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RPH1 and *GIS1* Are Damage-Responsive Repressors of *PHR1*

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The *Saccharomyces cerevisiae* DNA repair gene *PHR1* encodes a photolyase that catalyzes the light-dependent repair of pyrimidine dimers. *PHR1* expression is induced at the level of transcription by a variety of DNA-damaging agents. The primary regulator of the *PHR1* damage response is a 39-bp sequence called URS_{PHR1} which is the binding site for a protein(s) that constitutes the damage-responsive repressor PRP. In this communication, we report the identification of two proteins, Rph1p and Gis1p, that regulate *PHR1* expression through URS_{PHR1}. Both proteins contain two putative zinc fingers that are identical throughout the DNA binding region, and deletion of both *RPH1* and *GIS1* is required to fully derepress *PHR1* in the absence of damage. Derepression of *PHR1* increases the rate and extent of photoreactivation in vivo, demonstrating that the damage response of *PHR1* enhances cellular repair capacity. In vitro footprinting and binding competition studies indicate that the sequence AG_4 (C_4T) within URS_{PHR1} is the binding site for Rph1p and Gis1p and suggests that at least one additional DNA binding component is present in the PRP complex.

In the yeast *Saccharomyces cerevisiae*, more than 20 different genes are induced in response to UV radiation and a variety of chemical agents that damage DNA (1, 16). Induction is the final step in a series of events that includes damage recognition, signal transduction, and modification of transcription factors regulating expression of damage-responsive genes. Damage recognition and/or early steps in signal transduction are carried out by proteins encoded by *RAD9*, *RAD17*, *RAD24*, and *MEC3*, while *MEC1*, *RAD53*, and *DUN1* encode downstream protein kinases that are required for most transcriptional induction (reviewed in reference 48). In contrast to the components of the signaling pathway, little is known about the transcription factors that act as downstream effectors of the pathway.

To date, two transcriptional regulators targeted by the *MEC1*/*RAD53* pathway have been identified: Swi6p and Crt1p (also known as Rfx1p). Swi6p is the regulatory subunit for the G_1-specific transcription factors MBF and SBF. In response to methyl methanesulfonate (MMS)-generated damage, Swi6p is phosphorylated and represses transcription of the cyclin genes *CLN1* and *CLN2*, thereby contributing to delay of G_1 progression (41). Crt1p represses transcription of the *RNR2*, *RNR3*, and *RNR4* genes by binding to X boxes found in the 5′ flanking regions of these genes. Hyperphosphorylation of Crt1p in response to DNA damage or replication stress leads to dissociation of Crt1p from the X boxes and derepression (20). Genes containing X boxes or binding sites for MBF or SBF make up only a small subset of the known damage-inducible genes in yeast. Thus, additional damage-responsive regulators remain to be identified. Of particular interest are regulators of genes encoding DNA repair enzymes.

PHR1 encodes the apoenzyme for the DNA repair enzyme photolyase (31). Transcription of the gene is induced in response to a large number of different DNA-damaging agents, as well as by passage through the diauxic shift (38, 44). Three promoter elements control basal-level and induced expression of *PHR1* (35). An upstream activation sequence, UAS_{PHR1}, is required for both basal-level and induced expression and is the promoter element responsible for induction at the diauxic shift (44). 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 (35, 39). Mutations within the palindrome reduce or abolish repression, as does deletion of the entire 39-bp region, while transfer of URS_{PHR1} into the context of a heterologous promoter both represses expression and confers a low level of damage inducibility (35, 39). Crude extracts from nonirradiated cells contain a protein(s), called PRP, that binds to this region, while extracts from irradiated cells do not (39). Efficient derepression requires a third promoter element called an upstream essential sequence which consists of three related elements (35). In this communication, we describe the isolation and initial characterization of two damage-responsive transcriptional regulators, *RPH1* and *GIS1*, that control *PHR1* expression by binding to URS_{PHR1}.

MATERIALS AND METHODS

Plasmids. Standard recombinant DNA techniques (25) were used to construct the plasmids described here. The structures of all plasmids were confirmed by restriction analysis and in many cases by DNA sequence analysis across crucial regions.

pGBS116 is a 2μm-based *PHR1-lacZ* reporter plasmid described previously (35, 38). pGBS408 is a derivative of pBM1499 (15) in which the *Eco*RI fragment containing UAS_{GAL} was replaced with a 53-bp oligonucleotide containing URS_{PHR1} and several flanking nucleotides (−32 to −83 of the *PHR1* promoter [35]), thus placing *HIS3* expression under the control of URS_{PHR1}. The URS fragment was generated by PCR using oligonucleotides EcoRI-URS_{top} (GAAG CAGTC*G*AA*TT*CAACCTTAAGG) and EcoRI-URS_{bot} (TGTTCTGTGAA*TT* CAATTGTAAAGAGG) as primers and pGBS116 as the template. (Oligonucleotide sequences are given only when they differ from the wild-type sequence, in which case alterations are indicated in italics. Numbering is relative to the first ATG in a given open reading frame [ORF]. A prime indicates a sequence on the noncoding strand.) pGBS116 was also used as the template in a PCR to produce pGBS759 and pGBS723. In pGBS759, the AG_4 sequence in the *RPH1* binding site in pGBS116 was mutated to TC_3G by using oligonucleotides mURS-TC_3 (TCGCTTTTACTGGCGCCACTTTTCTTCCTCGTTTTTCGAGGAAGCAG TCAAATTAAACCTTA*CTTT*GTGAAAGTATGCTTACTT) and $BglII_{bot}$ (*PHR1* 66′→34′). pGBS723 is a derivative of the CEN-ARS *lacZ* reporter plasmid pRW95-3 (49). It was constructed by using primers Bam-URS_{top} (*CGGG*

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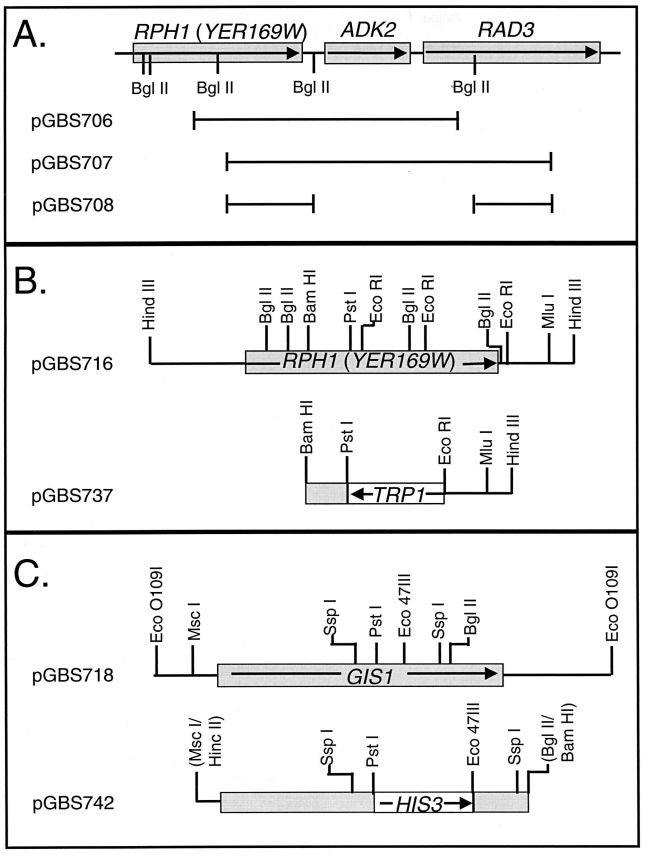


