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Two Distinct Upstream Regions Are Involved in Expression of the Catalase Gene in *Schizosaccharomyces pombe* in Response to Oxidative Stress

Chiaki W. Nakagawa, Kenichiro Yamada, and Norihiro Mutoh[1]

Department of Genetics, Institute for Developmental Research, Kagiya-cho, Kasugai, Aichi 480-0392

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The DNA region responsible for the induction of the catalase gene of *Schizosaccharomyces pombe* in response to oxidative stress was determined by constructing a series of deletions in the 5′-flanking region of the gene. Cells having deletion −672 (numbered with the transcription start site as +1) to −111 showed no significant difference in catalase expression from the wild-type cells. Cells having deletion −672 to −89 showed reduced basal expression of the catalase mRNA, but retained the ability of induction in response to oxidative stress. Cells having deletion −672 to −55 completely lost the ability to express the catalase mRNA. These results suggested that two regions, −89 to −55 and −111 to −89, are involved in expression of the catalase gene. The DNA region of −89 to −55 overlapped with the Atf1 binding sequence. The Atf1 is a bZIP transcription factor with an important role in stress response under the control of the Spc1 mitogen activated protein (MAP) kinase. Introduction of the *atf1*$^-$ or *spc1*$^-$ mutation into the mutant having a deletion in −672 to −89 completely abolished the expression of the catalase mRNA. This result indicated that the Spc1-Atf1 cascade is involved in expression of the catalase gene through the region of −89 to −55. In mutants *spc1*$^-$ and *atf1*$^-$, basal expression and induction by hydrogen peroxide of catalase mRNA were observed. These results revealed that not only the Atf1 binding site but also another DNA element independent of the Spc1-Atf1 pathway is involved in the expression of the catalase gene in response to oxidative stress in *S. pombe*. Proteins that bound specifically to each DNA element existed in the cell extract of the wild-type *S. pombe*.

Key words: catalase gene promoter, hydrogen peroxide, *Schizosaccharomyces pombe*, stress response, transcriptional regulation.

Reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxy radical are the most common destructive agents in living organisms. They are not only present in the environment, but also generated as by-products of respiration. All living organisms have evolved a wide range of mechanisms to protect against reactive oxygen species. These include reduction of reactive oxygen species by low molecular weight compounds, decomposition of reactive oxygen species by enzymes and repair or degradation of damaged molecules. Induction of molecules to defend against reactive oxygen species after exposure to low levels of oxidative stress has been reported in several species (for example, see Refs. *1-3*) and some transcription factors involved in the induction have been identified (*4-6*).

We previously reported that *Schizosaccharomyces pombe* cells exposed to low concentrations of hydrogen peroxide acquire resistance to several stresses and that catalase activity is induced in this process (*7*). Induction of catalase activity is regulated at the mRNA level and *de novo* protein synthesis is not required for induction of catalase mRNA (*8*). Recent studies have revealed that the Wak1-Wis1-Spc1 MAP kinase pathway is involved in several stress responses including oxidative stress and high osmolarity stress in *S. pombe* (*6, 9-11*). Atf1 bZIP transcription factor (*12*) is a target of the Wak1-Wis1-Spc1 MAP kinase pathway and is phosphorylated by Spc1 MAP kinase (*6, 10*). Expression of the catalase gene is also regulated by the Atf1 protein (*6, 10, 11*). Unphosphorylated Atf1 protein is a repressor of the catalase gene expression and is converted to an activator by phosphorylation with the Spc1 MAP kinase (*13*). Induction of the catalase gene has been adopted as an oxidative stress response in these studies (*6, 10, 11*) but no studies on the structure of the catalase gene promoter, which should be the target of the stress signal, have been carried out.

Here we describe the structure of the catalase gene promoter and show that two discrete elements are involved in the induction of the catalase gene by hydrogen peroxide. One is the Atf1 binding site and the other is the sequence −111 to −89 (numbered with the transcription start site as +1) of the catalase gene. We propose that the Atf1 binding site is the target of the general stress response signal through the Wak1-Wis1-Spc1 MAP kinase pathway and the other site is the specific target of oxidative stress that modulates the stress response of *S. pombe*.

[1] To whom correspondence should be addressed.

Abbreviations: MAP kinase, mitogen activated protein kinase; STREs, stress response elements.

