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GSH1, Which Encodes γ-Glutamylcysteine Synthetase, Is a Target Gene for yAP-1 Transcriptional Regulation

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Changes in gene dosage of the *YAP1* gene, encoding the yAP-1 transcriptional regulatory protein, cause profound alterations in cellular drug and metal resistance. Previous studies on yAP-1 action in yeast cells have used the AP-1 response element (ARE) from simian virus 40 as an artificial site for yAP-1-mediated transcriptional activation. No authentic yeast target sites for control of gene expression by yAP-1 are known. Here we show that the *GSH1* gene, encoding γ-glutamylcysteine synthetase, is transcriptionally responsive to the yAP-1 protein. *GSH1* encodes the rate-limiting step in yeast glutathione biosynthesis and contains within its promoter region a DNA element that matches the ARE in 11 of 12 positions. The *GSH1* yAP-1 response element (YRE) was recognized by yAP-1 protein in vitro. Northern (RNA) blot analysis showed that *GSH1* mRNA levels were responsive to *YAP1* gene dosage. A site-directed mutation in the YRE that blocked yAP-1 binding in vitro prevented the mutant *GSH1* promoter from responding to elevation in *YAP1* gene dosage. A Δ*gsh1* mutant strain was constructed and unable to grow in the absence of exogenous glutathione. A mutant *GSH1* gene lacking the YRE was unable to confer normal cadmium tolerance, although other yAP-1-mediated phenotypes remained normal. Thus, *GSH1* is one of several genes that are transcriptionally controlled by yAP-1 and influence drug resistance.

The *Saccharomyces cerevisiae* AP-1 protein (yAP-1) was originally identified as a biochemical homolog of mammalian AP-1 (7, 13). While this biochemical similarity allowed the isolation of the *YAP1* gene, the function of the yeast protein was initially difficult to assess. Work from a variety of laboratories has since shown that high-copy-number plasmids carrying the *YAP1* gene provide dramatic increases in resistance to drugs such as sulfometuron methyl, cycloheximide, 1,10-phenanthroline, 4-nitroquinoline, and cadmium (3, 8, 16, 27, 35). Strains lacking a functional *YAP1* allele are hypersensitive to oxidative stress (27), 4-nitroquinoline (8), and cadmium (35). Clearly, changes in the copy number of the *YAP1* gene have pronounced effects on the ability of yeast cells to tolerate drug challenges.

While a wide variety of phenotypes that are associated with yAP-1 have been found, none of these phenotypes are thought to be a consequence of the direct action of this factor. The phenotypic influence of yAP-1 on cells is believed to be mediated via this protein acting as a transcriptional regulatory molecule. We have found that mutations in the *YAP1* gene that inactivate the ability of the gene product to serve as a positive regulator of transcription also block the normal appearance of drug resistance mediated by yAP-1 (34). Thus, the effects of yAP-1 on drug resistance are likely to be mediated by the products of yAP-1-regulated genes. The large number of phenotypes produced by changes in *YAP1* gene dosage suggests that several different genes are under of yAP-1 control. However, no yAP-1 target gene has yet been identified.

In the work described here, we find that the *GSH1* gene, encoding γ-glutamylcysteine synthetase, is under yAP-1 regulation. In *S. cerevisiae*, the *GSH1* gene product catalyzes the first and rate-limiting step in the biosynthesis of glutathione (20). The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is present in animals, plants, and microorganisms and plays an important role in protection against cellular damage caused by carcinogens, xenbiotics, radiation, or oxidative agents (18). Yeast *GSH1* contains a DNA element in its 5′ noncoding sequence matching the simian virus 40 (SV40) AP-1 response element (ARE) in 11 of 12 positions. We have previously shown that the ARE is an effective site for yAP-1 transcriptional control in yeast cells (7). The *GSH1* DNA segment, designated a yAP-1 response element (YRE), can be bound by yAP-1 in vitro and is required for yAP-1 modulation of *GSH1* gene expression in vivo. Transcriptional activation of *GSH1* mediated by yAP-1 is essential for normal cadmium tolerance in *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains and methods. Genotypes of the yeast strains used in this study are listed in Table 1. PB1 was constructed by transformation of SEY6210 with pPB5. YAW10 was constructed by transformation of PB1 with *Eco*RI-cleaved pSM27. YAW11 was formed by transformation of SEY6210 with *Bam*HI-*Eco*RI-cut pAW20 in the presence of glutathione. Yeast transformations were carried out by the lithium acetate technique (12), and β-galactosidase assays were performed as described previously (6). YPD medium consists of 2% yeast extract, 1% peptone, and 2% glucose. Yeast cells were routinely grown in SD medium (29) containing appropriate supplements for RNA preparation, β-galactosidase assays, or glutathione level determination. Crude extracts were prepared as described previously (28) for use in the glutathione level assay. Glutathione levels were measured by the technique of Tietze (31). Glutathione levels were measured three times on two independent extracts from each strain and had an error of <5%.

