen peroxide resistance, being controlled by these three transcription factors (*32*, *33*, *36*, *40*). Both MN602 and DP1 were sensitive to CHP. This means that the gene whose expression is controlled by both Pap1 and Prr1 is involved in resistance to CHP. Since *sod1* was expressed at similar levels in these mutants as the wild type (*22*), though weak induction of *sod1* by MD or hydrogen peroxide was not observed in the mutants (*22*), genes other than *sod1* should be responsible for sensitivity in the mutants of transcription factors. Thorpe *et al*. (*54*) showed that distinct mechanisms work to maintain protection against different reagents generating ROS in *S. cerevisiae* by using a complete set of viable deletion mutants. Two phylogenically different species showed similar phenomena indicating that distinct genes are involved in resistance to reagents exerting oxidative stress. This indicates that a difference in cellular response to reagents used to exert oxidative stress could be widely observed in living cells.

At lower concentrations of CDNB, the cellular GSH content rapidly decreased, but *S. pombe* remained viable. The induction of oxidative stress defensive genes occurred at the concentration at which GSH depletion did not occur in cells treated with MD, hydrogen peroxide or CHP (Fig. 5). These results indicated that GSH depletion was neither the cause of loss of viability in oxidatively stressed *S. pombe* nor involved in the induction of genes by oxidative stress. Modification of cysteine residues in Pap1 by oxidants, which is important for nuclear localization and activation of Pap1, occurs without GSH depletion. This indicated that cysteine residues involved in oxidative modification to activate Pap1 should be more reactive to oxidants than GSH, at least in the case of MD and hydrogen peroxide.

In the survival assay, MD killed *S. pombe* cells at the lowest concentration of the reagents tested. However, in the spot assay, CDNB acted at the lowest concentration. These results might indicate that CDNB worked as a fungistatic agent at a wide range of concentrations, while MD acted as a fungicidic agent at relatively low concentrations, though it functioned as a fungistatic agent at 100 μM (Fig. 4). Otherwise, cells grown on plates and cells grown in liquid culture might differ in physiological state, giving rise to different responses to these reagents.

Oxidative stress has attracted much attention because it causes several diseases and aging. In the study of oxidative stress, several agents are used to exert oxidative stress on cells. However, the difference in cellular responses derived from the use of different reagents has not attracted much attention. The results presented here showed that cellular response differs according to the reagents causing oxidative stress. These differences should be considered carefully when interpreting the results obtained in an experiment using a specific reagent. What causes these differences is an interesting problem. One possibility is that these reagents generate ROS at different places in the cell. The generation of ROS in the cell was microscopically investigated. *S. pombe* cells treated with these reagents were stained with dihydrofluorescein diacetate (*55*) to visualize where the ROS were generated. *S. pombe* cells treated with these reagents produced brighter fluorescent images than untreated cells, but in any case the fluorescent signal was observed in the cytoplasm, and no distinctive structure with strong fluorescence was found (data not shown). Another possibility is that the different reagents generate ROS by different mechanisms. Since MN415 (a *sod1* mutant) was hypersensitive to these reagents, superoxide radical generated in the cell should be one of the causes of toxic effects of these reagents. MD produces superoxide radical by redox cycling. CHP is a organic peroxide that produces superoxide radical by initiating radical reaction in the presence of transition metals. CDNB shifts intracellular redox balance to a more oxidized state by lowering GSH level, which favors production of superoxide radical. Hydrogen peroxide is a ROS that does not directly produce superoxide radical but oxidizes cellular components and makes the intracellular environment favorable to superoxide radical production. Hydrogen peroxide is also converted to the more active hydroxyl radical in the presence of transition metals by the Harber-Weiss reaction. Indirect production of superoxide radical could be the reason that NAC did not restore the resistance to CDNB. MD and CHP produce superoxide radical in parts of the cell where these molecules are distributed. On the other hand, superoxide radical could be generated over a