MATERIALS AND METHODS

Organisms and Growth Conditions—*S. pombe* strain JY741 and JY746 were gifts from Dr. Y. Hotta of Nara Institute of Science and Technology. HM123 was a gift from Dr. M. Yamamoto of the University of Tokyo. CN513 is a catalase gene disruptant obtained from JY741 by inserting the *ura4* gene between a *Kpn*I site and a *Bam*HI site of the catalase gene. CN803 is an *atf1* gene disruptant constructed by inserting the *ura4* gene into the two *Hin*dIII sites of the *atf1* gene. CN710 was obtained from a genetic cross between JY746 and MN510, which is an *spc1*$^-$ mutant isolated from HM123 in this laboratory. The genetic backgrounds of the strains used in this paper are listed in Table I. *S. pombe* cells were grown in SD medium (0.67% yeast nitrogen base, 2% glucose) supplemented with required nutrients at the concentration of 50 μg/ml or YPD medium (2% polypeptone, 1% yeast extract, 2% glucose) at 30°C with vigorous shaking. An MEA plate (3% malt extract, 2% agar) was used for mating and sporulation of *S. pombe*. For hydrogen peroxide treatment, hydrogen peroxide was added to logarithmically growing cells at the concentration of 0.2 mM and cultivation was continued for another 30 min (for RNA analysis) or 60 min (for enzyme assay or gel electrophoresis DNA binding assay). *Escherichia coli* DH5α was used for propagation of plasmids. JM110 (*dam*$^-$) was used to prepare a plasmid for construction of the deletion mutant with exonuclease III because of utilization of an unmethylated *Cla*I site. BMH7-18MutS and MV1184 (*sup*^{0}) were used for site-directed mutagenesis. *E. coli* cells were grown in L broth (1% Bacto trypton, 0.5% yeast extract, 0.5% NaCl) at 37°C.

Construction of Deletion Mutants—A series of mutants of *S. pombe* having a deletion in the promoter region of the catalase gene was constructed by homologous recombination between a catalase gene having a deletion in the upstream region in the plasmid and a catalase gene disrupted by the *ura4* gene in CN513. Plasmid pCN9, which is a derivative of pUC19 harboring a 3.5 kb *Hin*dIII fragment containing a catalase gene of *S. pombe* (*8*), was used to construct plasmids having deletions in the upstream region of the catalase gene. Deletion mutants were constructed by using exonuclease III or site-directed mutagenesis. For exonuclease III deletion, plasmid pCN9 digested with *Cla*I restriction enzyme was treated with exonuclease III and then mung bean nuclease. The exonuclease-treated plasmid was digested with *Bgl*II restriction enzyme and 1.4 kb to 1.0 kb DNA fragments were isolated by agarose gel electrophoresis. Isolated fragments were then ligated to the 4.3 kb *Cla*I-*Bgl*II fragment of pCN9 to construct deletion plasmids. For site-directed mutagenesis, a 3.5 kb *Hin*dIII fragment containing the catalase gene was introduced into the *Hin*dIII site of pKF19 (purchased from Takara Shuzo). Site-directed mutagenesis was carried out by the oligonucleotide-directed dual amber method of Hashimoto-Gotoh *et al.* (*14*), using oligonucleotides TTTCCAAACTAATCGGCCAATGGAATCTCG, TTTCCAAACTAATCGGCGTTGGTAAGAGTT, TTTCCAAACTAATCGACGTTGGTAATTCTA, which correspond to deletions from the *Cla*I site (−672) to −111, −89, and −55 of the catalase gene, respectively. The catalase gene deleted in the upstream region in the plasmid was digested by *Hin*dIII and introduced into CN513. *S. pombe* cells having a deletion in the upstream region of the catalase gene were selected as 5-fluoroorotic acid-resistant cells by *ura4*$^-$ mutation. To confirm the establishment of the expected deletion in the cells, DNA was extracted from the cells by the method of Beach *et al.* (*15*) and amplified by PCR using two oligonucleotides flanking the deletion region, GAAATATACAATGCGCCCAT (corresponding to nucleotides −715 to −696) and TTAGTGTCGAGTCTTTAACT (nucleotides +155 to +136) as primers. The deletion mutants were named as CNd-XX (Fig. 1A), where XX represents the nucleotide number upstream from the transcription start site (+1).

Construction of the spc1$^-$ *and atf1*$^-$ *Mutants*—MN510 is a *spc1*$^-$ mutant of HM123, isolated as a hydrogen peroxide-hypersensitive mutant. MN510 has a mutation which changes the glutamine codon (CAA) for the 8th amino acid of the Spc1 protein to a stop codon (TAA). The mutation point was confirmed by PCR using primers, one of which forms a new *Dra*III restriction site on the PCR product from wild-type DNA, but abolishes the site on the PCR product from MN510 DNA. Details of isolation of the mutant will be described elsewhere (Mutoh *et al.*, manuscript in preparation). The *h*$^+$ strain with *spc1*$^-$ mutation was obtained by genetic crossing between JY746 and MN510. The resultant strain was used to introduce *spc1*$^-$ into strains JY741 and CNd-89 by genetic crossing. The *spc1*$^-$ mutants with the same genetic background as JY741 and CNd-89 were isolated and named CN710 (JY741 with *spc1*$^-$) and CNd-89S (CNd-89 with *spc1*$^-$). The *atf1* gene of *S. pombe* was disrupted by inserting the *ura4* gene between two *Hin*dIII sites of the *atf1* gene cloned on the plasmid pGEM-5Zf(+). The disrupted *atf1* gene was replaced with the chromosomal *atf1* gene of JY741 by homologous recombination to construct CN803 (JY741 with *atf1*$^-$) and with that of CNd-89 to construct CNd-89A (CNd-89 with *atf1*$^-$).

