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YAP1 dependent activation of *TRX2* is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides

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The role of the YAP1 transcription factor in the response of *Saccharomyces cerevisiae* cells to a variety of conditions that induce oxidative stress has been investigated. Cells deficient in YAP1 were found to be hypersensitive to hydroperoxides and thioloxidants, whereas over-expression of *YAP1* conferred hyper-resistance to the same conditions. These treatments resulted in an increase in YAP1-specific binding to DNA together with an increase in YAP1 dependent transcription. Our results indicate that this increase is not due to an increase in synthesis of YAP1 protein, but rather results from modification of pre-existing protein. Using a specific genetic screen, the *TRX2* gene, one of two genes of *S.cerevisiae* that encode thioredoxin protein, was identified as being essential for YAP1 dependent resistance to hydroperoxides. Furthermore, efficient expression of *TRX2* was dependent on YAP1 and enhanced under conditions of oxidative stress.

Key words: hydroperoxides/oxidative stress/thioredoxin/transcription factors/YAP1

Introduction

The AP-1 family of eukaryotic transcription factors binds specifically to DNA sequences that have the consensus TGAGTCA (for reviews see Abate and Curran, 1990; Angel and Karin, 1991). In mammalian cells these factors consist of homo- or heterodimers of various members of the Jun and Fos families of proteins. They all possess a bZ1P structural motif, which consists of a leucine zipper dimerization domain adjacent to a basic region that interacts directly with DNA. In the yeast, *Saccharomyces cerevisiae*, two factors have been identified that belong to this family, namely GCN4 (Struhl, 1987) and YAP1 (Moye-Rowley *et al.*, 1989). They both contain the bZIP motif but are distinguishable from each other by having a slightly different DNA binding specificity. The GCN4 recognition element (GCRE) is identical to the consensus AP-1 site but is recognized inefficiently by the YAP1 protein (Harshman *et al.*, 1988; Jones *et al.*, 1988). In contrast, the AP-1 recognition element of simian virus 40 (SV40; TTAGTCA) is efficiently recognized by YAP1 but not by GCN4 (Harshman *et al.*, 1988; Jones *et al.*, 1988). These proteins appear to play different regulatory roles in yeast; GCN4 is a regulator of amino acid biosynthesis genes whereas the exact cellular role of YAP1 remains unclear. It is, however, a transcriptional activator directing efficient transcription from a heterologous promoter containing the SV40 AP-1 site (Harshman *et al.*, 1988; Jones *et al.*, 1988). To date, no endogenous genes regulated by this factor have been identified. It is probable, however, that such genes are not essential since a yap1$^-$ disruption mutant can grow well under normal growth conditions (Moye-Rowley *et al.*, 1989).

Although *YAP1* was originally identified and cloned by virtue of its ability to bind specifically to AP-1 sites, three groups have identified this gene in high copy number plasmids that can confer resistance to a number of unrelated drugs. *PDR4* was isolated by virtue of its ability to confer resistance to sulfometuron methyl and cycloheximide (Hussain and Lenard, 1991), *SNQ3* by giving resistance to 4-nitroquinoline-*N*-oxide, trenimon and MNNG (Hertle *et al.*, 1991) and *PAR1* by giving resistance to the iron chelators 1,10-phenanthroline and 1-nitroso-2-napthol (Schnell and Entian, 1991). All these three are identical to *YAP1*. Just as overexpression of *YAP1* enhances the resistance of yeast cells to a variety of toxic compounds, deletion of *YAP1* can result in increased sensitivity to toxic drugs. Schnell *et al.* (1992) described the increased sensitivity of yap1$^-$ cells to hydrogen peroxide and chemicals which generate superoxide anion radicals. They further suggested that this phenotype resulted from decreased specific activity of enzymes involved in oxygen detoxification such as glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase and glutathione reductase. Whether the genes encoding these enzymes were targets of the YAP1 transcription factor, however, was not addressed.