PCR amplification of *GSH1* gene fragments. Yeast genomic DNA from SEY6210, prepared by the method of Hoffman and

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TABLE 1. Genotypes of yeast strains used in this study

Designation	Genotype	Reference
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9, Mel⁻*	35
SM10	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ yap1-Δ1::HIS3*	35
PB1	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ cad1-Δ1::hisG*	This study
YAW10	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ yap1-Δ1::HIS3 cad1-Δ1::hisG*	This study
YAW11	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ gsh1-Δ1::hisG*	This study
EE7	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ gcn4-Δ1::hisG*	This study
EE8	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ gcn4-Δ1::hisG yap1-Δ1::HIS3*	This study

Winston (11), was used as the template for PCR amplification. Two different PCR products were made to obtain different segments of the *GSH1* gene (21). A 900-bp fragment was produced by using an upstream primer (CCCGAATTCGC TATCCTGTACCATACTAATTATCT) corresponding to positions −280 to −261 (numbers are relative to the transcription start site) and a downstream primer (ATTCCACGGATCCT TAATGTT) corresponding to +657 to +677. The resulting product was cleaved with *Eco*RI and *Bam*HI, cloned into pBluescript SK−, and designated pAW11. This 900-bp segment of the *GSH1* gene contains 172 codons of the coding sequence and was used as a probe for Northern (RNA) blots. A 500-bp fragment, which was used for construction of the *GSH1-lacZ* gene fusion, was produced by using the upstream primer described above and a downstream primer (GCCG GATCCTTAATGTTAATAAAGTCGGGGCATCC) that introduces a *Bam*HI site directly following the ATG codon for *GSH1*. This 500-bp product was also cleaved with *Eco*RI and *Bam*HI, cloned into pUC19, and named pAW13. The 900-bp fragment was transferred into pUC19 as an *Eco*RI-*Sal*I fragment, and the resulting recombinant was designated pUC19-*GSH1*.

Plasmids. pSEY18-R2.5 and YEp351-YAP1 are 2μm vectors containing the wild-type *YAP1* gene cloned into pSEY18 or YEp351, respectively (35). Plasmid rescue was performed with pAW21, which contained the 900-bp PCR fragment of *GSH1* cloned as *Eco*RI-*Sal*I in pRS306 (30). Plasmid pAW22 contains the complete *GSH1* gene recovered by plasmid rescue. A 507-bp *Bgl*II fragment at the 3′ end of *GSH1* in pAW22 was replaced with a *Kpn*I linker to form pAW23. A *Kpn*I-*Xho*I fragment containing *GSH1* gene sequences from +54 to +2582 was cloned into both the 2μm vector pRS426 and centromeric vector pRS316 to form pAW25 and pAW24, respectively. Then a fragment containing the wild-type *GSH1* promoter (obtained as a blunt-ended *Eco*RI-to-*Xho*I fragment) was inserted into pAW24 or pAW25, both of which had been cleaved with *Not*I, Klenow repaired, and then digested with *Xho*I. Thus, pAW26 is a low-copy-number plasmid containing the wild-type *GSH1* gene, while pAW27 is a 2μm plasmid containing the same *GSH1* segment. The point mutants that were constructed in the *GSH1* promoter were placed upstream of the *GSH1* structural gene in the same fashion as described above for the wild-type promoter.

The wild-type *GSH1-lacZ* fusion plasmid was constructed by transferring an *Eco*RI-*Bam*HI fragment from pAW13 into *Eco*RI-*Bam*HI-cleaved pSEYC102 (4) to produce pAW14. The *lacZ* fusion plasmids containing the mutant *GSH1* promoters were shifted into pAW14 as *Eco*RI-*Hin*dIII fragments. Substitution mutations were constructed in the *GSH1* promoter by using a PCR-based strategy (25). Primers used to alter the YRE (AACGACGGCTGCCATTctagAGCATGGC GCGCACGT) or the general control recognition elements (GCRE) (AGCATGGCGCGCACGTcgacACAACTGTGGC TGGAA) incorporated a new *Xba*I site (YRE) or *Sal*I site (GCRE) to facilitate identification of the mutant promoters. In each oligonucleotide, the residues altered by each mutant and the locations of the new restriction sites are indicated by lowercase letters and underlining, respectively.

A plasmid containing a *CYC1-lacZ* gene fusion lacking the normal *CYC1* upstream activation sequence (UAS) elements was used to analyze the UAS function of the YRE. This plasmid, pLGΔBS, was cleaved with *Bgl*II and treated with alkaline phosphatase, and oligonucleotides corresponding to the wild-type or mutant YRE were cloned upstream of the *CYC1-lacZ* fusion gene. The sequences of the oligonucleotides were GAT CTG CCA TTA GTC AGC ATG GCG for the wild-type YRE and GAT CTG CCA TTc tag AGC ATG GCG for the mutant YRE. The YRE is underlined, and the altered positions in the mutant site are indicated by lowercase letters. Appropriate recombinants were identified by restriction mapping, and the sequence of the oligonucleotide was confirmed by DNA sequencing.

Plasmid rescue of the *GSH1* gene. YAW9 was obtained by transformation of SEY6210 with *Hin*dIII-cut pAW21. Integration of pAW21 into the *GSH1* locus was confirmed by Southern blotting. Isolated yeast genomic DNA was cut by *Sph*I, an enzyme that cuts outside the *GSH1* structural gene, religated, and transformed into *Escherichia coli* DH5α. Ampicillin-resistant transformants were analyzed by restriction mapping, and a clone containing the *GSH1* locus was designated pAW22.