Genetic Methods for S. pombe—Standard genetic methods for *S. pombe* described by Alfa *et al.* (*16*) were used.

Transformation of S. pombe—Transformation of *S. pombe* was carried out by electroporation as described by Ishiguro and Kobayashi (*17*) using a Cell Porator (BRL).

Hybridization Analysis of RNA—Total cellular RNA was extracted from logarithmically growing cells by the method of Elder *et al.* (*18*). RNA was electrophoresed in 0.8% agarose gel containing formaldehyde. The gel was stained with ethidium bromide to confirm that the same amount of RNA was electrophoresed in each lane by visualizing the ribosomal RNA. RNA was transferred to a nitrocellulose membrane and hybridized with a ^{32}P-labeled probe (*19*), which was synthesized by the random primed labeling

TABLE I. ***Schizosaccharomyces pombe*** **strains used.**

Strain	Genotype
JY741	*h*$^-$, *ade6-M216, leu1, ura4-D18*
JY746	*h*$^+$, *ade6-M210, leu1, ura4-D18*
HM123	*h*$^-$, *leu1*
CN513	*h*$^-$, *ade6-M216, leu1, ura4-D18, catalase::ura4*
MN510	*h*$^-$, *leu1, spc1*$^-$
CN710	*h*$^-$, *ade6M216, leu1, ura4-D18, spc1*$^-$
CN803	*h*$^-$, *ade6-M216, leu1, ura4-D18, atf1::ura4*
CNd-89S	*h*$^-$, *ade6-M216, leu1, ura4-D18, spc1*$^-$, *deletion −672 to −89 in catalase*
CNd-89A	*h*$^-$ *ade6-M216, leu1, ura4-D18, atf1::ura4, deletion −672 to −89 in catalase*

method using [α-^{32}P]dCTP (111 TBq/mmol) and a 3.5 kb *Hin*dIII fragment containing the catalase gene.

Assay for Catalase Activity—Cells were grown to mid logarithmic phase and harvested by centrifugation. Cell extract was prepared by vortexing the cell pellet with glass beads as described previously (*8*). Catalase activity was assayed by the method of Beers and Sizer (*20*). One unit of catalase was defined as the activity that decomposes 1 μmol of hydrogen peroxide in 1 min at 25°C. Protein concentration of the cell extract was estimated by the method of Lowry *et al.* (*21*) using bovine serum albumin as a standard.

Gel Electrophoresis DNA Binding Assay—Gel electrophoresis DNA binding assay was performed as described by Watanabe *et al.* (*22*) with slight modifications. Cells grown in 50 ml of SD medium with required nutrients to the mid logarithmic phase were harvested by centrifugation, washed twice with extraction buffer (30 mM Tris-HCl pH 8.0, 30 mM KCl, 0.6 mM EDTA, 12% glycerol), and suspended in an equal volume of extraction buffer. Cells were disrupted by agitating with a vortex mixer in the presence of 4 volumes of glass beads (0.3 mm diameter). Cell debris and glass beads were removed by centrifugation at 25,000$\times\dot{g}$ for 5 min, then the supernatant was centrifuged at 80,000$\times g$ for 60 min at 4°C to remove the particulate fraction. Probes were prepared by end-labeling DNA fragments with polynucleotide kinase and [γ-^{32}P]-ATP (148 TBq/mmol). The 111-90 probe was a 22 bp synthetic double-stranded oligonucleotide encompassing the sequence -111 to -90 of the catalase gene (the sequence of the top strand of the oligonucleotide is GCCAA-TGGAATCTCGGCCATTT). The Atf1 probe was a 20 bp synthetic double-stranded oligonucleotide encompassing the sequence -64 to -45 of the catalase gene that contains the putative Atf1 binding site (the sequence of the top strand of the oligonucleotide is GCAAATATGACGTTG-GTAAT). The labeled probes were purified by gel filtration through a Sephadex G-25 column (1$\times$4 cm) equilibrated with 10 mM Tris-HCl pH 7.6, 1 mM EDTA. Sixty picomoles of end-labeled probe (300,000 cpm) was incubated with cell extract (80 μg protein) for 30 min at 25°C in 25 μl of binding buffer (30 mM Tris-HCl pH 8.0, 30 mM KCl, 0.6 mM EDTA, 0.6 mM DTT, 12% glycerol) in the presence of 0.75 mg/ml of poly(dI-dC)•poly(dI-dC) and 1 mg/ml of bovine serum albumin. For competition experiments, a 150-fold excess of unlabeled DNA was added prior to the binding reaction. The sequence of the top strand of the mutated competitor for the Atf1 binding site was GCAAA-TAGGTGGTAGGTAAT (bases changed from the wild-type sequence are underlined). DNA-protein complexes