Mammalian cells have protective mechanisms to minimize cell injury that could result from toxic compounds or toxic oxygen species produced by normal cell metabolism. In addition to enzymatic defences against active oxygen, cells also employ non-enzymatic organic antioxidants, the two most important being the low molecular weight dithiols glutathione (GSH) and thioredoxin. These molecules maintain a strong reducing environment in the cell. GSH is synthesized by glutathione synthetase and acts both as a nucleophilic 'scavenger' of many compounds and their metabolites through enzymatic and chemical mechanisms and as a substrate in the GSH peroxide-mediated destruction of hydroperoxides. Thioredoxin is a small (mol. wt. 12 000), ubiquitous protein which has a redox active disulfide bond; accordingly the protein exists either in reduced form as a dithiol or in an oxidized form when the half-cysteine residues form an intramolecular disulfide bridge. This bridge can be reduced by thioredoxin reductase and NADPH (Holmgren, 1985; Wollman *et al.*, 1988). It has been suggested that thioredoxin protects living organisms from oxidative stress by scavenging reactive oxygen species as well as regenerating proteins inactivated by such stress (Fernando *et al.*, 1992; Mitsui *et al.*, 1992). Thioredoxin has also been

Table I. Effect of drugs causing oxidative stress on YAP1 wild-type and yap1$^-$ mutant yeast

	Half-drug concentration of maximal inhibition [μM][a] or (IC_{50})[b]				
	H_2O_2	*t*BOOH[c]	Paraquat[d]	Diamide	Diethylmaleate
YAP1$^+$	>1000 (870)	125 (170)	ND (4670)	880 (930)	940 (2450)
yap1[1]	440 (320)	<50 (60)	ND (1410)	380 (780)	<750 (920)

[a]Log phase cultures of DY(YAP1$^+$) or DWYU(yap1$^-$) were patched onto YPD agar plates containing the indicated drugs at various concentrations and cultured at 30°C for 2 days. The concentration indicated in the table represents 50% of the average concentration between the maximum concentration tested upon which the yeast could grow and the lowest concentration tested which completely inhibited growth.
[b]Log phase cultures of DY or DWYU growing in SD medium with added amino acid supplements were harvested and inoculated into the same medium containing the indicated drugs at various concentrations. Following incubation at 30°C for 15 h, growth of the cultures was monitored by OD measurement. The IC_{50} value was calculated from the probit curve.
[c]Abbreviation for *t*-butyl hydroperoxide.
[d]With this particular drug, consistent results from the plate assay could not be obtained.
ND = not determined.

implicated in the functional regulation of a number of different proteins through modifying their redox status (Grippo *et al.*, 1985; Okamoto *et al.*, 1992).

We have investigated further the role of YAP1 in the response of yeast cells to oxidative stress. To study such stress in intact cells hydroperoxides or thiol oxidants are commonly used (Plummer *et al.*, 1981; Sies, 1985). We found that resistance to hydroperoxides and thiol oxidants was YAP1 dependent; overexpression of *YAP1* gave hyper-resistance whereas deletion of *YAP1* resulted in hyper-sensitivity. Furthermore, YAP1 activity was stimulated by these compounds as measured by the increased transcription of a reporter gene driven by the SV40 AP-1 binding site. This stimulation did not result from increased expression of the *YAP1* gene but rather from an increase in the binding of pre-existing YAP1 protein to DNA. The results imply that YAP1 controls the expression of a gene(s) encoding a product that is critical for the anti-oxidant defence of the cell. Using a high copy plasmid library screen we identified a gene that conferred YAP1 dependent resistance to hydrogen peroxide and *t*-butyl hydroperoxide (*t*BOOH). The gene was identical to the previously described *TRX2* gene which is one of two genes in *S.cerevisiae* that encode thioredoxin. We show that *TRX2* is a direct downstream target of YAP1.

Results

Sensitivity of yap1$^-$ cells to oxidative stress

The function of the YAP1 transcription factor has not been clearly elucidated, mainly because of the lack of any obvious phenotype of a yap1 disruption mutant. Recently, however, it has been implicated in various defence mechanisms of the cell and has been recloned on numerous occasions as a multi-drug resistance gene. One such defence mechanism that appears to require YAP1 is the oxidative stress response. We began an investigation of the role of YAP1 in this response by comparing the growth of wild-type and yap1$^-$ cells on solid and in liquid media containing a variety of compounds that can artificially induce oxidative stress. These results, shown in Table I, demonstrated that yap1$^-$ cells showed a significantly increased sensitivity to all the treatments we tested. For example, yap1$^-$ cells were considerably more sensitive to H_2O_2 and *t*BOOH, a simple organic hydroperoxide which can be reduced by glutathione peroxidase but not catalase. Additionally, yap1$^-$ cells showed increased sensitivity to paraquat, at least in liquid medium. This is in agreement with the previous results of Schnell *et al.* (1992).