FIG. 1. Restriction maps of yeast chromosomal inserts in selected plasmids used in cloning and disruption of *YER169w* (*RPH1*) and *YDR096w* (*GIS1*). Arrows indicate the direction of transcription of genes indicated by boxes. (A) Sketch of the region of chromosome V carrying *YER169w* and adjacent genes which were included in the $GAL4_{AD}$-*YER169w* fusions that activated the UR-S_{PHR1} reporter constructs. The chromosomal DNA carried by plasmids pGBS706, pGBS707, and pGBS708 are indicated by the black lines beneath the map. (B) Restriction map of the yeast chromosomal DNA fragments carried by pGBS716 and by the derivative plasmid pGBS737 which was used to disrupt *YER169w*. (C) Restriction map of the 4.6-kbp chromosomal DNA fragment carried by pGBS718 and of the gene disruption in plasmid pGBS742. Among the *Ssp*I and *Eco*47III sites in the fragments, only those sites used in subcloning and directed homologous recombination are shown. Restriction sites in parentheses were lost during subcloning.

*ATCC*ACCTTAAGGGGTGAAAGTATGC) and Bam-URS_{bot} (*CGGGATCC*T GTAAAGAGGAATAAGTGTCAA) to generate a 65-bp fragment containing URS_{PHR1} which was inserted into *Bgl*II-digested pRW95-3. pLG669Z contains the *CYC1* promoter fused to *lacZ* and has been described previously (18).

pGBS706 and pGBS707 (Fig. 1A) are plasmids recovered from the yeast genomic library screen described below and contain $GAL4_{AD}$-*RPH1* translational fusions. pGBS708 (Fig. 1A) is a derivative of pGBS707 from which a 2.2-kbp *Bgl*II fragment of yeast genomic DNA was removed. A size-selected yeast genomic DNA library containing *Hin*dIII restriction fragments from strain GBS76 (38) inserted into pBlueScript SK(+) was screened by colony hybridization (3) for clones containing *RPH1*. Plasmid pGBS716 (Fig. 1B) was isolated in this screen and contains the entire *RPH1* ORF and approximately 1,500 bp of 5′ and 3′ flanking sequences. pGBS737 (Fig. 1B) contains *TRP1* flanked by 557 bp of *RPH1* coding sequence and 473 bp of *RPH1* 3′ flanking sequence and was used for targeted disruption of *RPH1*. pGBS712 contains the 3.8-kbp *Hin*dIII fragment from pGBS716 cloned into the *Hin*dIII site of pRS415 (42). PCR amplification using primers 096Eco_{top} (*GIS1* −604→−585) and 096Eco_{bot} (*GIS1* 4078′→4059′) and GBS76 (38) genomic DNA yielded a 4.5-kbp *GIS1*-containing fragment which was cloned into the *Eco*O109I site in pBlueScript SK(+), generating pGBS718 (Fig. 1C). pGBS718ΔCT contains a 2.4-kbp *Msc*I-*Bgl*II fragment from pGBS718 inserted into the *Bam*HI and *Hin*cII sites of pBlueScript SK(+). Subsequently, a 186-bp *Pst*I-*Eco*47III fragment was deleted from this construct and a 975-bp *Eco*47III-*Pst*I fragment containing *HIS3* from pJJ217 (22) was inserted, yielding pGBS742 (Fig. 1C). pDB81 (a kind gift from Hans Ronne) contains the entire *GIS1* gene, including promoter sequences. A *GIS1*-containing *Mlu*I-*Sac*I fragment from pDB81 was inserted into unique *Sma*I and *Sac*I sites in pRS415, yielding pGBS207.

Plasmids expressing glutathione *S*-transferase (GST)-Rph1p fusion proteins were constructed in pGEX18 (30). pGBS727 contains a 0.9-kbp *Eco*RI-*Bgl*II fragment from *RPH1* (Fig. 1B) subcloned into pBlueScript SK(+). pGBS731, which expresses the C-terminal third of Rph1p fused to GST (Rph1p-CT), was constructed by inserting a 0.9-kbp *Eco*RI-*Xba*I fragment from pGBS727 into pGEX18. pGST169w contains the entire *RPH1* ORF fused to GST. The plasmid was constructed in two steps. The first 340 bp of the coding sequence of *RPH1* were amplified in a PCR using *PFU* polymerase, primers GBT169-Bam CG*GG ATCC*CGATGACGAAACTAATC) and GBT169-BglII (GA*AGATCT*TCCGG AGGCACATAGTCC), and pGBS716 as the template. After digestion with *Bam*HI and *Bgl*II, the PCR product was subcloned into *Bam*HI-digested pGBS716. The resulting plasmid, pGBS733, contains *RPH1* flanked by a unique *Bam*HI site 6 nucleotides 5′ to the first ATG and a *Sal*I site immediately 3′ to the yeast genomic insert. In the second step, this *Bam*HI-*Sal*I fragment was ligated to pGEX18 digested with the same enzymes.

pGBS763 carries a portion of *RAD2* and was constructed by insertion of a 1.9-kbp *Eco*O109I-*Sac*I fragment from pNF2005 (28) into pBlueScript SK(+). A 2.0-kbp fragment containing the *LEU2* gene from pJJ283 (22), flanked by a filled-in *Hin*dIII site and a *Bam*HI site, was ligated into *Bgl*II-*Eco*RV-digested pGBS763, yielding the *RAD2* knockout plasmid pGBS764.