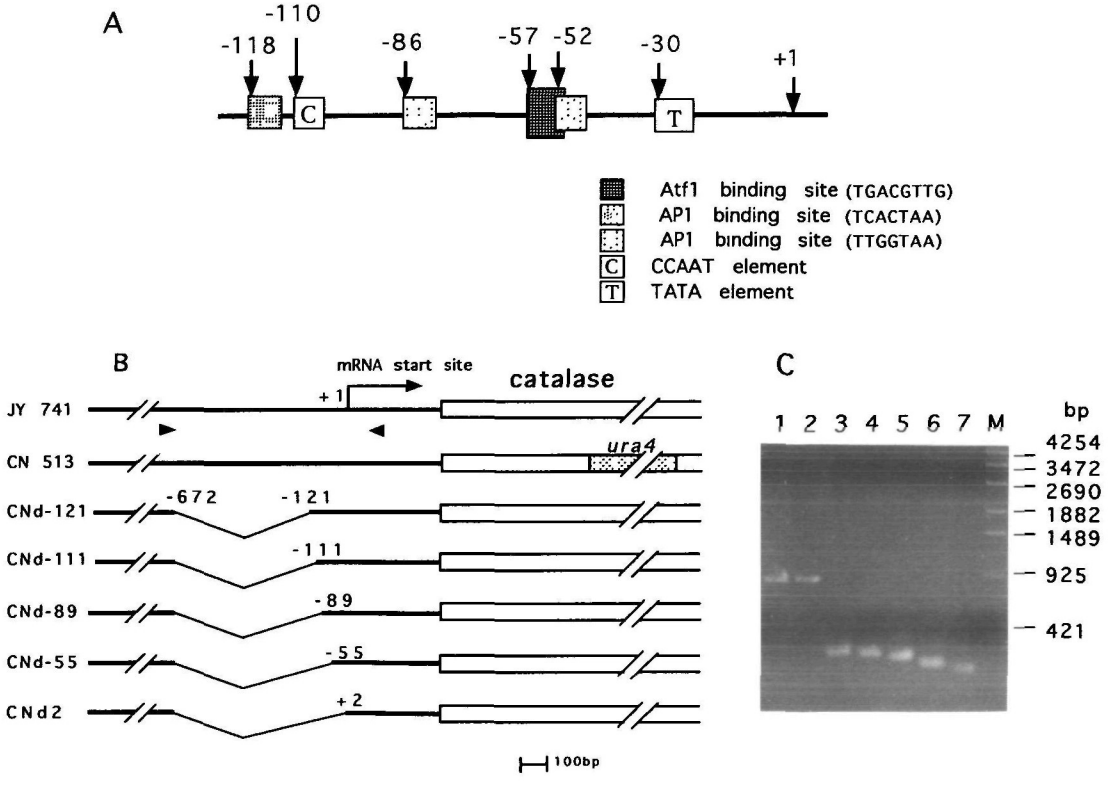

Fig. 1. **The promoter analysis of the catalase gene.** (A) The 5′-flanking region of the catalase gene. Numbers indicate the positions of the extreme nucleotide of each element relative to the transcription initiation site (+1). DNA sequences homologous to the Atf1 binding site and AP1 binding site, a CCAAT sequence and a TATA box are shown. (B) Deletion mutants of catalase promoter region. The catalase gene in wild-type JY741 is described in the upper line. Open squares and solid bars indicate the coding region and the 5′-flanking region of the catalase gene, respectively. Numbers on the solid bars indicate the nucleotide positions relative to the transcription initiation site. The deletion mutants were constructed from CN513, a catalase gene disruptant of JY741. Arrowheads represent the oligonucleotides used as primers for PCR to confirm the deletion. (C) PCR products from the deletion mutants. 1, JY741; 2, CN513; 3, CNd-121; 4, CNd-111; 5, CNd-89; 6, CNd-55; 7, CNd2. DNA size marker (M) is λDNA digested with *Sty*I. Numbers at the right margin indicate size in base pairs.

were separated on a 4% polyacrylamide gel in 1×TGE buffer (50 mM Tris base, 0.4 M glycine, 2.7 mM EDTA) at 20 mA for 90 min. The gel was then transferred to 3MM paper, dried and autoradiographed.