Gene disruption mutations. Gene disruptions were constructed by the one-step disruption method of Rothstein (24). A *GSH1* gene disruption plasmid was constructed by deleting the DNA present in pUC19-*GSH1* between positions +54 and +510 and then replacing this segment with a *Bgl*II linker. A 4.3-kb *Bgl*II-*Bam*HI fragment containing the *hisG-URA3-hisG* (1) construct was then inserted into the *Bgl*II site. The subsequent chimera was designated pAW20 and was digested with *Eco*RI and *Bam*HI prior to transformation into SEY6210. The *CAD1* disruption plasmid was generated by replacing *CAD1* DNA from +10 to +692 (35) with the *hisG-URA3-hisG* fragment. The resulting chimera was named pPB5. This plasmid was cleaved with *Eco*RI and *Sph*I before transformation into SEY6210. The *GCN4* disruption plasmid was constructed by replacing *GCN4* coding sequence DNA (10) between the internal *Xba*I and *Kpn*I sites with the *hisG-URA3-hisG* fragment. The resulting recombinant was designated pEAE3 and was cleaved with *Sal*I prior to transformation. Construction details for pPB5 and pEAE3 are available on request. Appropriate recombinants were identified by Southern blotting, and the *URA3* gene was cured by treatment with 5-fluoro-orotic acid as described previously (2). The *cad1-Δ1::hisG* and *gsh1-Δ1::hisG* strains were designated PB1 and YAW11, respectively. The *cad1-Δ1::hisG* and *yap1-Δ1::HIS3* double mutant strain (YAW10) was constructed by disruption of the *YAP1* gene in PB1 background, using pSM27 (35).

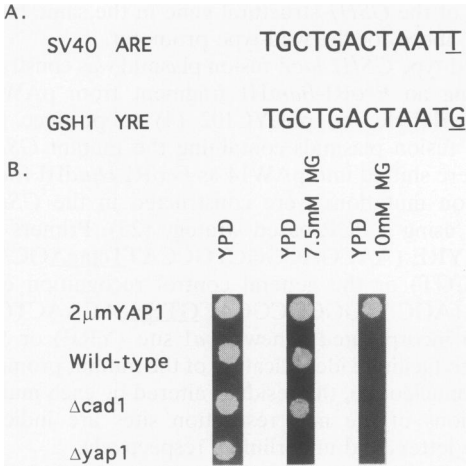

FIG. 1. Evidence for yAP-1 involvement in *GSH1* gene expression. (A) Presence of a potential YRE in the *GSH1* promoter region. The SV40 ARE is shown as defined by MPE (methidiumpropyl-EDTA) protection experiments (7). Residues in the ARE are numbered 1 to 12 from left to right. A sequence motif matching the ARE in 11 of the 12 residues was found in the 5′ noncoding DNA of the *GSH1* gene from positions −234 to −223. The sequence of the antisense strand of the *GSH1* gene is shown. (B) Methylglyoxal (MG) resistance is responsive to *YAP1* gene dosage. Yeast strains with the indicated relevant genotypes were tested for the ability to grow on the various media shown by spot test assay (35).

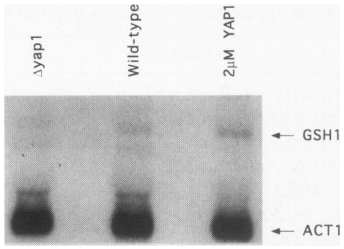

FIG. 2. Northern blot assay of *GSH1* mRNA levels. Total RNA was prepared from yeast strains of the indicated genotypes grown in SD medium with appropriate supplements (29). Twenty micrograms of each RNA was electrophoresed through a formaldehyde-agarose gel and then transferred to a nylon membrane (Nytran; Schleicher & Schuell). The resulting blot was probed with ^{32}P-labeled DNA probes specific for *GSH1* and *ACT1*. The *GSH1* probe was the 900-bp PCR fragment; the *ACT1* probe corresponded to a cDNA clone of the mature transcript. The location of each transcript was determined by comparison with the migration of the 28S and 18S rRNA subunits as well as the known sizes of *ACT1* (19) and *GSH1* (21) mRNAs.

RESULTS

Initial evidence that *GSH1* is a target gene for yAP-1. In other organisms, glutathione and glutathione-derived peptides are known to be important in detoxification of cadmium (18, 22). Since Δ*yap1* mutants are hypersensitive to cadmium (35), we searched the promoter region of *GSH1* (21), a gene involved in glutathione biosynthesis, for potential yAP-1 binding sites. Inspection of the sequence indicated that the promoter region of *GSH1* has an element matching the SV40 ARE in 11 of 12 positions that is located approximately 380 bp upstream of the ATG for the *GSH1* open reading frame (Fig. 1A). This element will be referred to as a YRE.