Strains. The parental *S. cerevisiae* strains used in this study are listed in Table 1 and were constructed and propagated by using standard techniques. RE1006 was transformed with *Pvu*II-digested pGBS408, thereby targeting insertion of the URS_{PHR1}-*HIS3* reporter gene to *LYS2*. Ura^+ transformants were subsequently subjected to selection on 5-fluoroorotic acid, and stable Ura^- derivatives were tested by Southern analysis to confirm integration of the reporter at *LYS2* and loss of *URA3*. The resulting strain, GBS157, was transformed with the *lacZ* reporter plasmid pGBS723, generating GBS1659. Strain GBS1391 carries a marked disruption of *RPH1* and was constructed by transforming YPH499 with a 1.8-kbp *Bam*HI-*Mlu*I fragment from pGBS737 (Fig. 1B). Replacement of *RPH1* was confirmed by PCR of DNA from Trp^+ transformants using primer KO169-5′ (*RPH1* 174→193) in combination with KOTRP-5′ (*TRP1* 305′→285′) or KO169-out (*RPH1* 2966′→2948′). A marked disruption of *GIS1* was constructed by transforming YPH500 with a 1.5-kbp *Ssp*I fragment from pGBS742 (Fig. 1C), yielding strain GBS1396. Gene replacement was verified in His^+ transformants by PCR using primers KO096-5′ (*RPH1* 1033→1050) and KO096-3′ (*RPH1* 2234′→2215′) or KOHIS-5′ (*HIS3* 611→628). GBS1406 is a diploid strain obtained by mating GBS1391 and GBS1396. Strains GBS1734, GBS1736, and GBS1738 are haploid meiotic segregants of GBS1406. Strains GBS1867, GBS1869, GBS1872, and GBS1875 contain marked deletions of *rad2* and were constructed by transforming YPH499, GBS1734, GBS1736, and GBS1738, respectively, with a 3.5-kbp *Eco*O109I-*Sac*I fragment from pGBS764, selecting for Leu^+ transformants. Replacement of *rad2* was confirmed by PCR using primers KO-rad2-5′ (*RAD2* 5→14) and KO-rad2-3′ (*RAD2* 1889′→1872′). All other strains are derivatives of these and were constructed by transformation with various plasmids as indicated in the figure legends.

Library screening. The $GAL4_{AD}$ fusion yeast genomic library constructed by Paetkau and coworkers (29) was screened for genes encoding proteins that bind to URS_{PHR1}. This library consists of high-copy-number *LEU2* plasmids carrying the Gal4 transcriptional activation domain fused to random yeast genomic DNA fragments. We used three libraries covering all three possible reading frames to transform GBS1659 and screened Leu^+ His^+ transformants for increased β-galactosidase activity by using a nonlethal colony assay (13). Plasmids from positive clones were recovered in *Escherichia coli* DH5α and used to transform naive GBS1659 to confirm the Leu^+ His^+ phenotype and increased β-galactosidase production.

Expression and purification of GST fusion proteins. *E. coli* BL21 was used for the expression of GST fusion proteins. Cells were grown in Luria broth to an A_{595} of 0.5, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and growth was continued for 2 h at 27°C. Cells were lysed, and the proteins were purified by glutathione affinity chromatography as described by the manufacturer (Pharmacia). Both the fusion protein containing only the Rph1p C-terminal region (Rph1p-CT) and the fusion protein containing full-length Rph1p (Rph1) were proteolyzed to a significant extent. Based upon the intensity of bands in sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue, we estimate that approximately 30% of the protein in the Rph1p-CT preparations was of the expected length while approximately 10% of the protein from the Rph1p preparation was full length.

EMSAs and footprinting. Radiolabeled substrate was prepared by hybridization of oligonucleotides URS_{top} (*PHR1* −85→−40) and URS_{bot} (*PHR1* −40′→−85′) followed by end filling using Klenow fragment and [α-^{32}P]dATP using conditions previously described (44). Unlabeled competitors were prepared by hybridization of oligonucleotide pairs AG_4TG (*PHR1* −85→−65 and *PHR1* −65′→−85′) or various derivatives (see Fig. 6). The buffer for Rph1 binding assays contained 4 mM Tris HCl (pH 8.0), 4 mM $MgCl_2$, 40 mM NaCl, 10 μM $ZnCl_2$, 10% glycerol, bovine serum albumin at 100 μg/ml, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin at 10 μg/ml, soybean trypsin inhibitor at 10 μg/ml, and leupeptin at 4 μg/ml. Rph1p or Rph1p-CT was

TABLE 1. Parental *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
RE1006	*MAT***a** *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52*	M. Johnston
GBS157	*MAT***a** *can1-100 his3-11,15 lys2::*URS_{PHR1}*-HIS3 leu2-3,112 trp1-1 ura3-52*	This work
GBS1391	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *rph1Δ::TRP1 trp1-Δ63 ura3-52*	This work
GBS1396	*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *gis1Δ::HIS3 trp1-Δ63 ura3-52*	This work
GBS1406	*MAT***a**/*MATα ade2-101*/*ade2-101 his3-Δ200*/*his3-Δ200 leu2-Δ1*/*leu2-Δ1 lys2-801*^{amber}/*lys2-801*^{amber} *rph1Δ::TRP1*/*RPH1 gis1Δ::HIS3*/*GIS1 trp1-Δ63*/*trp1-Δ63 ura3-52*/*ura3-52*	This work
GBS1734	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *rph1Δ::TRP1 trp1-Δ63 ura3-52*	This work
GBS1736	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *gis1Δ::HIS3 trp1-Δ63 ura3-52*	This work
GBS1738	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *rph1Δ::TRP1 gis1Δ::HIS3 trp1-Δ63 ura3-52*	This work
GBS1867	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *rad2Δ::LEU2 trpl-Δ63 ura3-52*	This work
GBS1869	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *rad2Δ::LEU2 rphΔ1::TrP1 trp1-Δ63 ura3-52*	This work
GBS1873	*MAT***a** *ade2-101 gis1Δ::HIS3 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *rad2Δ::LEU2 trp1-Δ63 ura3-52*	This work
GBS1875	*MAT***a** *ade2-101 gis1Δ::HIS3 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *rad2Δ::LEU2 rphΔ1:: TRP1 trp1-Δ63 ura3-52*	This work
YPH499	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *trp1-Δ63 ura3-52*	42
YPH500	*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801*^{amber} *trp1-Δ63 ura3-52*	42

incubated on ice for 20 min with the various oligonucleotides at the concentrations indicated in the figure legends. Free and bound DNAs were separated by electrophoresis through 6% polyacrylamide gels in 1× Tris-borate-EDTA and quantitated by PhosphorImager analysis or an Ambis Radioanalytic System as previously described (44).

^{32}P-labeled substrates for footprinting were prepared by using kinase-treated oligonucleotide UES_{top} (*PHR1* −155→−134) or PHR_{10} (*PHR1* 10′→−10′) as the primer in a PCR (25) in which pGBS116 was the template. Copper phenanthroline (OP-Cu) footprinting was performed as previously described (39). For DNase I footprinting, 2 ng of probe was incubated with various concentrations of Rph1p-CT or Rph1p at concentrations sufficient to produce 60 to 80% bound substrate as judged by electrophoretic mobility shift assay (EMSA). The binding buffer used was the same as that described above, except that 0.5 μg of poly(dA-dT) was included. Following a 20-min incubation on ice, 1 U of DNase I (Promega) and 1 μl of 50 mM $CaCl_2$ were added, the reaction was allowed to proceed at room temperature for 45 s to 2 min, and then 20 μl of stop solution (1% sodium dodecyl sulfate, 200 mM NaCl, 20 mM EDTA, 40 μg of tRNA per ml) was added. The products were purified by phenol extraction and ethanol precipitation and displayed on 8% polyacrylamide–7 M urea gels (39).