RESULTS

Catalase Expression of Deletion Mutants in Response to Hydrogen Peroxide Stress—Induction of the catalase activity of *S. pombe* under stress conditions is controlled at the transcriptional level (*8*). DNA sequences that have homologies with some transcription factor binding sites were found in the upstream region of the catalase gene (Fig. 1A). To determine the DNA element involved in the expression of the catalase gene, we constructed a series of deletions in the upstream region of the catalase gene as described in "MATERIALS AND METHODS." The mutated genes were introduced into chromosomal DNA of CN513 by homologous recombination. A series of deletion mutants was obtained (Fig. 1B). To confirm the size and site of the deletions in the mutated cells, the regions of the deletion were amplified by PCR (Fig. 1C) and sequenced. The expression of the catalase mRNA of the deletion mutants was assessed by RNA hybridization analysis (Fig. 2A). In CNd-121 and CNd-111, the basal expression and induction by hydrogen peroxide of the catalase mRNA were similar to those of the wild-type JY741. In the more deleted mutant CNd-89, these were decreased but still apparent. Expression of the catalase mRNA in strains CNd-55 and CNd2 was hardly detectable even in the presence of hydrogen peroxide. Figure 2B shows the catalase activity of the deletion mutants under the same conditions used to assess the amount of mRNA. Basal catalase activity of deletion mutants CNd-121 and CNd-111 was the same as that of the wild type, and induction of the catalase activity in CNd-121 and CNd-111 was gradually decreased with deletions, but the activity was more than 2 times the basal level of each strain. Catalase activity of CNd-89 was reduced compared with that of CNd-111, but was induced by hydrogen peroxide. The catalase activity of the deletion mutants CNd-55 and CNd2 was not detectable even after hydrogen peroxide treatment. These results suggested that two elements in the upstream region of the catalase gene are involved in the gene expression. We tentatively named

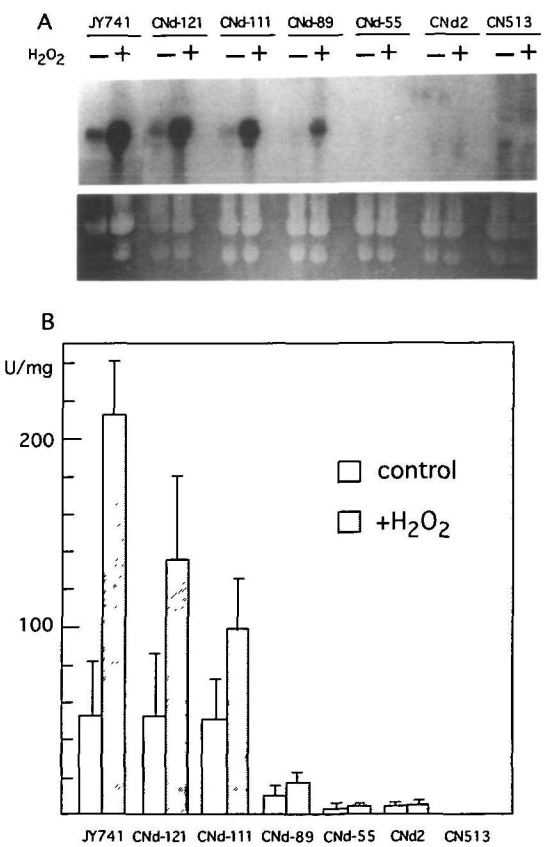

Fig. 2. **RNA hybridization analysis (A) and catalase activity (B) of the deletion mutants.** Cells were grown in SD medium supplemented with required nutrients. For hydrogen peroxide stress, cells were treated with hydrogen peroxide for 30 min (A) or 60 min (B). The lower panel of (A) shows ethidium bromide-stained ribosomal RNA, demonstrating that an equal amount (40 μg) of total cellular RNA was loaded in each lane. Catalase activity of each strain was expressed by open bars (basal activity) and stippled bars (hydrogen peroxide-induced activity). Vertical bars denote 1 SE for at least 3 experiments.