GSH1, encoding γ-glutamylcysteine synthetase, catalyzes the first step in the biosynthesis of glutathione (18). Cells lacking the *GSH1* gene show hypersensitivity to methylglyoxal (20). Interestingly, we found that overexpression of yAP-1 elevated methylglyoxal resistance, while a Δ*yap1* strain is hypersensitive to methylglyoxal (Fig. 1B). We also tested the effect of deletion of the *CAD1* gene, a locus that encodes a transcription factor related both structurally and functionally to yAP-1 (3, 35). Cad1p, yAP-1, and the *GCN4* gene product are DNA-binding proteins that employ a basic region-leucine zipper (bZip) (15) DNA binding domain for their characteristic sequence specificity. All of these three proteins are able to bind to the ARE and mediate transactivation from this site (5, 35). Despite the similarity of these proteins, loss of the *CAD1* gene from either the wild type or a Δ*yap1* strain did not show any effect on methylglyoxal resistance. *GSH1* expression was also found to be Gcn4p independent (see below). These data suggest the possibility that *GSH1* transcription is under the control of yAP-1. To directly confirm this suggestion, we assayed *GSH1* steady-state transcription levels in backgrounds containing different gene dosages of *YAP1*.

The mRNA level of *GSH1* is elevated by increasing the copy number of the *YAP1* gene. RNA was isolated (26) from wild-type cells carrying either a 2μm vector containing the *YAP1* gene or the 2μm vector alone. RNA was also prepared from an isogenic strain lacking the *YAP1* locus. The RNA was then subjected to Northern blot analysis using a *GSH1*-specific probe. The RNA analysis (Fig. 2) indicated that the level of *GSH1* mRNA was responsive to the gene dosage of *YAP1*. Steady-state level of *GSH1* mRNA was highest in the strain overexpressing yAP-1 and lowest in the Δ*yap1* strain. Equivalent RNA loading was demonstrated by the strong but invariant signal for *ACT1* mRNA (19). Densitometric quantitation of this and other autoradiograms indicated that *GSH1* mRNA levels were two- to threefold higher in a yAP-1-overproducing strain than in wild-type cells, while loss of the *YAP1* gene reduced *GSH1* transcription to 33% of normal.

Along with the analysis of *GSH1* mRNA levels in strains containing different *YAP1* gene dosages, we also measured the amount of glutathione in each of these strains. Previously, others have shown that glutathione levels are proportional to the copy number of the *YAP1* gene (28). Using our genetic backgrounds, we also found that the glutathione content of the cells increased as the copy number of the *YAP1* gene was increased. The glutathione content of a *YAP1* strain was 37 nmol/mg of protein, which was decreased to 30 nmol/mg of protein when the Δ*yap1* allele was present. Overproduction of yAP-1, elicited by the presence of the *YAP1* gene on a 2μm plasmid, increased to 55 nmol/mg of protein. These values agree well with those reported earlier (28) and are consistent with the belief that increasing the gene dosage of *YAP1* increases the biosynthesis of glutathione in vivo.

To determine whether the YRE is in a reasonable location to serve as a UAS for *GSH1*, we mapped the start point of *GSH1* gene transcription by primer extension. Primer extension analysis (25) was performed with RNA isolated from a strain containing the *GSH1* gene on a 2μm plasmid. The major extension product comigrated with a G residue which was located 152 bp upstream of translation start codon (Fig. 3). Results of an RNase protection analysis (data not shown) were consistent with the primer extension analysis.

All of the data presented above are consistent with the notion that yAP-1 regulates the expression of *GSH1* at the transcriptional level. To confirm that yAP-1 acts by binding the putative YRE in the *GSH1* promoter, we analyzed the physical contact between yAP-1 and the *GSH1* YRE in vitro.

Bacterially produced yAP-1 binds to a YRE. The similarity

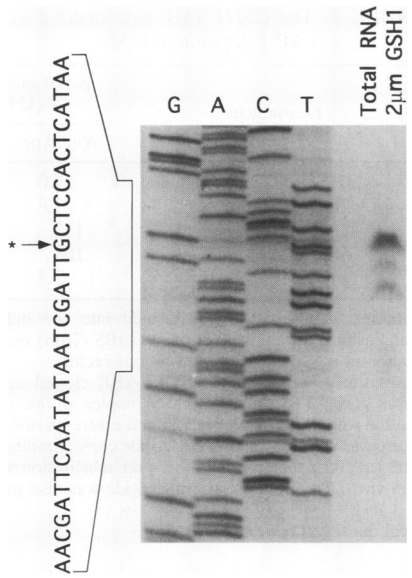

FIG. 3. Primer extension mapping of the 5′ end of *GSH1* mRNA. Total RNA was prepared from a wild-type strain carrying a 2μm plasmid containing the *GSH1* structural gene (pAW27) by a rapid technique (26). A 5′-end-labeled primer was annealed to this RNA and treated with reverse transcriptase and the four deoxynucleoside triphosphates. The extended products were run on a denaturing gel next to a dideoxy sequence ladder generated with the same primer. The asterisk and arrow indicate the major start site for *GSH1* mRNA. Control experiments established that no extension product was seen if an equal amount of yeast tRNA was used in place of total RNA (data not shown).