In vivo expression and UV survival studies. β-galactosidase assays were performed as previously described (44). Cells were grown in liquid YPAD or synthetic complete medium lacking appropriate components to maintain plasmid selection (40), and 1-ml samples were harvested at an A_{600} of 0.1 to 0.5. The damage response was assessed by using MMS (2.3 mM final concentration) or UV irradiation. MMS was added to cultures at an A_{600} of 0.1 to 0.2, and cells were incubated at 30°C for 3 h prior to harvesting. To correct for variations in reporter plasmid copy number, DNA was extracted from control cultures (2), digested with *Eco*RI, and subjected to Southern analysis (25). Probe for plasmid-borne *lacZ* was synthesized in a PCR using pGBS116 as the template and oligonucleotides lac-top (*lacZ* 571→592) and lac-bot (*lacZ* 2700′→2681′). Probe for the single-copy chromosomal gene *ACT1* was obtained by PCR of YPH499 genomic DNA using the primers act-top (*ACT1* 405→428) and act-bot (*ACT1* 1414′→1393′). Probes were labeled with either [^{32}P]dATP (random primer method [25]) or horseradish peroxidase (ECL; Amersham life Science). Band intensity was determined by using a Molecular Dynamics Storm 860 PhosphorImager and ImagQuant software.

For UV survival and photoreactivation experiments, cultures were harvested in early log phase (A_{600} of <0.3), washed with and suspended in phosphate-buffered saline, and irradiated at 254 nm as previously described (36). Following irradiation, aliquots of cells were transferred to culture tubes on a tissue culture roller drum placed 9 in. from a bank of two 15-W Cool White fluorescent lamps. Cells were sampled at various times, diluted, and plated on YPAD, and surviving colonies were counted after 3 days of growth at 30°C in the dark.

RESULTS

Identification of *YER169w* and *GIS1* as putative regulators acting through URS_{PHR1}. We utilized the one-hybrid method to identify putative PRP-encoding genes. URS_{PHR1} was inserted into the promoter regions of two reporter genes, *HIS3* and *lacZ*, in the reporter strain GBS1659. Because both reporter genes are devoid of upstream activation sequences, GBS1659 is a histidine auxotroph and produces extremely low levels of β-galactosidase regardless of whether URS_{PHR1} is present. In principle, expression of a gene encoding the DNA binding domain of PRP fused to the transcriptional activation domain of *GAL4* should confer high-level expression of the reporter genes. We transformed GBS1659 with a series of *GAL4* fusion yeast genomic libraries carried on the 2μm *LEU2* plasmids pDP4, pDP7, and pDP12 (29). Approximately two million Leu^+ transformants from each library were tested for histidine prototrophy, and a total of 85 His^+ Trp^+ Leu^+ clones were obtained. In a secondary screening for increased β-galactosidase activity using a colony color assay (13), four of these clones (URS39, URS48, URS67, and URS72) consistently produced dark blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) indicator plates. Plasmids carrying the *GAL4* fusion genes were rescued from these clones, and the DNA was sequenced at the 5′ and 3′ fusion sites. Each plasmid carried *GAL4* fused in frame to sequences from the carboxy-terminal half of yeast ORF *YER169w* (17), followed by an intact copy of *ADK2* and variable amino-terminal portions of *RAD3* (Fig. 1). Plasmids from transformants URS48 and URS67 were identical to one another and were designated pGBS706; similarly, plasmids from URS39 and URS72 were identical and were designated pGBS707. To confirm that the Gal4-Yer169w fusion protein was responsible for enhanced expression from the reporter genes, a 2.2-kbp *Bgl*II fragment containing the entire *ADK2* gene and the *RAD3* promoter and translational start site was removed from pGBS707. The resulting plasmid (pGBS708, Fig. 1) conferred histidine prototrophy and high-level β-galactosidase expression on naive GBS1659, whereas the vector alone had no effect on expression (data not shown).

YER169w is a 2,388-bp ORF with an unknown function that was identified in the course of the *S. cerevisiae* genome sequencing project (9). It encodes a highly basic 90-kDa protein containing, near the carboxy terminus, a classical C_2H_2 zinc finger followed by a C_2HC zinc finger (6) (Fig. 2). Deletion of the zinc fingers abolishes transcriptional activation by the Gal4-Yer169w fusion protein in vivo (data not shown), suggesting that the Zn fingers are required for binding to URS_{PHR1}. ORF *YER169w* has been renamed *RPH1* (regulator of PHR1). Comparison of the predicted amino acid sequence of *RPH1* to all other yeast ORFs revealed striking homology to the protein encoded by *GIS1* (9). *GIS1* has been previously isolated as an overexpression suppressor of *gig1-2* (5), a mutation in the *SRB8* gene encoding a subunit of the cyclin C-

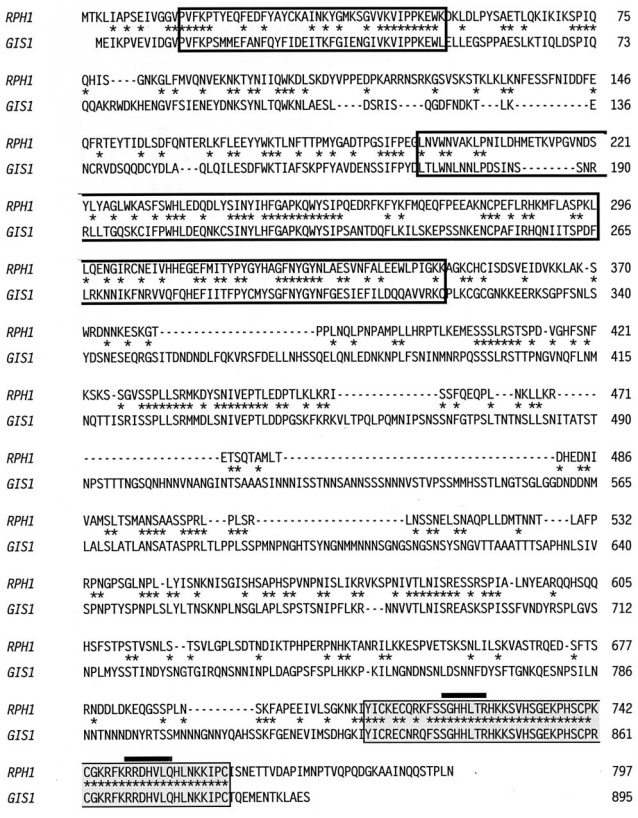


FIG. 2. Alignment of the Rph1p and Gis1p proteins. The predicted amino acid sequences of the proteins were aligned by using the program WU-BLAST 2.0 (30a). Open boxes indicate the regions of homology to RBP2, while filled boxes indicate the region containing the two zinc finger motifs (6). The amino acids within the zinc fingers thought to be involved in DNA binding are overlined. Asterisks indicate identical amino acids.