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wider area in the cells treated with CDNB, which lowers GSH level and makes a wider area of the cell more oxidized. In cells treated with MD and CHP, superoxide radical might be produced in areas that exogenously added NAC could easily reach, but in CDNB-treated cells, superoxide radical was generated over a wider area including parts where exogenously added NAC did not reach, though microscopically no difference in the area of ROS generation was detected as described above. Restoration of hydrogen peroxide resistance by NAC, though hydrogen peroxide produces superoxide radical indirectly, was probably because NAC reacted directly with hydrogen peroxide and reduced the concentration of hydrogen peroxide. Direct oxidation of cellular components by hydrogen peroxide or hydroxyl radical produced from hydrogen peroxide by the Harber-Weiss reaction could be the main basis of toxicity of hydrogen peroxide, and superoxide radical was produced secondarily by oxidation of cellular components. This might be why MN415 was less sensitive to hydrogen peroxide but very sensitive to CDNB, which produces superoxide radical indirectly.

Elucidation of the precise mechanisms by which these reagents work is required to clarify the basis of differences of cellular responses to these reagents. This work is now under way in our lab.

REFERENCES

1. Floyd, R.A. (1990) Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB J.* **4**, 2587–2597
2. Crawford, D.W. and Blankenhorn, D.H. (1991) Arterial wall oxygenation, oxyradicals, and atherosclerosis. *Atherosclerosis* **89**, 97–108
3. Jennings, B.J., Ozanne, S.E., and Hales, C.N. (2000) Nutrition, oxidative damage, telomere shortening, and cellular senescence: individual or connected agents of aging? *Mol. Genet. Metab.* **71**, 32–42
4. Schrauwen, P. and Hesselink, M.K. (2004) Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. *Diabetes* **53**, 1412–1417
5. Warner, D.S., Sheng, H., and Batinic-Haberle, I. (2004) Oxidants, antioxidants and the ischemic brain. *J. Exp. Biol.* **207**, 3221–3231
6. Arner, E.S. and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267**, 6102–6129
7. Noctor, G. and Foyer, C.H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 249–279
8. Keppler, D. (1999) Export pumps for glutathione S-conjugates. *Free Radic. Biol. Med.* **27**, 985–991
9. Wu, G., Fang, Y.-Z., Yang, S., Lupton, J.R., and Turner, N.D. (2004) Glutathione metabolism and its implications for health. *J. Nutr.* **134**, 489–492
10. Toone, W.M., Kuge, S., Samuels, M., Morgan, B.A., Toda, T., and Jones, N. (1998) Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. *Genes Dev.* **12**, 1453–1463
11. Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., and Toledano, M.B. (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J. Biol. Chem.* **274**, 16040–16046
12. Carmel-Harel, O. and Storz, G. (2000) Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol.* **54**, 439–461
13. Lee, J.M., Calkins, M.J., Chan, K., Kan, Y.W., and Johnson, J.A. (2003) Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. *J. Biol. Chem.* **278**, 12029–12038