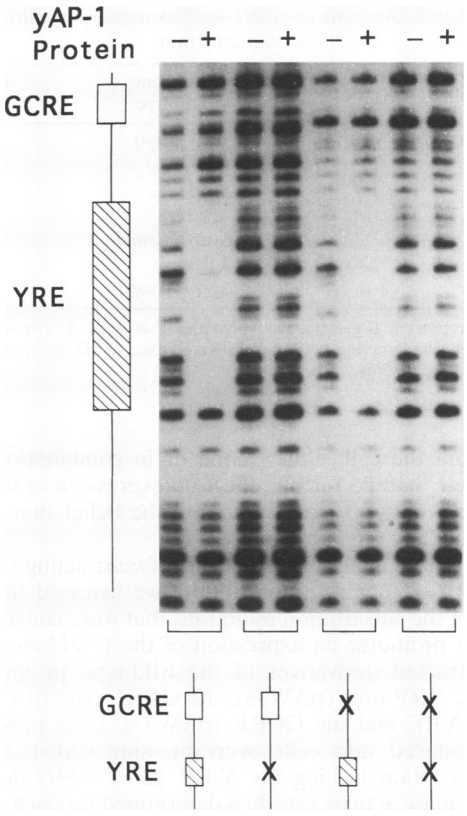

FIG. 4. DNase I protection analysis of yAP-1 protein binding to wild-type and mutant *GSH1* promoters. DNase I digestions were performed on a 250-bp *Eco*RI-*Hin*dIII DNA fragment isolated from the wild-type or mutant *GSH1* promoters. Each fragment was 5′ end labeled at the *Eco*RI site only. DNase I digestions were carried out either in the presence (+) or absence (−) of bacterially produced yAP-1 as indicated above each lane. The identity of the specific template used in each DNase I reaction is shown at the bottom. The cross-hatched box indicates the presence of a wild-type YRE; the open box indicates the presence of the wild-type GCRE. Replacement of either box with an X denotes a mutation in the respective DNA recognition element.

between the SV40 ARE and *GSH1* YRE suggested that yAP-1 could recognize and physically bind to the YRE. Furthermore, a potential GCRE was located only 10 bp away from the YRE. An authentic GCRE would serve as a binding site for Gcn4p (9) and would likely contribute to the transcriptional control of *GSH1*. We produced both yAP-1 and Gcn4p in bacteria to assess their abilities to bind to these potential recognition elements in the *GSH1* promoter.

A DNase I footprint assay was performed with a radiolabeled probe from the *GSH1* gene (Fig. 4). This probe was end labeled 51 bp upstream of the YRE and 73 bp upstream of the GCRE. The proximity of these two sites allowed the examination of protein binding to both elements on the same gel. Bacterially produced yAP-1 was able to bind to the YRE but not the GCRE. Twenty-four base pairs were protected by yAP-1 binding to the YRE corresponding to nucleotides −238 to −215. Both the YRE and GCRE were recognized by bacterially derived Gcn4p (data not shown). No protection was seen when extracts from cells carrying the expression vector with no yeast insert were used in the DNase I footprint experiment.

To explore the roles of the YRE and GCRE in control of *GSH1* expression, site-directed mutations were made in each element. The goal of the mutagenesis was to generate mutations that would eliminate the ability of either yAP-1 or Gcn4p to bind to the resulting altered site. To enhance the likelihood that neither factor could recognize the mutant site, four residues were changed in the YRE and the GCRE. The ability of yAP-1 and Gcn4p to recognize the mutant sites was then assessed by DNase I footprinting as described for the wild-type promoter.

The changes made in the YRE prevented either yAP-1 or Gcn4p from recognizing this mutant site. Similarly, the mutant GCRE inactivated Gcn4p binding to this element (data not shown). Thus, both mutant sites had the desired effect, at least in vitro, of preventing factor binding. The effects of these altered promoters on *GSH1* gene expression were then assessed in vivo.

Transactivation of *GSH1* by yAP-1 in *S. cerevisiae*. To facilitate measurement of *GSH1* gene expression, we constructed a gene fusion between *GSH1* and *E. coli lacZ*. This *GSH1-lacZ* fusion was then introduced into strains with various genotypes of three known yeast bZip protein-encoding genes: *YAP1*, *CAD1*, and *GCN4*. The resulting β-galactosidase activity was determined as a measure of the effect of these loci on the expression level of *GSH1* (Table 2).

Presence of the *GSH1-lacZ* fusion in wild-type cells led to the production of 1.4 U of β-galactosidase activity. If this same plasmid was introduced into a strain lacking the *YAP1* gene, the β-galactosidase activity was reduced to 0.5 U. Finally, *GSH1*-dependent enzyme activity was elevated to 6.2 U when the multicopy plasmid containing the *YAP1* gene was present in the same cell as the *GSH1-lacZ* fusion. Loss of *GCN4* or

TABLE 2. Analysis of *GSH1-lacZ* expression in wild-type and mutant strains

Strain	Relevant genotype	*GSH1-lacZ* expression[a]
SEY6210/YEp351-*YAP1*	2μm *YAP1*	6.2
SEY6210	*YAP1*	1.4
SM10	Δ*yap1*	0.5
PB1	Δ*cad1*	1.7
YAW10	Δ*yap1* Δ*cad1*	0.5
EE7	Δ*gcn4*	1.2
EE8	Δ*gcn4* Δ*yap1*	0.4

[a] Values represent β-galactosidase activities that were determined and reported as described previously (35). Cells were grown in SD medium containing appropriate supplements.