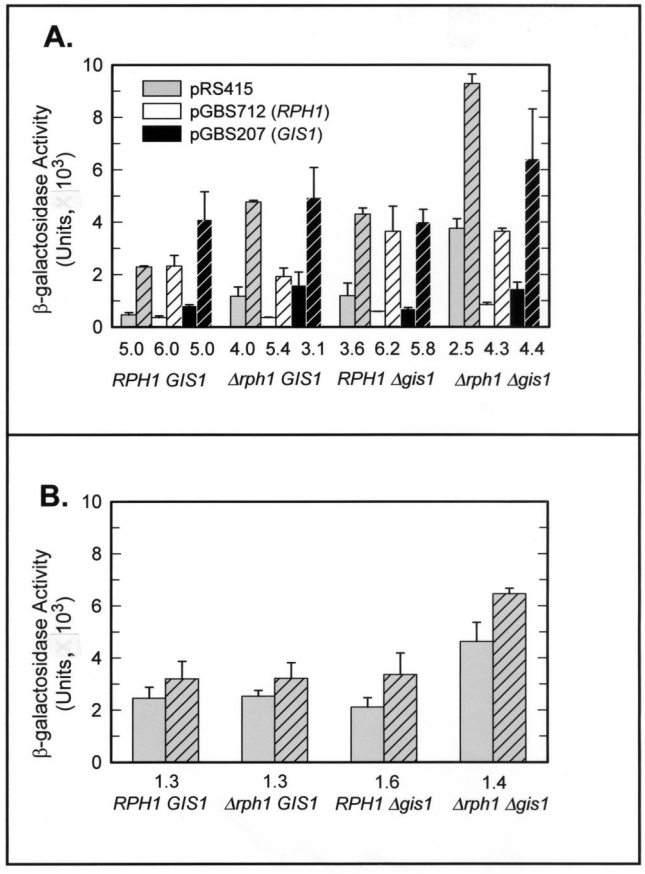


FIG. 3. Effects of deletion of *RPH1* and *GIS1* on basal-level expression and damage induction of *PHR1*. Strains YPH499, GBS1734, GBS1736, and GBS1738 were transformed with a *PHR1-lacZ* reporter plasmid and with pRS415, pGBS712 (*RPH1*), or pGBS207 (*GIS1*), and the effect on expression was assessed with (cross-hatched bars) or without (open bars) MMS treatment. The chromosomal genotypes are indicated below the ordinate, and the induction ratio following MMS treatment is indicated immediately above the chromosomal genotype. Error bars show the standard deviations from three or four independent determinations. (A) Effects on expression from a reporter plasmid (pGBS116) that contains the intact *PHR1* promoter. (B) Effect on expression of a pGBS116 derivative (pGBS759) in which the AG_4 sequence has been mutated.

dependent protein kinase complex (4). As is shown in Fig. 2, the two proteins are 92.7% identical over the 55-amino-acid region comprising the zinc fingers of the two proteins, 100% identical in the regions of the zinc fingers thought to interact with DNA, and 34.7% identical overall. In addition to the zinc finger region, scattered regions of homology are found throughout the molecules. Two particularly interesting regions near the amino terminus also show 30 to 40% identity with human retinoblastoma binding protein 2 (14), human cDNA XE169 (50), the mouse *jumonji*-encoded protein (45), and the product of ZK593.4, a gene with an unknown function identified during the *Caenorhabditis elegans* genome sequencing project (8). While the function of this region is not known, its conservation across phylogenetic lines suggests it is an important structural or functional motif.

***RPH1* and *GIS1* are required for repression of *PHR1*.** We constructed targeted disruptions of *RPH1* and *GIS1* to assess the effect of loss of function on cell growth and viability and on *PHR1* expression. Disruption of either *RPH1* or *GIS1* in haploid strains of either mating type had no discernible effect on the viability of log-phase cells grown in YPAD at 30°C (data not shown), indicating that neither *RPH1* nor *GIS1* is an essential gene under these conditions. This was confirmed by tetrad analysis of sporulated GBS1406, a diploid strain in which a single copy of each gene was disrupted; all four expected classes of segregants were recovered, and there was no consistent difference in viability on YPAD of any segregant class (data not shown). The effect of *RPH1* and *GIS1* disruption on *PHR1* expression was assessed by using pGBS116, which contains the intact *PHR1* promoter, including URS_{PHR1}, fused to *lacZ*. As can be seen in Fig. 3A, strains containing a disruption of either *RPH1* or *GIS1* displayed a modest increase in basal-level expression, as well as a decrease in the induction ratio (defined as the ratio of damage-induced expression to basal-level expression), following treatment with the DNA-damaging agent MMS. Simultaneous disruption of both *RPH1* and *GIS1* had a synergistic effect, producing a sixfold increase in basal-level expression and a 50% decrease in the induction ratio. Both the increase in basal-level expression and the decrease in the induction ratio upon deletion of either or both genes are consistent with the encoded proteins acting as damage-responsive negative regulators of *PHR1*. The synergistic effect observed when both genes are disrupted suggests that the proteins are redundant with respect to *PHR1* repression. It is somewhat surprising, then, that while multiple copies of *RPH1* complement a deletion of *GIS1*, multiple copies of *GIS1* do not complement an *RPH1* deletion (Fig. 3). It is unlikely that this reflects a unique requirement for *RPH1* in *PHR1*

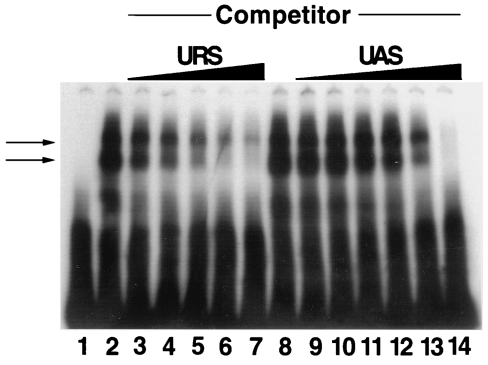


FIG. 4. EMSA testing the affinity and binding specificity of Rph1p-CT for URS_{PHR1}. ^{32}P-labeled URS oligonucleotide (20 nM), either without (lane 1) or incubated with Rphp-CT (100 nM; lanes 2 to 14), was electrophoresed as described in Materials and Methods. In lanes 3 to 7 and 9 to 14, the indicated unlabeled competitor oligonucleotide was present during the incubation. Competitor concentrations (lanes): 3 and 9, 200 nM; 4 and 10, 400 nM; 5 and 11, 1 μM; 6 and 12, 2 μM; 7 and 13, 4 μM; 14, 8 μM. Arrows indicate the major Rph1p-URS complexes which appear as a doublet. We believe this is due to partial proteolysis of Rph1p (see Materials and Methods).

expression or *GIS1* function, since *GIS1* alone partially restores repression in a Δ*rph1* Δ*gis1* mutant (Fig. 3A). At present, we believe that the failure of multiple copies of *GIS1* to complement an *RPH1* deletion may be due to differences in the expression levels of the two genes or in the strength of repression conferred by the two proteins. *RPH1* mRNA is approximately threefold more abundant in undamaged *S. cerevisiae* cells than is *GIS1* mRNA (19). In these experiments, extra copies of *RPH1* and *GIS1* are expressed from their own promoters and are carried on centromeric plasmids that average one to two copies per haploid genome (46). Thus, in all likelihood, *GIS1* was overexpressed only two- to threefold, a level that is apparently insufficient to fully repress *PHR1*.