A number of previous studies using intact mammalian cells or organs employed strategies that modulated the GSH state of the cell in order to study the oxidative stress response. For example, GSH can be oxidized by diamide treatment or depleted by conjugation with diethylmaleate (Boyland and Chasseaud, 1967). Since GSH is an important antioxidant and helps to maintain a strong reducing environment in the cell, its oxidation or depletion should mimic some of the effects of exerting oxidative challenge. We therefore tested the importance of YAP1 in cell resistance to both diamide and diethylmaleate treatment. In both cases yap$^-$ cells were significantly more sensitive than wild-type cells (Table I).

Induction of YAP1 dependent transcription

Previous studies have shown that YAP1 is a DNA binding factor that can activate transcription from a promoter containing an appropriate binding site such as the SV40 AP-1 site (Harshman *et al.*, 1988; Jones *et al.*, 1988). Therefore, if YAP1 is playing an important role in the oxidative stress response it is likely to be doing so by stimulating the transcription of one or more genes whose products protect the cell from the potentially damaging effects of such stress. It would follow therefore, that the activity of YAP1 may increase in response to stress conditions. To test this possibility directly we studied the expression of a heterologous gene comprising the bacterial *LacZ* coding region fused to a promoter containing three SV40 AP-1 binding sites. As shown in Figure 1, the expression of this gene is dependent upon the presence of YAP1, *LacZ* transcripts being undetectable in yap1$^-$ cells. Transcription was dramatically activated in the presence of 1 mM H_2O_2, 1.5 mM diamide or 2 mM diethylmaleate; in contrast, however, little increase was evident by treatment with 0.2 mM *t*BOOH or 1 mM paraquat. Slightly elevated transcript levels were detected with much higher concentrations of *t*BOOH (0.6 mM; data not shown). These results demonstrate that the induction of oxidative stress is often accompanied by an elevation of YAP1 activity. Therefore, genes that are dependent upon YAP1 for their expression are likely to be components of the cellular response to such stress conditions.

Induction of YAP1 DNA binding activity

The increase in YAP1 activity could result from an increase in synthesis of the protein itself or an increase in function