TABLE 3. The *GSH1* YRE can function as a yAP-1-dependent UAS

Plasmid present[a]	Fusion gene[b]	β-Galactosidase activity (U/OD_{600}[c])	
		Wild type	Δ*yap1*
pSMC188	*TRP5-lacZ*	14.0	12.8
pLGΔBS	*CYC1-lacZ*	3.8	2.2
pEPC11	ARE-*CYC1-lacZ*	132	5.0
pAW62	YRE-*CYC1-lacZ*	168	0.7
pAW64	mYRE-*CYC1-lacZ*	3.3	2.5

[a] *URA3*-containing plasmids were transformed into the indicated genetic backgrounds. All plasmids are derivatives of pLGΔBS (2μm) except pSMC188, which is constructed in a pSEYC102 (centromeric) vector.

[b] ARE indicates the presence of the SV40 ARE cloned upstream of the *CYC1-lacZ* fusion gene. YRE denotes three copies of an oligonucleotide corresponding to the wild-type *GSH1* YRE cloned upstream of *CYC1-lacZ*. The same nomenclature was used for an oligonucleotide corresponding to the mutant copy of the YRE (mYRE) that contains the 4-bp substitution shown to block yAP-1 binding in vitro. The mutant oligonucleotide was also present in three copies.

[c] OD_{600}, optical density at 600 nm.

CAD1 from the cell, either alone or in combination with a Δ*yap1* allele, had no further effect on expression of the fusion gene. These data are consistent with the belief that *GSH1* is transcriptionally regulated by yAP-1.

In addition to analyzing the effects of *trans*-acting factors on control of *GSH1* gene transcription, we assessed the consequences of the substitution mutations that were constructed in the *GSH1* promoter on expression of the *GSH1-lacZ* fusion. We constructed derivatives of the wild-type promoter that lacked the YRE only (pAW35), the GCRE only (pAW36), or both the YRE and the GCRE (pAW37). Each plasmid was then introduced into cells overexpressing yAP-1, wild-type cells, or a strain lacking the *YAP1* gene. *GSH1*-dependent β-galactosidase activity was then determined for each transformant (Fig. 5).

The wild-type promoter and the promoter lacking the GCRE responded indistinguishably to changes in *YAP1* gene dosage. This finding indicates that loss of the GCRE alone from the *GSH1* promoter has no significant effect on promoter function. In opposition to these results, the mutant promoters lacking either the YRE alone or both the YRE and the GCRE showed a total inability to respond to the presence of yAP-1. Even the presence of the high-copy-number plasmid containing the *YAP1* gene did not enhance β-galactosidase activity from the mutant promoters lacking the YRE. Furthermore, there was no difference in β-galactosidase produced from these mutant promoters in the wild-type cell. Therefore, even in the absence of the YRE, the presence of the GCRE had no significant effect on *GSH1* transcription. This finding provides further evidence that the GCRE is not required for normal *GSH1* expression.

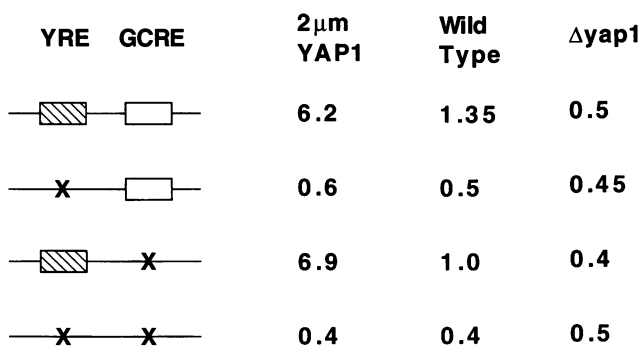

FIG. 5. *GSH1-lacZ* fusion expression driven by wild-type and mutant promoters. Yeast strains of the indicated genotypes were transformed with *GSH1-lacZ* fusion plasmids, and the β-galactosidase activity produced in each transformant was determined (6). The promoter mutations present in each chimera are indicated as in Fig. 4. Values reported are the averages of at least two determinations for two transformants of each type and have an error of <20%.

Upstream activation function of the *GSH1* YRE. To provide further support for the idea that the effect of yAP-1 on *GSH1* was via the YRE, the ability of this DNA element to act as a UAS was tested. Oligonucleotides corresponding to the wild-type YRE or the 4-bp substitution mutant form of the YRE were cloned upstream of a *CYC1-lacZ* gene fusion lacking the normal *CYC1* UAS information. The resulting plasmids were introduced into wild-type cells or an isogenic Δ*yap1* strain. *CYC1-lacZ*-dependent β-galactosidase activity was then determined as a measure of the ability of the YRE to act as a UAS.

The wild-type YRE was able to strongly stimulate *CYC1-lacZ* expression in cells containing the *YAP1* gene (Table 3). However, introduction of the YRE-*CYC1-lacZ* fusion gene into Δ*yap1* cells resulted in very low levels of β-galactosidase activity, demonstrating the yAP-1 dependence of this UAS element. The mutant version of the YRE (containing the 4-bp substitution mutation that blocked in vitro binding) was unable to drive significant production of β-galactosidase, irrespective of genetic background.