Rph1p binds to URS_{PHR1} in vitro. While the simplest interpretation of the in vivo data is that *RPH1* and *GIS1* encode DNA-binding proteins that recognize sequences within URS_{PHR1}, secondary or indirect effects cannot be ruled out by these studies. Therefore, we expressed the protein encoded by *RPH1* in *E. coli* and tested whether the purified protein binds specifically and with high affinity to URS_{PHR1}. EMSAs shown in Fig. 4 demonstrate that this is indeed the case. Rph1p bound to an oligonucleotide containing URS_{PHR1} (Fig. 4, lanes 2 and 8). Sequence-specific binding was confirmed by competition studies in which a homologous oligonucleotide competed much more efficiently for binding of Rph1p than did a heterologous oligonucleotide (Fig. 4). Thus far, excessive proteolysis and insolubility have made it impossible to perform similar binding experiments with purified Gis1p.

DNase I footprinting was used to determine the region within URS_{PHR1} that is bound by Rph1p. The 39-bp region footprinted by PRP contains a 22-bp palindrome, as well as flanking sequences (39). Surprisingly, Rph1p protected only the 5′ portion of the URS from attack by DNase I (Fig. 5). It should be noted that full-length Rph1p and Rph1p-CT, which contains only the C-terminal one-third of Rph1p, including the zinc fingers, yielded identical DNase I footprints (Fig. 5A), thereby validating the use of Rph1p-CT for DNA binding and footprinting experiments. DNase I overestimates the region of DNA in intimate contact with binding proteins, and therefore a more accurate estimation of the DNA binding site was obtained by using OP-Cu as a footprinting agent. Rph1p protected an 8-bp region, TAAGGGGT, from attack on the top strand and a 10-bp region, CCCCTTAAGG, on the bottom strand (Fig. 5B). The protected region partially overlaps the 39-bp region protected by partially purified PRP (39). A likely explanation for the smaller footprint compared to PRP is that the latter is composed of proteins in addition to Rph1p and/or Gis1p. This is supported by previous work demonstrating that changing the four central GC base pairs within the URS_{PHR1} palindrome to AT base pairs abolishes repression of *PHR1* in vivo (35). However, currently we cannot rule out effects of proteolysis on the extent of the footprint (see Materials and Methods). That the Rph1p footprint extends outside of the previously footprinted region may be due to the relatively weak OP-Cu cleavage at the boundary regions or may reflect conformational differences between Rph1p in isolation versus Rph1p in a multisubunit complex.

Delineation of Rph1p binding specificity. To further define the binding specificity of Rph1p, we compared the ability of oligonucleotides containing mutations within URS_{PHR1} to compete with the wild-type sequence for binding of Rph1p in vitro. As can be seen in Fig. 6, oligonucleotides containing either a deletion or a point mutation outside of the AG_4 sequence were still able to compete effectively for binding of Rph1p (oligonucleotides AG_4TG, URS406, and AG_4TA). In contrast, oligonucleotides containing mutations within the AG_4 sequence reduced competition to undetectable levels (oligonucleotides CT_3GTG, CT_5G, AG_2AGTG, TG_4TG, and $AGAG_2TG$). The one exception to this pattern was the oligonucleotide AC_4TG, in which the AG_4 sequence was switched to the bottom strand while retaining the same polarity. We conclude that the AG_4 sequence is both necessary and sufficient for binding by Rph1p in vitro.

To determine whether AG_4 is the sequence through which Rph1p and Gis1p act in vivo, we constructed pGBS759, which contains a *PHR1-lacZ* fusion in which the AG_4 sequence in URS_{PHR1} was mutated to TC_3G, and assayed expression of the reporter gene in various genetic backgrounds (Fig. 3B). This mutation reduced induction in response to MMS by 70 to 75% in strains with intact *RPH1* and *GIS1* genes and rendered expression of the reporter gene almost completely insensitive to loss of either or both genes. Together, these data strongly argue that Rph1p and Gis1p regulate the damage response of *PHR1* by binding to the AG_4 sequence in URS_{PHR1}.

Derepression of PHR1 enhances UV survival. To determine whether derepression of *PHR1* results in enhanced repair capacity, we tested the survival of wild-type, Δrph1, Δ*gis1*, and Δ*rph1* Δ*gis1* strains following UV irradiation, with or without subsequent photoreactivation. Strains bearing deletions of *rad2* were used because the effect of photoreactivation on survival is often difficult to see in cells with an intact nucleotide excision repair pathway. As can be seen in Fig. 7, deletion of *rph1*, *gis1*, or both genes enhanced both the rate and extent of light-dependent repair and the relative enhancement of survival mirrored the enhanced *PHR1* expression seen in these strains. It should be noted that under these experimental conditions, both the rate and extent of the light-dependent increase in survival are decreased by the presence of 6-4 photoproducts which are lethal lesions that are not repaired by the Phr1 photolyase (7, 32). Thus, the survival data underestimate the extent of *PHR1* derepression.