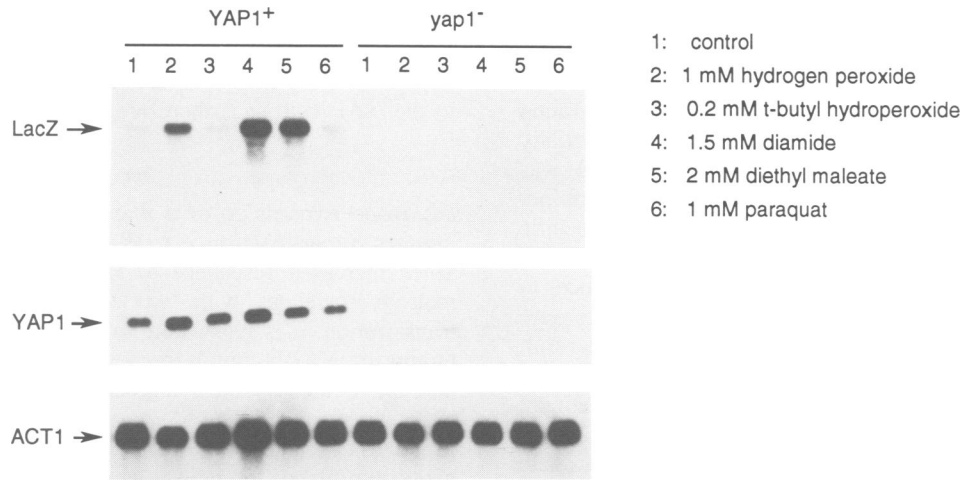

Fig. 1. YAP1 dependent transcription in response to oxidative stress. YAP1$^+$ (DY) or yap1$^-$ (DWYU) yeast strains were cultured in minimal medium (lane 1), or minimal medium containing 1 mM H_2O_2 (lane 2), 0.2 mM *t*BOOH (lane 3), 1.5 mM diamide (lane 4), 2 mM diethylmaleate (lane 5) or 1 mM paraquat (lane 6) and total RNAs were prepared as described in Materials and methods. RNA samples (5 μg/slot) were separated on a 1.2% formaldehyde gel and transferred to Hybond-N (Amersham) filters. The filters were hybridized with radioactive probes specifically containing *LacZ*, *YAP1* or *ACT1* (yeast actin).

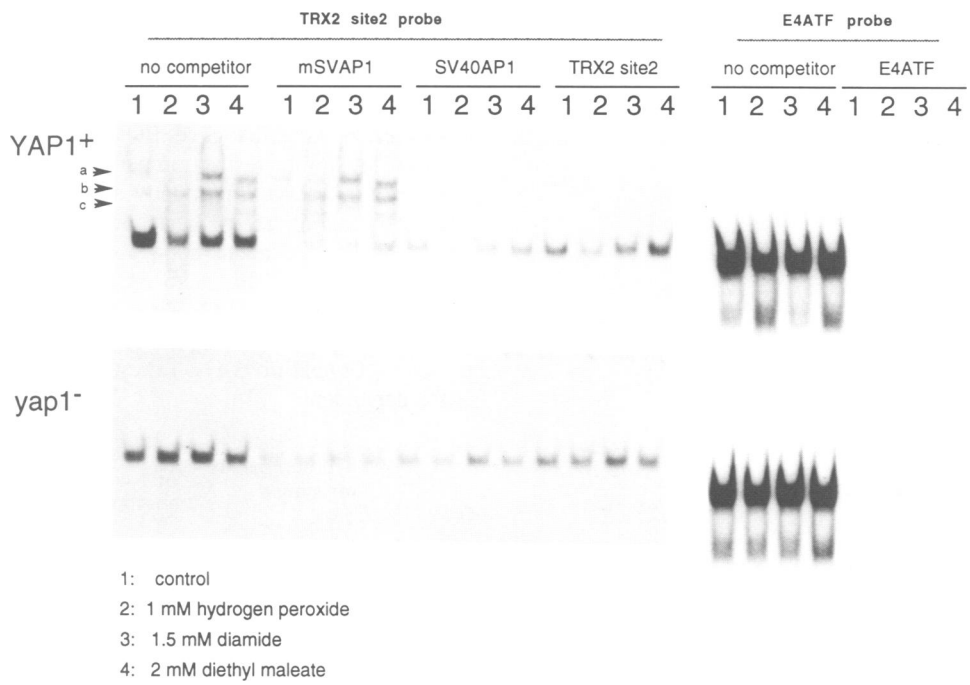

Fig. 2. Enhanced DNA binding activity of YAP1 protein following drug treatment. The YAP1$^+$ or yap1$^-$ yeast strains were cultured in SD medium (lanes 1), or SD medium containing 1 mM H_2O_2 (lanes 2 of each set), 1.5 mM diamide (lanes 3) or 2 mM diethylmaleate (lanes 4). Extract preparation and the EMSA were carried out as described in Materials and methods. The radioactive probe used for the assay was an oligonucleotide (*TRX2* site 2, see Materials and methods) containing a YAP1 binding site (TTAGTAA). Competitors were used at 50-fold excess as indicated in the figure. In the case of mSVAP1 the SV40 AP-1 binding site has been mutated so that it no longer binds the YAP1 protein. A control mobility shift assay was carried out using an oligonucleotide containing the E4ATF binding site which is bound by a specific yeast protein, yATF (Jones and Jones, 1989). Unlabelled E4ATF competitor DNA was added at a 50-fold molar excess.