These data indicate that the *GSH1* YRE is capable of acting as a yAP-1-dependent UAS element. This finding is consistent with our previous demonstration that yAP-1-dependent control of *GSH1* expression requires the presence of the YRE (Fig. 5). Coupled with the Northern blot analysis of *GSH1* mRNA levels responding to *YAP1* gene dosage, these data are entirely consistent with the belief that yAP-1 transcriptionally regulates the expression of *GSH1*.

Phenotypes of *GSH1* promoter mutants. The use of the *lacZ* gene fusions allowed the facile assessment of changes in *GSH1* gene expression in response to the various promoter mutations that were generated. However, to analyze the effects of these promoter lesions in terms of their effects on phenotypes mediated by *GSH1*, each mutation was reconstructed upstream of the normal *GSH1* gene.

A yeast strain (YAW11) that lacked a functional copy of the *GSH1* gene was constructed. We found that this mutant was unable to grow in the absence of exogenous glutathione supplementation to the growth medium. Introduction into this Δ*gsh1* strain of either low-copy-number or multicopy plasmids

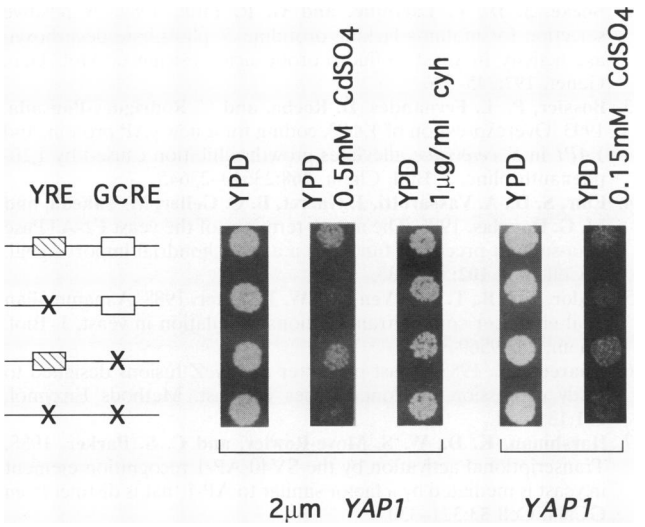

FIG. 6. Phenotypic effect of *GSH1* promoter mutations. YAW11 (relevant genotype, *ura3-52* Δ*gsh1-Δ1::hisG*) cells were transformed with low-copy-number plasmids containing the *GSH1* gene with the indicated wild-type or mutant promoters. The promoter mutations are denoted as in Fig. 4. Each *GSH1*-containing plasmid was cotransformed with either a 2μm vector plasmid or the same high-copy-number plasmid containing the *YAP1* gene. 2μm *YAP1* denotes the presence of a high-copy-number plasmid containing *YAP1*, while *YAP1* indicates the presence of the high-copy-number vector plasmid only. Appropriate transformants were tested for cycloheximide or cadmium resistance on YPD plates containing 1 μg of cycloheximide (cyh) per ml or the indicated concentration of $CdSO_4$.

carrying *GSH1* relieved the resulting transformants of the dependence on exogenous glutathione. Neither the low- or high-copy-number vector plasmid alone could confer the ability of the Δ*gsh1* strain to grow on YPD alone. This result indicated that a plasmid-borne copy of *GSH1* could complement the loss of the chromosomal locus and provided a simple assay for assessment of the effect of the promoter mutations on *GSH1* gene function.

Each of the different promoter mutations, in the context of an otherwise normal *GSH1* gene, was then introduced into the Δ*gsh1* strain. These *GSH1* derivatives were transformed along with either a high-copy-number vector plasmid or a high-copy-number plasmid carrying *YAP1*. The resulting transformants were then assayed initially for glutathione independence. The results of this assay are shown in Fig. 6. All of the *GSH1* constructs were able to confer glutathione independence, irrespective of the presence of the multicopy *YAP1*-containing plasmid. Thus, the level of *GSH1* gene expression provided by the various promoter mutants is adequate to support levels of glutathione biosynthesis sufficient to permit wild-type growth.

Our previous work showed that yAP-1 played an important physiological role in mediating resistance to cadmium (35). The finding that yAP-1 could serve to control *GSH1* transcription led us to test the effect of the *GSH1* promoter mutations on yAP-1-mediated cadmium resistance. For this analysis, all *GSH1* gene derivatives were carried on low-copy-number plasmids in the presence of either a 2μm vector or a 2μm plasmid containing *YAP1*. Transformants were grown in the absence of cadmium and then placed on cadmium-containing media and examined for the ability to grow.

Transformants containing the wild-type *GSH1* gene (pAW26) were able to grow normally in the presence of cadmium. Overproduction of yAP-1 led to an enhanced ability to tolerate cadmium, as was seen previously (35). This same growth behavior was observed for transformants containing a *GSH1* gene with a mutant GCRE (pAW33). As in the quantitative assay of expression using the *lacZ* fusion genes, there was no detectable effect on *GSH1* promoter function that arose when the GCRE was mutated.

Interestingly, the mutant promoters lacking the YRE (pAW32 and pAW34) were unable to support the same level of growth in the presence of cadmium as the promoters containing the YRE. Overproduction of yAP-1 did not correct this growth defect. Thus, a cell containing a *GSH1* gene unable to respond to yAP-1 is hypersensitive to cadmium. These data provide strong evidence that normal cadmium tolerance requires yAP-1 transactivation of *GSH1* gene expression.