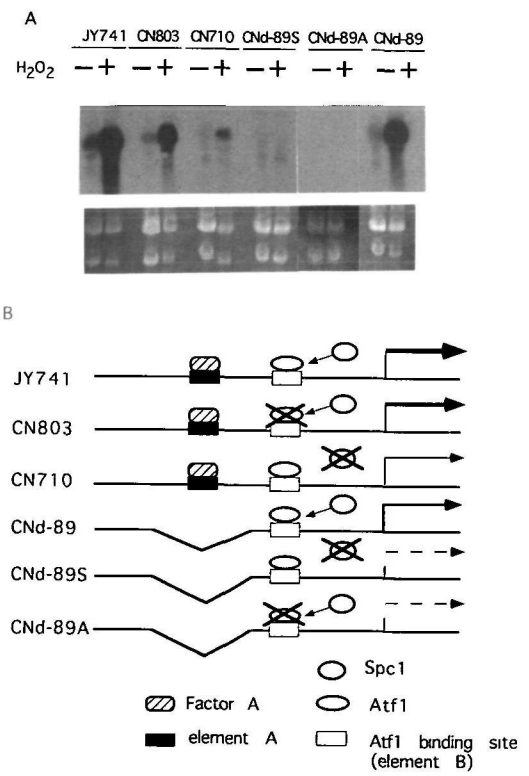

Fig. 3. **(A) RNA hybridization analysis of the catalase mRNA expression in strains JY741 (wild type), CN803 (*atf1*⁻), CN710 (*spc1*⁻), CNd-89 (deletion of nucleotides −672 to −89 in the promoter region), CNd-89S (double mutant with *spc1*⁻ and the same deletion as CNd-89), and CNd-89A (double mutant with *atf1*⁻ and the same deletion as CNd-89).** Cells were grown in SD medium with required nutrients. In the case of hydrogen peroxide treatment, hydrogen peroxide was added 30 min prior to harvest. The lower panel shows ethidium bromide-stained ribosomal RNA, demonstrating that an equal amount (40 μg) of total cellular RNA was loaded in each lane. **(B) Schematic representation of the promoter region of the catalase gene of each strain.** X denotes the inactivation of the protein by a mutation. Size of arrows shows the relative amount of the transcript.

them element A (−111 to −89) and element B (−89 to −55).

Involvement of the Spc1-Atf1 Cascade in the Expression of the Catalase Gene under Oxidative Stress—The Wak1-Wis1-Spc1 MAP kinase pathway is involved in the stress responses of *S. pombe* (*6, 9-11*) and expression of the catalase gene is also under the control of this pathway (*6, 11, 13*). We isolated CN710, which is *spc1*$^-$ and has the same genetic background as JY741, as described in "MATERIALS AND METHODS." RNA hybridization analysis showed that the basal expression of catalase mRNA was reduced in this mutant, but the induction by hydrogen peroxide was still observed (Fig. 3A). Atf1 transcription factor is phosphorylated and activated by Spc1 MAP kinase (*6, 10*). The sequence TGACGTTG, similar to the consensus sequence of the Atf1 binding site (*12*), was found at −57 to −50 of the catalase gene, a position which overlaps with element B. We supposed that the expression of catalase mRNA in CNd-89, in which element A is deleted, is dependent solely on the Spc1-Atf1 cascade. Mutation in the *spc1* or *atf1* gene introduced into CNd-89 was expected to repress the catalase expression completely. We constructed such double mutants, CNd-89S (CNd-89 with *spc1*$^-$) and CNd-89A (CNd-89 with *atf1*$^-$), as described in "MATERIALS AND METHODS." These double mutants showed no expression of the catalase mRNA (Fig. 3A), as expected. These results indicated that the Spc1-Atf1 cascade is involved in the expression of the catalase gene through the region of −89 to −55.

Involvement of Element A in the Oxidative Stress Response in S. pombe—Expression of mRNA in CNd-89 was less than that of CNd-111 (Fig. 2). This suggests that a factor binds element A, region −111 to −89 of the catalase gene, and activates transcription. Since the nucleotide sequence of element A is not homologous to the Atf1 binding site, Atf1 should not be able to bind element A. Expression of the catalase mRNA was assessed in strains JY741 (wild type), CN803 (*atf1*$^-$), CN710 (*spc1*$^-$), CNd-89A (CNd-89 with *atf1*$^-$), and CNd-89S (CNd-89 with *spc1*$^-$) (Fig. 3A). RNA hybridization analysis showed that the *spc1*$^-$ mutant CN710 has reduced catalase mRNA expression, but showed apparent induction of the mRNA on hydrogen peroxide treatment. In the *atf1*$^-$ mutant CN803, the basal expression and induction by hydrogen peroxide of catalase mRNA were similar to those of the wild-type JY741. However, CNd-89S or CNd-89A, which has a deletion in element A in addition to *spc1*$^-$ or *atf1*$^-$ mutations, did not show basal expression or induction by hydrogen peroxide of catalase mRNA. These results revealed that element A plays an important role in both the basal and hydrogen peroxide induced expression of the catalase gene, independently of the Spc1-Atf1 cascade.

Specific Binding of Atf1 Protein in Region −64 to −45 of the Catalase Gene—A DNA sequence homologous to the Atf1 binding site, TGACGTTG, was found in region −57 to −50 of the catalase gene. CNd-55, a mutant partially deleted in this region, completely loses the ability to synthesize the catalase mRNA. To confirm that Atf1 binds this region, we carried out DNA binding assay using cell extract of JY741 (wild type) and CN803 (*atf1*$^-$) (Fig. 4). Specific binding complexes were observed when cell extract of JY741 and ^{32}P-labeled 20 bp double strand probe, which corresponds to −64 to −45 of the catalase gene, were used (Fig. 4, lanes 2-4). They did not disappear when Atf1 binding site-mutated oligonucleotide was used as a competitor (data not shown). The electrophoretic mobility of the DNA binding complexes formed with CN803 extract (Fig.