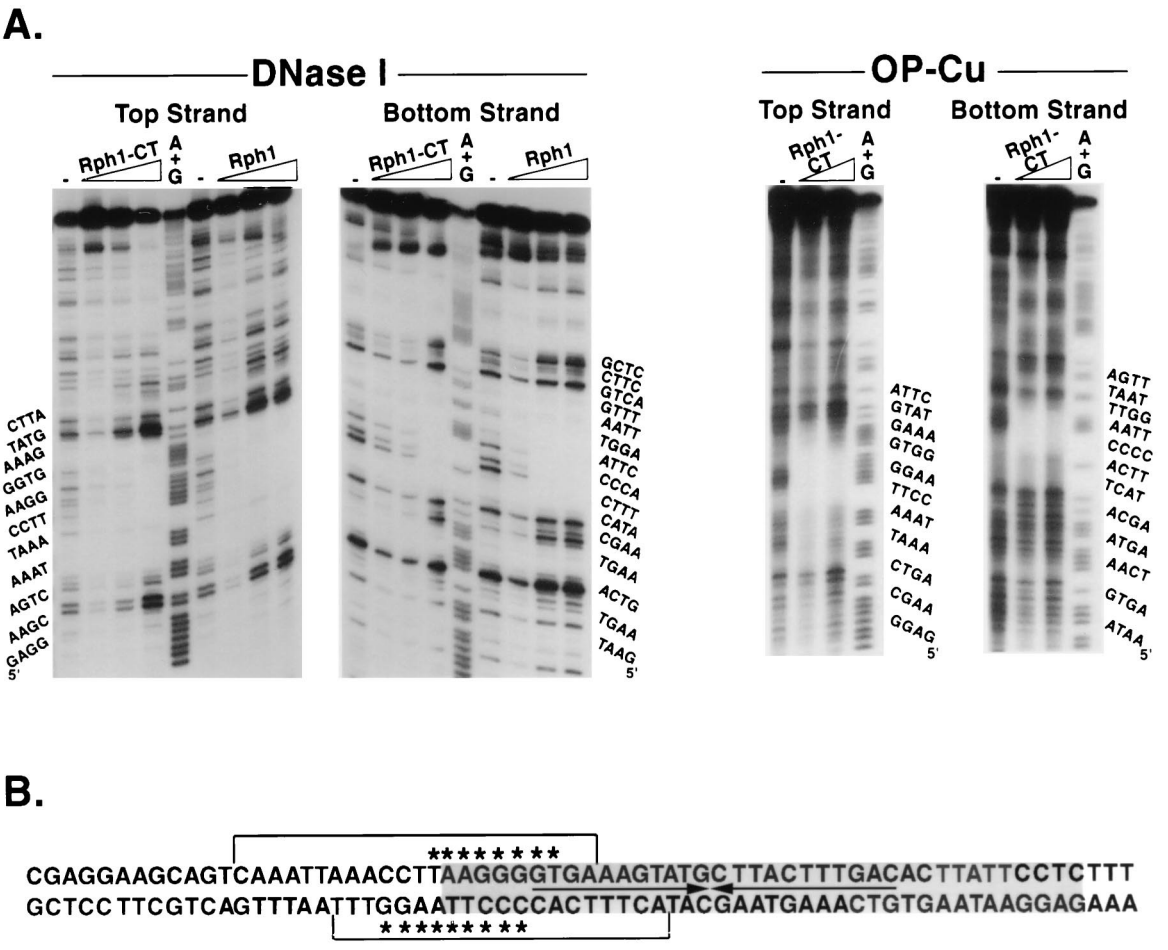
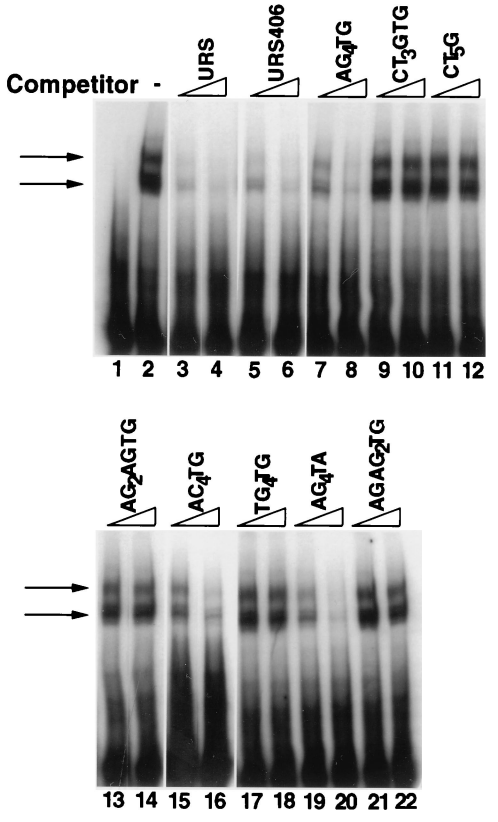


FIG. 5. Footprinting of Rph1p on the *PHR1* transcriptional regulatory region. Oligonucleotides containing the *PHR1* transcriptional regulatory region and labeled at the 5′ end on either the top or bottom strand were exposed to DNase I or OP-Cu in the absence or presence of increasing concentrations of Rph1p-CT or Rph1p as described in Materials and Methods. (A) Autoradiograms of the partial digestion products separated on denaturing acrylamide gels. The sequence of the oligonucleotide in the region of the footprint is shown to the left of each autoradiogram. Lanes: −, no protein added; A + G, products of a Maxam-Gilbert reaction which cleaves at A's and G's. (B) Sequence within and surrounding the region footprinted by Prp (gray area) and the region protected by Rph1p and Rph1p-CT from attack by DNase I (brackets above and below the sequence) and by OP-Cu (asterisks above and below the sequence).

DISCUSSION

In this work, we have identified the proteins encoded by *RPH1* and *GIS1* as DNA damage-responsive repressors of *PHR1* transcription and have demonstrated that derepression of *PHR1* enhances light-dependent repair of UV-induced DNA damage. Rph1p recognizes a single AG_4 sequence found in previously defined URS_{PHR1}, and Rph1p binding to this site requires the two zinc fingers near the carboxy terminus of the protein. The key residues for sequence-specific binding by zinc fingers are at positions −1, 2, 3, and 6 relative to the beginning of the finger helix (reviewed in reference 21). These residues, and indeed all amino acids in the helical domain of the fingers, are identical in Rph1p and Gis1p, strongly suggesting that these two proteins recognize identical sequences. Additionally, altering the AG_4 sequence in URS_{PHR1} eliminates Rph1p binding in vitro, derepresses *PHR1* expression, and almost entirely eliminates the effects of deletion of *RPH1* and *GIS1* in vivo. Together with the observation that both *RPH1* and *GIS1* must be deleted to fully derepress *PHR1* expression, the data indicate that *RPH1* and *GIS1* are functionally redundant with respect to *PHR1* repression.