A variety of resistance phenotypes emerge in cells overexpressing yAP-1 (3, 8, 16, 28, 35). We tested the effect of the *GSH1* promoter mutations on cycloheximide, 1,10-phenanthroline, zinc, and methylglyoxal resistance. Resistance to each of these compounds was elevated when yAP-1 is overproduced, irrespective of the presence of the YRE in the *GSH1* promoter (data not shown). We conclude from this analysis that the yAP-1-dependent increase in resistance to these toxic chemicals does not come about through activation of the *GSH1* promoter by this transcription factor.

DISCUSSION

The physiological role of a transcription factor is defined through the nature of the genes that it regulates. An important gap in our knowledge of the function of yAP-1 has now been filled with the identification of *GSH1* as a gene responsive to transcriptional control by this yeast bZip protein. It is very likely that *GSH1* is not the only gene under control of yAP-1, as the pleiotropic effect of this transcription factor on drug resistance cannot be fully explained in terms of regulation of *GSH1* gene expression. Identification of the other loci under yAP-1 control will provide a more complete understanding of the in vivo function of this protein.

GSH1 was identified as a candidate yAP-1 target gene through inspection of the DNA sequence of the presumptive promoter region. The sequence motif that was used as the query corresponded to an element from SV40 that we had previously shown to be yAP-1 responsive in a yeast cell (7). It is interesting that the *GSH1* YRE differs from the SV40 ARE in only 1 of 12 positions. In previous searches, we have found two other 11-of-12 matches to the ARE present in a likely promoter region of other yeast genes. These genes, *RPC40* (17) and *TRP2* (36), were examined and found to be unaffected by *YAP1* gene dosage (our unpublished data). *RPC40* differs from the ARE at position 2 in the sequence shown in Fig. 1, while *TRP2* differs at position 9. The *RPC40* variation changes a residue that corresponds to a position that shows reduced dimethyl sulfate (DMS) reactivity upon yAP-1 binding to the ARE (7). Likewise, the *TRP2* change alters a residue immediately adjacent to a position that is made more accessible to DMS reaction on yAP-1 binding. The *GSH1* YRE differs at position 12 which is a nucleotide at the extreme end of the recognition element and is not adjacent to any residue showing altered DMS reactivity. These data suggest that ARE-like sequences that do not conserve the key residues defined by DMS protection are unlikely to bind yAP-1 in *S. cerevisiae*.

A protein showing sequence similarity to the DNA binding domain of yAP-1 has been isolated from *Schizosaccharomyces pombe*. This factor, designated pap1 (33), was found to be able to bind DNA elements related to the ARE as well as a palindromic sequence that shows no obvious similarity to the

ARE (32). This finding makes it premature to conclude that all yAP-1 recognition elements resemble the ARE. Identification of the structural determinants recognized by yAP-1 during site-specific DNA binding will require a systematic study of the requirements of this protein for DNA recognition.

The function of the yAP-1 transcriptional regulatory protein appears to be as a central modulator of drug and metal resistance. Along with the many other resistance phenotypes that have previously been described for changes in *YAP1* gene dosage, we have found that resistance to methylglyoxal is also yAP-1 dependent. Mutant yeast strains with reduced glutathione biosynthetic capabilities are hypersensitive to this compound (20). Surprisingly, yAP-1 activation of *GSH1* transcription is not required for elevated methylglyoxal resistance. Overproduction of yAP-1 in a strain containing, as its sole source of γ-glutamylcysteine synthetase activity, a *GSH1* gene lacking a functional YRE still increases the resistance to methylglyoxal (data not shown). This finding strongly suggests that the gene under yAP-1 control that is responsible for the increase in methylglyoxal resistance is not *GSH1* and remains to be identified. However, we cannot exclude a minor role for yAP-1-mediated transactivation of *GSH1* in methylglyoxal resistance.

Glutathione is the most prevalent thiol-containing molecule in the cell and is involved in a vast array of functions (18). The ubiquitous nature of glutathione action in the cell complicates the assignment of a specific role of this molecule in cadmium resistance. However, glutathione and glutathione polymers have been shown to directly bind or at least detoxify cadmium in plants, *Schizosaccharomyces pombe*, and mammals (18). This is an attractive possibility to explain the action of glutathione in *S. cerevisiae*, but proof of this suggestion requires biochemical assessment of proteins bound to cadmium in this organism as has been performed for *Schizosaccharmoyces pombe* (23).

In summary, we have found that production of a key enzyme in glutathione biosynthesis is transcriptionally controlled by the yeast AP-1 protein. *GSH1* encodes the rate-limiting step in yeast glutathione biosynthesis and increases in levels of γ-glutamylcysteine synthetase activity increase glutathione production (20). Previously, it was demonstrated that elevation of *YAP1* gene dosage led to increased intracellular glutathione levels (28). Our determination that *GSH1* is under control of yAP-1 provides a molecular rationale for this finding. Elucidation of the other genes that are responsive to yAP-1 will provide the framework for understanding the mechanisms through which this transcription factor is able to alter drug and metal resistance.

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ADDENDUM

While this report was being revised, a report indicating that yAP-1 controls the expression of a yeast thioredoxin gene, *TRX2*, appeared (14).

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