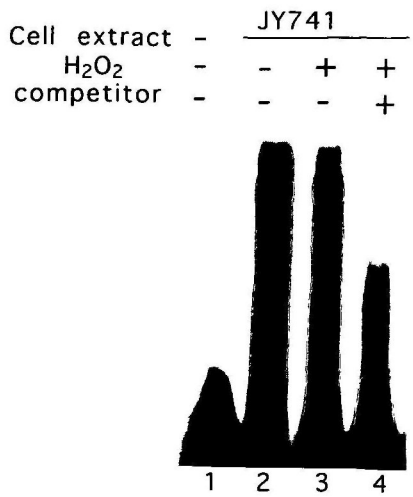

Fig. 4. **Oligonucleotide (corresponding to −64 to −45 of the catalase gene)-binding activity detected in the wild-type cells (JY741) and the *atf1*$^-$ cells (CN803).** Cell extracts were prepared from cells growing logarithmically in SD medium with required nutrients. In the case of hydrogen peroxide treatment, hydrogen peroxide was added 60 min prior to harvest. Cell extracts (80 μg protein) from JY741 (lanes 2, 3, and 4) and CN803 (lanes 5 and 6) treated (lanes 3, 4, and 6) or not treated (lanes 2 and 5) with hydrogen peroxide were incubated under standard reaction conditions with 60 pmol of ^{32}P-labeled 20 bp probe (300,000 cpm) encompassing the Atf1 binding site. A reaction with a 150-fold excess of an unlabeled competitor DNA (lane 4) was carried out. The reaction mixture without cell extract shown in lane 1 served as a control.

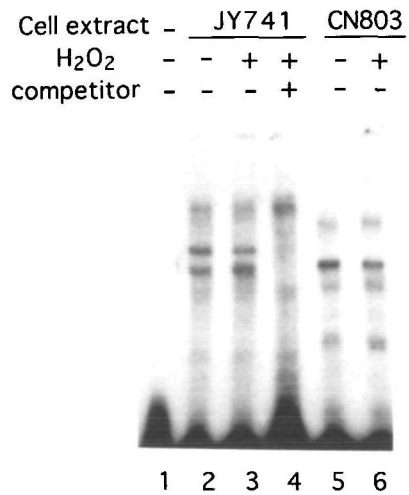

Fig. 5. **Oligonucleotide (corresponding to −111 to −90 of the catalase gene) binding activity detected in the wild-type cells (JY741).** Cells were grown in SD medium with required nutrients. Cell extract (80 μg of protein) prepared from cells not treated (lane 2) or treated (lanes 3 and 4) with hydrogen peroxide were incubated with a standard reaction mixture containing 60 pmol of ^{32}P-labeled 22 bp probe (300,000 cpm). A reaction with a 150-fold excess of an unlabeled competitor DNA (lane 4) was carried out. The reaction mixture without cell extract shown in lane 1 served as a control.

4, lanes 5 and 6) was different from that formed with JY741 extract, though we did not pursue the nature of the DNA binding complexes found in CN803. These results indicate that Atf1 protein binds specifically to this region of the catalase gene. Atf1 binding activity did not change in response to hydrogen peroxide stress (Fig. 4, lanes 2 and 3). This result is similar to that observed with high osmolarity stress (*6*).

DNA Binding Assay Using 22 mer of the Upstream Region of the Catalase Gene—DNA binding assay was performed to examine whether some proteins bind to the DNA sequence of element A. Specific binding complexes were formed when a 22 bp of ^{32}P-labeled oligonucleotide corresponding to element A and JY741 extract were used (Fig. 5).

DISCUSSION

The catalase gene of *S. pombe* is considered to be a typical gene that responds to oxidative stress and has been used as a model in the study of the intracellular signaling mechanism for stresses (*6, 11, 13*). However, the structure of the promotor of the catalase gene, which is the target of the signaling system, has not been investigated. We started the analysis of the promotor structure of the catalase gene by introducing various sized deletions into the 5′-flanking region of the genomic catalase gene. We found that two discrete elements, which we called element A and element B, are involved in the expression of the catalase gene.