Several pairs of transcription factors that recognize identical sequences have been identified in yeast; however, functional redundancy of the type seen for *RPH1* and *GIS1* is unusual. The repressors Mig1p and Mig2p regulate *SUC2* expression, but unlike Rph1p and Gis1p, Mig1p alone is sufficient to confer complete repression and Mig2p activity is only seen in strains lacking Mig1p (24). Ace2p and Swi5p activate the *CTS1* and HO promoters, respectively, and can substitute for one another only when present in high copy number or in specific genetic backgrounds (11, 12). Perhaps the closest parallel to the functional redundancy of *RPH1* and *GIS1* is the situation observed with Msn2p and Msn4p, two activators of the multistress response in yeast that bind to the STRE (stress response element) (26, 37). While deletion of *MSN2* reduces expression from an STRE-driven reporter gene by 80% (37), deletion of both genes is required to observe the full repertoire of phenotypes associated with loss of the multistress response (17, 26). A further similarity among Msn2p, Msn4p, Rph1p, and Gis1p is that each of these proteins binds specifically to the sequence AG_4 (26, 37, and this work). Preliminary results indicate that deletion of *RPH1* and *GIS1* derepresses basal-level expression from an STRE-driven reporter gene (33). This suggests either that there is cross talk between the multistress response and the *RPH1*/*GIS1* DNA damage response pathway or that dele-



URS	AAACCTTAAGGGGTGAAAGTATGCTTACTTTGAC
URS406	AAACCTTAAGGGGTGAAATTATAATTATTTTGAC
AG_4TG	AAACCTTAAGGGGTGAAAGTA
CT_3GTG	AAACCTTACTTTGTGAAAGTA
CT_5G	AAACCTTACTTTTTGAAAGTA
AG_2AGTG	AAACCTTAAGGAGTGAAAGTA
AC_4TG	AAACCTTAACCCCTGAAAGTA
TG_4TG	AAACCTTATGGGGTGAAAGTA
AG_4TA	AAACCTTAAGGGGTAAAAGTA
$AGAG_2TG$	AAACCTTAAGAGGTGAAAGTA

FIG. 6. Binding competition assays to determine the sequences required for Rph1p binding. Radiolabeled URS oligonucleotide was incubated with Rph1p-CT as described in the legend to Fig. 4, either in the absence or in the presence of the indicated competing unlabeled double-stranded oligonucleotides, and the bound and free portions of the substrate were separated by electrophoresis and autoradiographed. Two concentrations are shown for each competitor, 1 μM (lanes 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21) and 4 μM (lanes 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22). Lane 1 contained the substrate only, and lane 2 contained the substrate and Rph1p-CT without a competitor. Arrows indicate the bound substrate. The sequences of the competitors are shown below the autoradiograms. Sites changed relative to the wild-type sequence are indicated by dots above the changed bases. The 12 bp 3′ to URS_{PHR1} are not shown for oligonucleotides URS and URS406; however, they are identical.

tion of *RPH1* and *GIS1* produces a signal that activates the stress response pathway.

An important question that remains to be addressed is whether *RPH1* and *GIS1* regulate DNA damage-responsive genes in addition to *PHR1*. The AG_4 sequence recognized by these proteins occurs much too often in the yeast genome for a search based simply on this sequence to be meaningful.

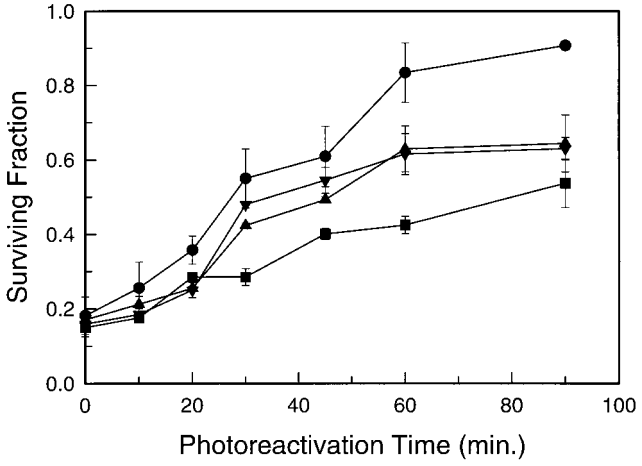


FIG. 7. Effect of derepression of *PHR1* on the UV survival of Δ*rad2* strains. Log-phase cells were exposed to 4.5 J of 254-nm radiation per m^2 and then to photoreactivating light as described in Materials and Methods. Samples were taken at the indicated times and plated for survival determination. The data points are averages from three independent experiments, and the error bars indicate the standard deviation. Symbols: ■, GBS1867 (*RPH1 GIS1*); ▲, GBS1869 (Δ*rph1 GIS1*); ▼, GBS1873 (*RPH1* Δ*gis1*); ●, GBS1875 (Δ*rph1* Δ*gis1*).

Nevertheless, it is probably significant that one or more AG_4 sequences are found within 500 bp of the translational start site of half of the 28 known damage-inducible DNA repair and metabolism genes of yeast (*PHR1*, *RAD5*, *RAD6*, *RAD7*, *RAD16*, *RAD27*, *RAD51*, *RAD54*, *DUN1*, *REV3*, *RFX1* [*CRT1*], *RNR2*, *RNR3*, and *RNR4* [1, 16, 20, 23, 27, 43, 47]), while less than 20% of noninducible repair genes contain this sequence. Since most of these damage-responsive genes are not induced by heat shock, it is unlikely that the AG_4 sequence is targeted by Msn2p and Msn4p in these promoters. The availability of *MSN2*, *MSN4*, *RPH1*, and *GIS1* deletion mutants makes it possible to test directly whether *RPH1* and *GIS1* control a damage response regulon and whether *MSN2* and *MSN4* contribute to this response.

Repression by *RPH1* and *GIS1* differs in at least two respects from that mediated by *CRT1*, a homolog of the mammalian RFX family of DNA binding proteins and the only other characterized regulator of damage-inducible DNA repair genes in yeast (20). Despite the fact that the canonical RFX-X box contains the AG_4 sequence recognized by Rph1p and Gis1p, none of the Crt1p binding sites thus far identified contain the AG_4 sequence. In addition, repression by Crt1p requires the corepressors Ssn6p and Tup1p. In contrast, repression by *RPH1* and *GIS1* is *TUP1* -independent (10). Another striking difference is that derepression of *CRT1*-regulated genes requires both the *RAD53* and *DUN1* protein kinases (20) while derepression of *PHR1* requires *RAD53* but not *DUN1* (34). Thus, it appears not only that there are multiple damage-responsive transcriptional regulators but also that the signal transduction pathway differs to some extent, depending upon the target. This conclusion is consistent with studies by Kiser and Weinert (23) that suggested that at least four transcriptional pathways are activated by the damage response in yeast.

URS_{PHR1} was originally identified by OP-Cu footprinting as a 39-bp region that is bound by a protein or proteins present in partially purified extracts from nonirradiated cells and absent from extracts from UV-irradiated cells (39). The binding site for Rph1p identified in the current studies lies at the extreme 5′ end of URS_{PHR1} and includes only 2 bp of a 22-bp palin-

drome which we have previously shown to be required for repression of *PHR1* (35). Taken together, these results indicate that an additional protein(s) is bound to URS_{PHR1} in vivo. This may explain the residual damage response of *PHR1* when both *RPH1* and *GIS1* are deleted (Fig. 3). In addition, the fact that mutations in the palindrome abolish repression (35) indicates that the protein functions synergistically with Rph1p and Gis1p to repress transcription of *PHR1*. Experiments are in progress to identify additional components of the repressor complex and to determine the mechanisms that govern loss of DNA binding in response to damage.

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