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AG$_4$ Sequence within *PHR1* Promoter Acts as a Gate for Cross-Talks between Damage-Signaling Pathway and Multi-Stress Response

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Rph1 and Gis1 are damage-responsive repressors involved in *PHR1* expression. They have two C_2H_2 zinc finger motifs as putative DNA binding domains and N-terminal conserved domain with unknown function. They are also found in the human retinoblastoma binding protein 2 and the mouse *jumonji*- encoded protein. The repressors are able to bind to AG$_4$ sequence within a 39-bp sequence called upstream repressing sequence of *PHR1* promoter (*URS$_{PHR1}$*) responsible for the damage-response of *PHR1*. We report here that Rph1 is predominantly localized in the nucleus as examined by fluorescence microscopic analysis with GFP-Rph1 fusion protein. On the basis of the fact that the AG$_4$ sequence that is recognized by Rph1 and Gis1 is also recognized by Msn2 and Msn4 in a process of stress response, we also tried to examine the *in vivo* function of AG$_4$ and the role of Msn2 and Msn4 in *PHR1* expression. Our results demonstrate that Msn2 and Msn4 are actually required for the basal transcription of *PHR1* expression but not for its damage induction. When AG$_4$ sequence was inserted into the minimal promoter of the *cyc1-LacZ* reporter, the increased *LacZ* expression was observed, indicating its involvement in transcriptional activation. The data suggest that the AG$_4$ is primarily required for basal transcriptional activation of *PHR1* or *CYC1* promoter through the possible involvement of Msn2 and Msn4. However, since the AG$_4$ is also involved in the repression of *PHR1* via Rph1 and Gis1, it is proposed that AG$_4$ functions as either URS or upstream activating sequence (UAS) depending on the promoter context.

In the budding yeast *Saccharomyces cerevisiae*, a variety of genes are transcriptionally induced in response to various DNA damaging agents including UV irradiation and methyl methanesulfonate (MMS) (Friedberg et al., 1995). *PHR1* encodes a photolyase that catalyzes the light-dependent repair of pyrimidine dimmers, and transcription of the gene is induced in response to a large number of different DNA-damaging agents (Sebastian et al., 1990). The basal level of transcription of *PHR1* gene is controlled by three promoter elements (Sancar et al., 1995; Sebastian et al., 1990). The damage response is regulated primarily through an upstream repressing sequence, *URS$_{PHR1}$*, which consists of a 39-bp region containing a 22-bp palindrome (Sancar et al., 1995; Sebastian and Sancar Sancar et al., 1995; Sebastian and 1991).

Recent report indicated that Rph1 and Gis1 are damage-responsive repressors of *PHR1* as isolated by one-hybrid screening. Rph1 recognized a single AG$_4$ sequence found in *URS$_{PHR1}$*, and Rph1 binding to this site required two zinc fingers in the carboxyl terminus of the protein. Altering the AG$_4$ sequence in *URS$_{PHR1}$* eliminated Rph1 binding *in vitro* and derepressed *PHR1* expression (Jang et al., 1999). *RPH1* and *GIS1* have demonstrated that the derepression of *PHR1* enhances light-dependent repair of UV-induced DNA damage (Jang et al., 1999).

Regulation of damage-responsive gene expression requires a series of events including damage recognition, signal transduction, and modulation of transcriptional factors as final effector molecules. Mec1/Rad53 pathway is largely responsible for the damage recognition and signal transduction (Friedberg et al., 1995). In *S. cerevisiae*, the DNA damage checkpoint pathway involves damage recognition by *RAD9*, *RAD17*, *RAD24*, *TEL1*, and *MEC3* and activation of downstream protein kinases encoded by *MEC1* and *RAD53* (Friedberg et al., 1995; Weinert, 1998). The recently discovered connection between 'checkpoint' pathways and DNA repairs and their physiological effects on the cell prompted us

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to re-evaluate the roles of checkpoint proteins within the context of the overall DNA damage responses.

A recent study argued for involvement of damage checkpoint pathway in damage-dependent *PHR1* induction. The induction is almost abolished in a *rad53* mutant cell. In contrast, *dun1* mutation has little or no effect on the induction. In the case of the best-known damage-inducible *RNR* genes, the Dun1 protein kinase functions downstream of *MEC1* and *RAD53* to down-regulate the damage-responsive transcriptional repressor Crt1 (Huang et al., 1998; Zhou and Elledge, 1993). These data indicate that *PHR1* induction is controlled by the Dun1-independent pathway for transducing the damage signal to Rph1-Gis1 repressors (Sancar, 2000). In the previous report, we have demonstrated that Rph1 was hyperphosphorylated by the Rad53-dependent checkpoint pathway but not by the Dun1 protein kinase, supporting that the regulation of *PHR1* expression by Rph1 is not Dun1-dependent (Kim et al., 2002).