of pre-existing protein. The latter possibility was suggested by the finding that transcription of the *YAP1* gene was not significantly altered in response to any of the conditions used to generate oxidative stress (Figure 1). Also, the addition of cyclohexamide (50 μg/ml) to the medium at the time of induction did not prevent enhanced transcription (data not shown). Therefore, *de novo* protein synthesis was not required, reinforcing the conclusion that the stimulation of YAP1 activity is mediated at a post-translational level. The function of pre-existing protein could be elevated in one of two ways: modulation of binding of the protein to DNA or modulation of the ability of the protein to interact with, and stimulate, the basal transcription machinery. In order to distinguish between these possibilities, extracts were prepared from treated cells and YAP1-specific binding activity measured with an electrophoretic migration shift assay using an oligonucleotide containing a YAP1 binding site (*TRX* site 2) as a probe. As shown in Figure 2, three YAP1-specific complexes designated a, b and c were detected. These complexes were not observed in the yap1$^-$

strain and they were efficiently competed with an excess of unlabelled homologous oligonucleotide and an oligonucleotide containing the SV40 AP-1 site but not by an oligonucleotide containing a mutant AP-1 site. A faster migrating non-specific complex was also detected; it was partially competed by all oligonucleotide competitors and was also present in yap1$^-$ cell extracts. It is therefore not YAP1-specific. Enhanced binding of the YAP1-specific complexes was observed in extracts from cells treated with H_2O_2, diamide or diethylmaleate (Figure 2). Differences in the relative ratio of the three different YAP1 complexes were evident; in particular, H_2O_2 treatment appeared to result preferentially in an increase in the b complex. Additionally, the complexes obtained following diethylmaleate treatment migrated slightly faster than those seen following treatment with H_2O_2 or diamide. Differences in the modification state of YAP1 probably account for the different complexes seen in the mobility shift assay. The different treatments may not affect such modifications equally. As a control, we examined complex formation of the ATF binding site which had been shown previously to interact with specific protein(s) in a yeast cellular extract (Lin and Green, 1989; Jones and Jones, 1989). Complex formation was unchanged following H_2O_2, diamide or diethylmaleate treatment. The increase in binding to the YAP1 site was therefore specific.

Table II. The ability of genomic clones to confer drug hyper-resistance in YAP1$^+$ and yap1$^-$ cells[a]

Genotype (plasmid clone)	None	2 mM diamide	0.6 mM *t*BOOH
YAP1$^+$ (YEp13)	+	−	−
(GDR2)	+	+	−
(GDR3)	+	+	+
(GDR19)	+	−	+
yap1$^-$ (YEp13)	+	−	−
(GDR2)	+	−	−
(GDR3)	+	+	+
(GDR19)	+	−	−

[a]The drug resistance phenotype was determined as described in Materials and methods. '+' indicates normal levels of growth; '−' indicates no growth.

Increased expression of a thioredoxin gene (TRX2) confers hyper-resistance to H_2O_2 and tBOOH.

Since increased resistance to H_2O_2, diamide or diethylmaleate correlates with the level of YAP1 dependent transcription, it is reasonable to assume that one or more targets of YAP1 confer the resistance phenotype. In an attempt to identify such targets we screened yeast cells transformed with a *S.cerevisiae* genomic library constructed in a high copy number vector for transformants that could grow on drug concentrations which inhibited the growth of wild-type, YAP1$^+$ cells. Transformants were selected that could grow on 0.6 mM *t*BOOH and 2 mM diamide; for technical reasons we could not screen for transformants resistant to high levels of H_2O_2. One clone, represented by GDR3, was repeatedly isolated with both screens (Table II). This clone was found to encode YAP1, indicating that overexpression of YAP1 resulting from increased copy number gave hyper-resistance to *t*BOOH and diamide. As expected, the same hyper-resistance phenotype was observed when GDR3 was transformed into yap1$^-$ cells. Seven clones were isolated that gave *t*BOOH resistance and which all showed a similar restriction pattern. GDR19 is a representative of this class. Importantly, GDR19 could not confer hyper-resistance in a yap1$^-$ background, suggesting that it is functionally dependent upon YAP1 and may therefore represent a target of this factor. GDR19 could also confer increased resistance to H_2O_2 but not to diamide. In contrast, another clone, GDR2 conferred hyper-resistance to diamide but not to *t*BOOH or H_2O_2. Like GDR19, its activity was YAP1 dependent.