14. Keller, G.A., Warner, T.G., Steimer, K.S., and Hallewell, R.A. (1991) Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblast and hepatoma cells. *Proc. Natl. Acad. Sci. USA* **88**, 7381–7382
15. Chang, L., Slot, J.W., Geuza, H.J., and Crapo, J.D. (1988) Molecular immunocytochemistry of the CuZn superoxide dismutase in rat hepatocytes. *J. Cell Biol.* **107**, 2169–2179
16. Weisiger, R.A. and Fridovich, I. (1973) Mitochondrial superoxide dismutase. site of synthesis and intramitochondrial localization. *J. Biol. Chem.* **248**, 4793–4796
17. Sturtz, L.A., Diekert, K., Jensen, L.T., Lill, R., and Culotta, V.C. (2001) A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. a physiological role for SOD1 in guarding against mitochondrial oxidative damage. *J. Biol. Chem.* **276**, 38084–38089
18. Okado-Matsumoto, A. and Fridovich, I. (2001) Subcellular distribution of superoxide dismutases (SOD) in rat liver Cu,Zn-SOD in mitochondria. *J. Biol. Chem.* **276**, 38388–38393
19. Chary, P., Dillon, D., Schroeder, A.L., and Natvig, D.O. (1994) Superoxide dismutase (sod-1) null mutants of Neurospora crassa: oxidative stress sensitivity, spontaneous mutation rate and response to mutagens. *Genetics* **137**, 723–730
20. San Mateo, L.R., Hobbs, M.M., and Kawula, T.H. (1998) Periplasmic copper-zinc superoxide dismutase protects *Haemophilus ducreyi* from exogenous superoxide. *Mol. Microbiol.* **27**, 391–404
21. Piddington, D.L., Fang, F.C., Laessig, T., Cooper, A.M., Orme, I.M., and Buchmeier, N.A. (2001) Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. *Infect. Immun.* **69**, 4980–4987
22. Mutoh, N., Nakagawa, C.W., and Yamada, K. (2002) Characterization of Cu, Zn superoxide dismutase deficient mutant of fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **41**, 82–88
23. Hwang, C.S., Rhie, G.E., Oh, J.H., Huh, W.K., Yim, H.S., and Kang, S.O. (2002) Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. *Microbiology* **148**, 3705–3713
24. Sansone, A., Watson, P.R., Wallis, T.S., Langford, P.R., and Kroll, J.S. (2002) The role of two periplasmic copper- and zinc-cofactored superoxide dismutases in the virulence of *Salmonella choleraesuis*. *Microbiology* **148**, 719–726
25. Ho, Y.S., Gargano, M., Cao, J., Bronson, R.T., Heimler, I., and Hutz, R.J. (1998) Reduced fertility in female mice lacking copper-zinc superoxide dismutase. *J. Biol. Chem.* **273**, 7765–7769
26. Blander, G., de Oliveira, R.M., Conboy, C.M., Haigis, M., and Guarente, L. (2003) Superoxide dismutase 1 knock-down induces senescence in human fibroblasts. *J. Biol. Chem.* **278**, 38966–38969
27. Seo, Y.J., Lee, J.W., Lee, E.H., Lee, H.K., Kim, H.W., and Kim, Y.H. (2004) Role of glutathione in the adaptive tolerance to H_2O_2. *Free Radic. Biol. Med.* **37**, 1272–1281
28. Berruyer, C., Martin, F.M., Castellano, R., Macone, A., Malergue, F., Garrido-Urbani, S., Millet, V., Imbert, J., Dupre, S., Pitari, G., Naquet, P., and Galland, F. (2004) Vanin-$1^{-/-}$ mice exhibit a glutathione-mediated tissue resistance to oxidative stress. *Mol. Cell. Biol.* **247**, 214–224
29. Jeong, J.H., Kwon, E.S., and Roe, J.H. (2001) Characterization of the manganese-containing superoxide dismutase and its