Rph1 and Gis1 belong to a group of transcriptional regulators targeted by the *MEC1/RAD53* pathway (Jang et al., 1999). Interestingly, recent report revealed that the AG_4 (or C_4T) sequence within *URS*$_{PHR1}$ is recognized by Rph1-Gis1, which is identical to the binding consensus sequences for two transcriptional activators (Msn2 and Msn4) in the multi-stress response. The data suggest that transcriptional repressors of Rph1 and Gis1 might compete with activators of Msn2 and Msn4 for the AG_4 binding sites (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996).

In the present study, we attempted to assess whether Msn2 and Msn4 are involved in the *PHR1* expression, and to elucidate the function of the AG_4 sequence within *URS*$_{PHR1}$ on a heterologous promoter context. In addition, we discuss the possible cross-talk between damage- signaling pathway and multi-stress response.

Materials and Methods

Plasmids and strains

The plasmid pGBS769 is a derivative of pGBS116, which has an additional selective marker of *LEU2* and used for *PHR1-LacZ* expression. pGBS743 is a derivative of pRW95-3 and constructed by insertion of *URA3* fragment (*Sma*I-*Hin*dIII) from pLG669Z into *Xba*I-*Hin*dIII sites within *TRP1* fragment of pRW95-3 (Wolf et al., 1996) to create *URA3* selective marker instead of *TRP1*. Therefore, like pRW95-3, the *cyc1* promoter in pGBS743 has a minimal size without *UAS* sequence. The plasmid pGBS746, pGBS747, pGBS758, and pGBS750 are derivatives of pGBS743, which were constructed by insertions of DNA fragments into *Spe*I-*Eco*RI sites of pGBS743 as described in Table 2. For β-galactosidase assay, derivatives of pGBDS743 were transformed into wild type YPH499 strain (*MATa ade2-101 his3-Δ200 leu2-Δ1 lus2-801 trp1-Δ63 ura3-52*). Yeast strains used in this study are as follows. GBS-1295 (*MATa ade2-101 ura3 leu2 his3 trp1*) is an isogenic wild type strain. GBS1289 (*MATa Δ(msn2::HIS3*), GBS1291 (*MATa Δmsn4::URA3*), and GBS1293 (*MATa Δmsn2::HIS3 Δmsn4::URA3*) are *Δmsn2*, *Δmsn4*, and *Δmsn2-Δmsn4* deletion derivatives of GBS1295, respectively (generous gift from Dr. Kevin McEntee). GBS1738 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) is *Δrph1* and *Δgis1* double mutant.

Construction of green fluorescent protein (GFP) expression vectors

The plasmid expressing GFP-Rph1 fusion protein was constructed by introducing end-filled 2.45-kb *Bam*HI-*Eag*I DNA fragment containing the full ORF of *RPH1* into end-filled *Xho*I site of the plasmid pGFP-N-FUS expressing N-terminal GFP (Niedenthal et al., 1996). The DNA structures of all plasmids were confirmed by restriction mapping and by DNA sequencing. The cells harboring GFP expressing vectors were grown exponentially in minimal media lacking methionine to induce high level of expression from the *MET25* promoter.

Fluorescence microscopy

Cells were fixed with 3.7% formaldehyde for 20 min. The fixed cells were washed three times in phosphate-buffered saline (PBS) and stained with 4,6′-diamidino-2-phenylin doledihydrochloride (DAPI). Fluorescence was observed with Olympus IX70-131 microscope with a 100W light source. Photographs were taken on Kodak Elite Chrome color slide film rated at 100 ASA.

In Vivo expression studies

β-galactosidase assay was performed as described previously (Jang et al., 1999).

Results

Rph1 is localized in the nucleus

RPH1 encodes a highly basic 90-kDa protein containing a classical C_2H_2 zinc finger followed by a C_2HC zinc finger near the carboxyl terminus. Two regions exist near the amino terminus, which show 30 to 40% identities with the human retinoblastoma binding protein 2.

To determine the subcellular localization of Rph1 *in vivo*, GFP-tagged Rph1 fusion protein was constructed by using pGFP-N-FUS vector as shown in Fig. 1.

The *rph1* deletion mutant cells expressing GFP-Rph1 protein were examined on fluorescence microscope. As shown in Fig. 2, the GFP-Rph1 fusion protein was exclusively localized in the nucleus as compared to DAPI stained image, whereas GFP control protein is distributed along the cytoplasm. These data indicate that Rph1 is primarily a nuclear protein.