A sequence similar to the Atf1 binding site was found at −57 to −50 of the catalase gene, which overlapped with element B. Atf1, which is the target of the Wak1-Wis1-Spc1 MAP kinase pathway (*6, 10*), is involved in the expression of the catalase gene (*6, 11, 13*). Specific binding of Atf1 to this region of DNA was shown by DNA binding assay (Fig. 4). The appearance of multiple bands in the DNA binding assay might indicate that Atf1 forms a homodimer and heterodimers with some other proteins. Analyses of the expression of the catalase gene in mutants CNd-89, CNd-89S, and CNd-89A revealed that the Spc1-Atf1 cascade is involved in the expression of the catalase gene through element B. This is the first report to specify the sequence bound to the Atf1 in *S. pombe*.

The deletion mutant CNd-89, in which element A is deleted, showed decreased expression of the catalase gene compared with the deletion mutant CNd-111 (Fig. 2). This suggested that element A is also involved in the expression of the catalase gene. We assessed the expression of the catalase gene in *spc1*$^-$ strain (CN710) and *atf1*$^-$ strain (CN803) to examine the effects of elimination of the Spc1-Atf1 pathway (Fig. 4). Although the catalase mRNA expression of CN710 (*spc1*$^-$) was very low, induction was observed. The basal expression and induction by hydrogen peroxide of the catalase mRNA in CN803 (*atf1*$^-$) were similar to those in JY741 (wild type). The reduced expression of the catalase mRNA in strain CN710 is probably due to unphosphorylated Atf1 working as a repressor for expression of the catalase gene, as described by Degols and Russell (*13*). Expression of the catalase gene without hydrogen peroxide administration, which we call basal expression, is expression under weak oxidative stress due to reactive oxygen species generated by intracellular metabolism. This could be the reason why CN803, which lacks the repressor for catalase expression, does not show increased expression compared with JY741. On the other hand, the expression of the catalase mRNA in CNd-89S or CNd-89A, in which element A is deleted and Spc1 function or Atf1 function is lost, was not detected in either the presence or absence of hydrogen peroxide. These results indicate that element A is involved in both the basal expression and induction of the catalase gene in response to hydrogen peroxide, independently of the Spc1-Atf1 pathway. Since catalase induction in response to UV irradiation at 254 nm and high osmolarity did not occur in the *atf1* disruptant (*6, 13*), induction of the catalase gene through element A appears to result only in response to oxidative stress. In *Saccharomyces cerevisiae*, the Yap 1 transcription factor, which is the *S. cerevisiae* homolog of mammalian AP1 protein, is activated primarily by oxidative stress (*5, 23*). Although sequences homologous to the AP1 binding site were found near element A, deletion analysis of the catalase promoter region revealed that such sequences are not required for the expression of the catalase gene. Element A contains a CCAAT sequence which is involved in the expression of a mitochondrial function gene in *S. pombe* (*24*). Mutagenesis of CCAAT to CCCAT did not affect the expression of the catalase gene (Nakagawa *et al.*, unpublished observation). DNA protein complexes were formed with element A and the cell extract of JY741 (Fig. 5), though the involvement of the complexes in the transcription activation has not been proved. The nature of the protein bound to element A and the manner in which the protein activates the catalase gene expression remain to be investigated.

The existence of two discrete elements for the expression of the catalase gene implies that two independent activation pathways work to induce the catalase gene in response to hydrogen peroxide in *S. pombe*. One is the Wak1-Wis1-Spc1 MAP kinase pathway that is involved in responses to several stresses, including high osmolarity, hydrogen peroxide and UV irradiation. The other is the signaling pathway that targets (a) protein(s) binding to element A, which probably responds only to oxidative stress. The cytosolic catalase gene (*CTT1*) of *Saccharomyces cerevisiae* is induced by several stresses including osmotic stress and oxidative stress (*2, 25-28*). Induction of the *CTT1* gene is mediated by stress response elements (STREs) (*2, 28*). The SSK2/SSK22-PBS2-HOG1 MAP kinase pathway (*29, 30*), which is a homolog of the Wak1-Wis1-Spc1 MAP kinase pathway, transduces high osmolarity signals to the STREs (*28*). Signals originating from other stresses are not transduced by the SSK2/SSK22-PBS2-HOG1 pathway, though STREs mediate the induction of *CTT1* gene expression by these signals (*2*). Transcriptional activation of the *CTT1* gene through STREs in *S. cerevisiae* is modulated by the Ras-protein kinase A system (*2*) to repress the induction in the logarithmic growth phase. Expression of the catalase gene in *S. pombe* is reduced in the early logarithmic growth phase compared with the late logarithmic growth phase (*31*, Nakagawa *et al.*, unpublished observation). This suggests that the stress-induced transcriptional activity of the catalase gene of *S. pombe* could be modulated by a system analogous to that of *S. cerevisiae*. While *S. pombe* cells respond to stresses by activating the Wak1-Wis1-Spc1 MAP kinase pathway to activate the general defensive mechanisms, modulation systems should work at

several levels of signal transduction to adapt the defensive mechanisms to the specific stress. Induction through element A could be one such modulation system.

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