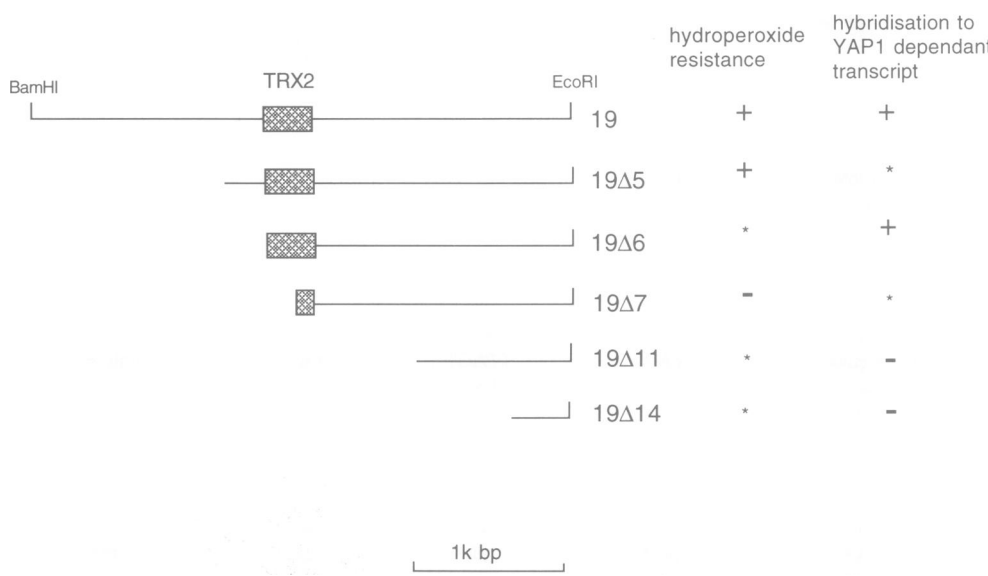

Fig. 3. Functional characterization of GDR19. GDR19 contains a 3.5 kb *Bam*HI−*Eco*RI genomic fragment that confers hyper-resistance to *t*BOOH and H_2O_2 in YAP1$^+$ cells. A number of deletions of this fragment were made (as described in Materials and methods) and characterized for their ability to confer increased resistance to hydroperoxides (as described in Table II), as well as their ability to hybridize to a transcript encoded by GDR19 that is dependent upon YAP1. The deleted fragments were used as hybridization probes in a Northern blot analysis of RNA isolated from YAP1$^+$ and yap1$^-$ cells. A number of transcripts are detected by the complete *Bam*HI−*Eco*RI fragment, only one of which was significantly dependent upon a YAP1$^+$ phenotype. Positive results are indicated as '+' and negative results as '−' in the figure. '*' indicates that the assay had not been done.

GDR19 contains the TRX2 gene

If GDR19 or GDR2 contained direct targets of YAP1 we would expect them to encode a transcript that was both dependent upon YAP1 and was enhanced in response to the same treatments that increased the activity of YAP1. This was found to be the case for a transcript present in GDR19. The location of the gene encoding this transcript was determined by analysing a series of deletions, correlating the ability of the mutant to hybridize to this transcript and its ability to confer hyper-resistance to *t*BOOH. In this way a region in the middle of GDR19 was identified as being critical (Figure 3). Upon sequencing, this region was found to contain the gene *TRX2*, one of two genes of *S.cerevisiae* that encode a thioredoxin protein (Gan, 1991). As shown in Figure 4, expression of *TRX2* was strongly enhanced by H_2O_2 and diamide, moderately enhanced by *t*BOOH and diethylmaleate but not enhanced by paraquat. In yap1$^-$ cells, the overall level of *TRX2* expression was very significantly reduced although some enhancement was evident, particularly with diamide. These results suggest that the regulation of *TRX2* expression is complex and that although YAP1 is an important factor in determining the overall level of expression, other regulatory factors must also be involved.

From the result described above it would appear that increased *TRX2* expression, emanating from enhanced YAP1 activity, confers hyper-resistance to *t*BOOH and H_2O_2. Surprisingly, the other thioredoxin gene, *TRX1*, was not isolated in the multicopy plasmid library screen.