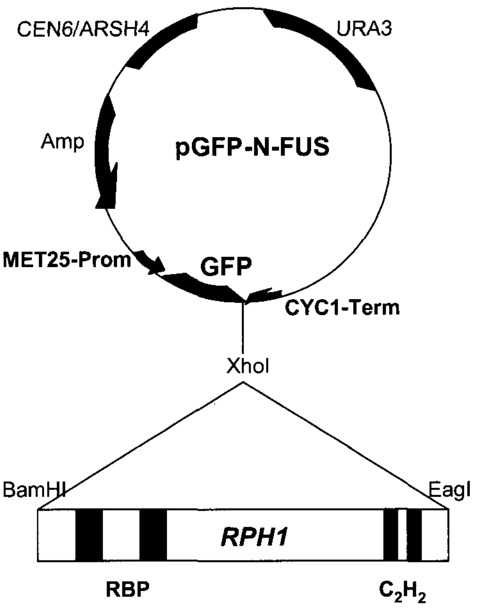


Fig. 1. Construction of GFP-Rph1. For construction of GFP-Rph1 in pGFP-N-FUS vector, the full ORF of *RPH1* gene digested with *Bam*HI-*Eag*I was end-filled and inserted into the end-filled *Xho*I site of the vector. This construct was confirmed by restriction enzyme mapping and DNA sequencing. Transcription of fusion constructs is from the *MET25* promoter and is terminated by the *CYC1* terminator. The construct with *MET25* promoter was transcriptionally induced by omission of methionine from culture media. RBP, retinoblastoma binding protein homology domain; C_2H_2, zinc finger motif.

Effect of Δmsn2 and Δmsn4 deletions on PHR1 expression

Previous studies demonstrated that the damage-responsive repressor of *PHR1*, Rph1, recognizes the AG$_4$ sequence which is identical to the consensus binding sequence of the transcriptional activators of the multistress response, Msn2 and Msn4 (Jang et al., 1999; Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996).

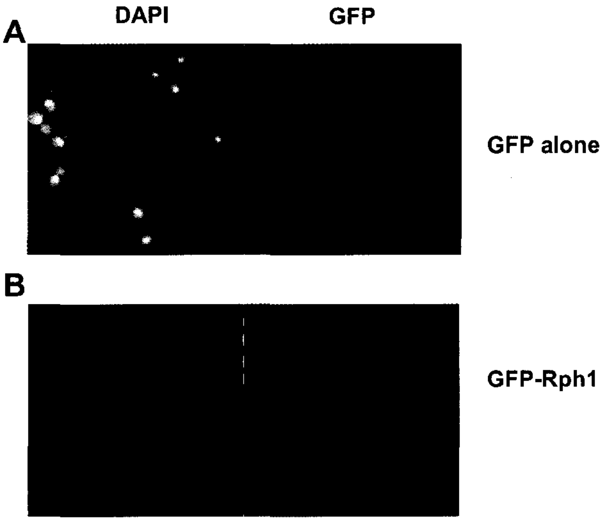


Fig. 2. Rph1 is a nuclear protein. GFP alone (A) and GFP-Rph1 (B) were expressed in *Δrph1* mutants by culturing in minimal media lacking methionine. Exponentially growing cells were fixed with 3.7% formaldehyde and then stained with DAPI. GFP alone (A) and GFP-Rph1 (B) were examined by fluorescence microscopy.

Table 1. Effect of *Δmsn2* and *Δmsn4* deletions on *PHR1* expression

Strain	*PHR1-LacZ* expression		Induction fold
	Control	MMS treatment	
Wild type	3,376	19,538	5.8
Δmsn2	1,834	11,551	6.3
Δmsn4	1,254	6,098	4.9
Δmsn2-Δmsn4	336	1,749	5.2
Δrph1-Δgis1	7,870	22,382	2.8

To investigate the potential involvement of the multistress response pathway in damage response of *PHR1* expression, we examined the effect of *Δmsn2* and *Δmsn4* deletions on *PHR1* expression. Wild type, *Δmsn2*, *Δmsn4*, *Δmsn2-Δmsn4*, and *Δrph1-Δgis1* derivatives were transformed with the pGBS769, which contains 2 µM based *PHR1-LacZ* reporter and *LEU2* marker. The β-galactosidase activity was measured for *PHR1* expression. Unexpectedly, the basal levels of transcription in *Δmsn2*, *Δmsn4* and *Δmsn2-Δmsn4* were significantly decreased relative to the isogenic wild type strain (Table 1). Interestingly, the levels of induction found in the mutants by MMS treatment were similar to that of wild type whereas induction in *Δrph1-Δgis1* was decreased. The data indicated that Msn2 and Msn4 are required for the basal transcription of *PHR1* but not for the damage response of *PHR1*.