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gene regulation in stress response of *Schizosaccharomyces pombe*. *Biochem. Biophys. Res. Commun.* **283**, 908–914
30. Kirby, K., Hu, J., Hilliker, A.J., and Phillips, J.P. (2002) RNA interference-mediated silencing of Sod2 in *Drosophila* leads to early adult-onset mortality and elevated endogenous oxidative stress. *Proc. Natl. Acad. Sci. USA* **99**, 16162–16167
31. Watson, W.H., Yang, X., Choi, Y.E., Jones, D.P., and Kehrer, J.P. (2004) Thioredoxin and its role in toxicology. *Toxicol. Sci.* **78**, 3–14
32. Shiozaki, K. and Russell, P. (1996) Conjugation, meiosis and osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Genes Dev.* **10**, 2276–2288
33. Wilkinson, M.G., Samuels, M., Takeda, T., Toone, W.M., Shieh, J.-C., Toda, T., Millar, J.B.A., and Jones, N. (1996) The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. *Genes Dev.* **10**, 2289–2301
34. Castillo, E.A., Ayte, J., Chiva, C., Moldon, A., Carrascal, M., Abian, J., Jones, N., and Hidalgo, E. (2002) Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. *Mol. Microbiol.* **45**, 243–254
35. Vivancos, A.P., Castillo, E.A., Jones, N., Ayte, J., and Hidalgo, E. (2004) Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. *Mol. Microbiol.* **52**, 1427–1435
36. Ohmiya, R., Kato, C., Yamada, H., Aiba, H., and Mizuno, T. (1999) A fission yeast gene ($prr1^{+}$) that encodes a response regulator implicated in oxidative stress response. *J. Biochem.* **125**, 1061–1066
37. Mutoh, N., Kawabata, M., Nakagawa, C.W., and Kitajima, S. (2005) Pro-oxidant action of phloxine B on fission yeast *Schizosaccharomyces pombe*. *Yeast* **22**, 91–97
38. Mutoh, N. and Hayashi, Y. (1988) Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesize cadystin, small cadmium-binding peptides. *Biochem. Biophys. Res. Commun.* **151**, 32–39
39. Mutoh, N., Nakagawa, C.W., and Yamada, K. (1999) The Role of Catalase in Hydrogen Peroxide Resistance in Fission Yeast *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **45**, 125–129
40. Nakagawa, C.W., Yamada, K., and Mutoh, N. (2000) Role of Atf1 and Pap1 in the induction of the catalase gene of fission yeast *Schizosaccharomyces pombe*. *J. Biochem.* **127**, 233–238
41. Toda, T., Shimanuki, M., and Yanagida, M. (1991) Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. *Genes Dev.* **5**, 60–73
42. Moreno, S., Klar, A., and Nurse, P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**, 795–823
43. Elder, R.T., Loh, E.Y., and Davis, R.W. (1983) RNA from the yeast transposable element Ty1 has both ends in the direct repeats, a structure similar to retrovirus RNA. *Proc. Natl. Acad. Sci. USA* **80**, 2432–2436
44. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, pp. 7.39–7.52 and 9.31–9.57, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
45. Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6–13
46. Kikuchi, Y., Kitazawa, Y., Shimatake, G., and Yamamoto, M. (1988) The primary structure of the $leu1^{+}$ gene of *Schizosaccharomyces pombe*. *Curr. Genet.* **14**, 375–379
47. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
48. Mutoh, N., Kawabata, M., and Hayashi, Y. (1991) Tetramethyllthiuram disulfide or dimethyldithiocarbamate induces the synthesis of cadystins, heavy metal chelating peptides, in *Schizosaccharomyces pombe*. *Biochem. Biophys. Res. Commun.* **176**, 1068–1073
49. Ellman, G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**: 70–77
50. Yamada, K., Nakagawa, C.W., and Mutoh, N. (1999) *Schizosaccharomyces pombe* homologue of glutathione peroxidase, which does not contain selenocysteine, is induced by several stresses and works as an antioxidant. *Yeast* **15**, 1125–1132
51. Quinn, J., Findlay, V.J., Dawson, K., Millar, J.B., Jones, N., Morgan, B.A., and Toone, W.M. (2002) Distinct regulatory proteins control the graded transcriptional response to increasing H_2O_2 levels in fission yeast *Schizosaccharomyces pombe*. *Mol. Biol. Cell.* **13**, 805–816
52. Veal, E.A., Toone, W.M., Jones, N., and Morgan, B.A. (2002) Distinct roles for glutathione S-transferases in the oxidative stress response in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **277**, 35523–35531
53. Nakagawa, C.W., Mutoh, N., and Hayashi, Y. (1995) Transcriptional regulation of catalase gene in the fission yeast *Schizosaccharomyces pombe*: Molecular cloning of the catalase gene and northern blot analyses of the transcript. *J. Biochem.* **118**, 109–116
54. Thorpe, G.W., Fong, C.S., Alic, N., Higgins, V.J., and Dawes, I.W. (2004) Cells have distinct mechanisms to maintain protection against different reactive oxygen species: Oxidative-stress-response genes. *Proc. Natl. Acad. Sci. USA* **101**, 6564–6569
55. Hempel, S.L., Buettner, G.R., O'Malley, Y.Q., Wessels, D.A., and Flaherty, D.M. (1999) Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. *Free Radic. Biol. Med.* **27**, 146–159

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