The consensus binding sequence of Rph1 protein including AG$_4$ induces the transcriptional activation of a heterologous cyc1-LacZ reporter gene

As previously mentioned (Sancar et al., 1995; Sebastian and Sancar, 1991), the primary regulator of the *PHR1* damage response is a 39-base sequence called *URS*$_{PHR1}$, which is the binding site for a protein(s) that constitutes the damage-responsive repressor Prp. Since the AG$_4$ sequence recognized by Rph1 is included in the Prp-binding sequence *URS*$_{PHR1}$, we expected that the AG$_4$ acts as a repressor binding sequence like *URS*$_{PHR1}$. Therefore, we examined the effect of the AG$_4$ sequence derived from *PHR1* promoter on a heterologous *cyc1-LacZ* expression. Wild type AG$_4$ sequence in Rph1 binding sequence, its CT$_3$G derivative, wild type *URS*$_{PHR1}$ sequence in Prp binding sequence and its CT$_3$G derivative were inserted into the *cyc1* minimal promoter of pRW95-3 which contains the *cyc1* promoter fused to *LacZ* (Wolf et al., 1996), respectively (Table 2).

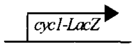
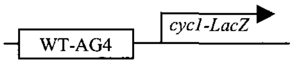
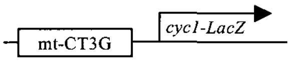
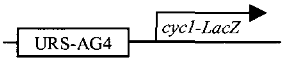
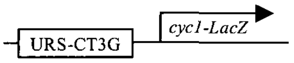
Surprisingly, the data indicate that the wild type AG$_4$ sequence derived from the Rph1 binding sequence caused a significant increase of *lacZ* expression as the AG$_4$ of the Msn2-Msn4 binding sequence did, while no increase was observed in its mutant derivative (compare pGBS743 & pBS746, pGBS747). There was little change observed in wild type *URS*$_{PHR1}$ construct.

Table 2. Oligomer sequences used in construction of plasmid for the promoter activity assay of a heterologous *cyc1-LacZ* system in Table 3

Name	Sequence	Location[a]
Wild type (WT) AG4	AAACCTTAAGGGGTGAAAGTA	-85 → -65
Mutant type (mt) CT3G	AAACCTTACTTTGTGAAAGTA	-85 → -65
URS-AG4	AAACCTTAAGGGGTGAAAGTA TGCTTACTTTGACACTTATTCCTCT	-85 → -40
URS-CT3G	AAACCTTACTTTGTGAAAGTA TGCTTACTTTGACACTTATTCCTCT	-85 → -40

[a] Adenine in first ATG codon indicates +1. Wild-type and mutant sequences of Rph1 binding consensus site were underlined in each construc

Table 3. Effect of Rph1-binding sequence on the promoter activity of a heterologous *cyc1-LacZ* system

Plasmid	*Promoter structure*	*cyc1-LacZ*[a]
pGBS743		127
pGBS746		1,644
pGBS747		188
pGBS758		147
pGBS750		93

[a] Cells were grown to early-log phase in selective medium lacking uracil. Numbers are the mean of determinations with at least independent colonies: standard errors were less than 10% of the mean values.

However, the moderate decrease in *LacZ* expression was observed when the AG$_4$ sequence in *URS*$_{PHR1}$ sequence was mutated to CT$_3$G, indicating that AG$_4$ sequence is involved in basal transcriptional activation (Table 3).

Discussion

In this communication, we report that Rph1 is a nuclear protein and that the AG$_4$ sequence found in the upstream repressor sequence *URS*$_{PHR1}$ acts as an upstream activating sequence on the heterologous promoter context. Unexpectedly, Msn2 and Msn4, the transcriptional activators of the multi-stress response, are involved in the basal transcription of photolyase gene. The data present the potential cross-talk between the multi-stress response and the damage-signaling pathway on *PHR1* gene expression.

In the previous report, we have shown that the sequence recognized by Rph1 is AG$_4$, which is identical to the sequence recognized by Msn2 and Msn4, transcriptional activators involved in stress response (Jang et al., 1999; Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). Therefore, we attempted to answer the following fundamental questions:

1) Are Msn2 and Msn4 involved in damage-response of *PHR1*?; (2) What is the activity of the AG$_4$ contained in the *URS*$_{PHR1}$ on a heterologous promoter context?

As shown in Table 2, Msn2 and Msn4 are actually required for the basal transcription of *PHR1*. However, loss of function in *msn2* and *msn4* did not affect the transcriptional induction of *PHR1* gene in response to DNA damaging agent such as MMS. Change of AG$_4$ sequence to CT$_3$G in the *PHR1* promoter alleviated the induction of *PHR1* by MMS treatment (Jang et al., 1999), indicating that the AG$_4$ is crucial for damage response. Therefore, the data have complicated the role of Msn2-Msn4 and Rph1-Gis1 acting through the AG$_4$ sequence.

Our data in Table 2 suggested that the AG$_4$ sequence within Rph1 binding site acts as an upstream activating sequence based on heterologous promote context. This implies that the dual functions of AG$_4$ as both *UAS* sequence and *cis*-acting factor for damage-induction are dependent on promoter context.

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