TRX2 is a target of YAP1 and is essential for hydroperoxide resistance

A direct effect of YAP1 on the expression of *TRX2* is suggested by the existence of two sites similar to the SV40 AP-1 binding site in a region upstream of the *TRX2* transcriptional start (Figure 5A). These sites are identical but inverted with respect to each other and differ from the SV40 AP-1 site by a single nucleotide (Figure 5B). A 200 nucleotide long probe from the upstream region of *TRX2* that contains both potential YAP1 binding sites produced two complexes in an electrophoretic mobility shift assay (Figure 5C). These complexes were not observed with extracts from yap1$^-$ cells, and were competed by oligonucleotides containing *TRX* site 1 or site 2 or by the SV40 AP-1 site but were not competed by an oligonucleotide containing a mutant SV40 AP-1 site. Furthermore, the complexes were significantly enhanced by treatment of the cells with diamide. Clearly therefore, these *TRX2* sites can function as binding sites for YAP1, suggesting that *TRX2* is a direct target of the YAP1 transcription factor.

In order to verify that the YAP1 binding sites are critical elements of the *TRX2* promoter, a chimeric gene was constructed containing the *LACZ* coding region fused to ~0.5 kb of *TRX2* 5′ sequences (TRXLACZ). This region contains both the YAP1 binding sites. A second construct was prepared that was identical except for specific point mutations within both YAP1 binding sites (mTRXLACZ) converting the sequence 5′-TTAGTAA-3′ to 5′-TTAGGAA-3′ (Figure 6A). These constructs were transformed into YAP1$^+$ yeast and *LacZ* activity measured following growth in the absence and presence of drugs that we showed previously (Figure 1) could induce *YAP1* activity. In the absence of drugs, a very low but detectable level of expression was obtained with TRXLACZ, which was stimulated ~40-fold by diamide and ~3-fold by H_2O_2 (Figure 6B). In contrast, expression of mTRXLACZ was very low under all conditions tested. These results demonstrate the importance of the YAP1 binding sites for both basal and induced activity of the *TRX2* promoter and strongly support the conclusion that *TRX2* is a direct target of the YAP1 transcription factor.

The interplay between *YAP1* and *TRX2* was also evident when strains lacking *YAP1*, *TRX2* or both were tested for drug sensitivity (Table III). Deletion of the *TRX2* gene resulted in hypersensitivity to H_2O_2, although sensitivity to *t*BOOH, diamide and diethylmaleate remained largely unaltered (Table III, line 2). This is in contrast to deletion of *YAP1* which, as described earlier, results in hypersensitivity to all four treatments (Table III, line 3). Deletion of both genes increased the sensitivity to H_2O_2 and *t*BOOH even further (Table III, line 4). This is consistent with the finding that some *TRX2* expression occurs in yap1$^-$ cells and therefore this low level of expression may be sufficient to mediate some response to the stress conditions, albeit an inefficient one. Deletion of the *TRX2* gene would eliminate this possibility. As described earlier, increased copy number of both *YAP1* and *TRX2* gave a hyper-resistance phenotype, in the case of *YAP1* to all four treatments and, in the case of *TRX2*, to H_2O_2 and *t*BOOH (Table III, lines 5 and 7). The effect of an increased copy number of *TRX2* was dependent upon the presence of YAP1 (Table II; Table III, compare lines 7 and 8). Similarly, we found that the effect of an increased copy number of *YAP1* was dependent upon the presence of *TRX2*; thus in a *TRX2* deletion strain, no increased resistance to H_2O_2 and *t*BOOH was seen, although there was increased resistance to diamide and diethylmaleate (Table III, compare lines 5 and 6).

Discussion

All organisms have evolved a number of cellular responses to allow them to cope with a variety of environmental stress conditions. These responses invariably involve the increased expression of a number of genes whose products counteract

Fig. 4. YAP1 dependent transcription of the *TRX2* gene. RNA samples from untreated and drug treated cells were prepared as described in Figure 1 and subjected to Northern blot analysis using a radioactive probe that specifically contained *TRX2* sequences. The same RNA samples were used for the analysis shown in this figure and in Figure 1.

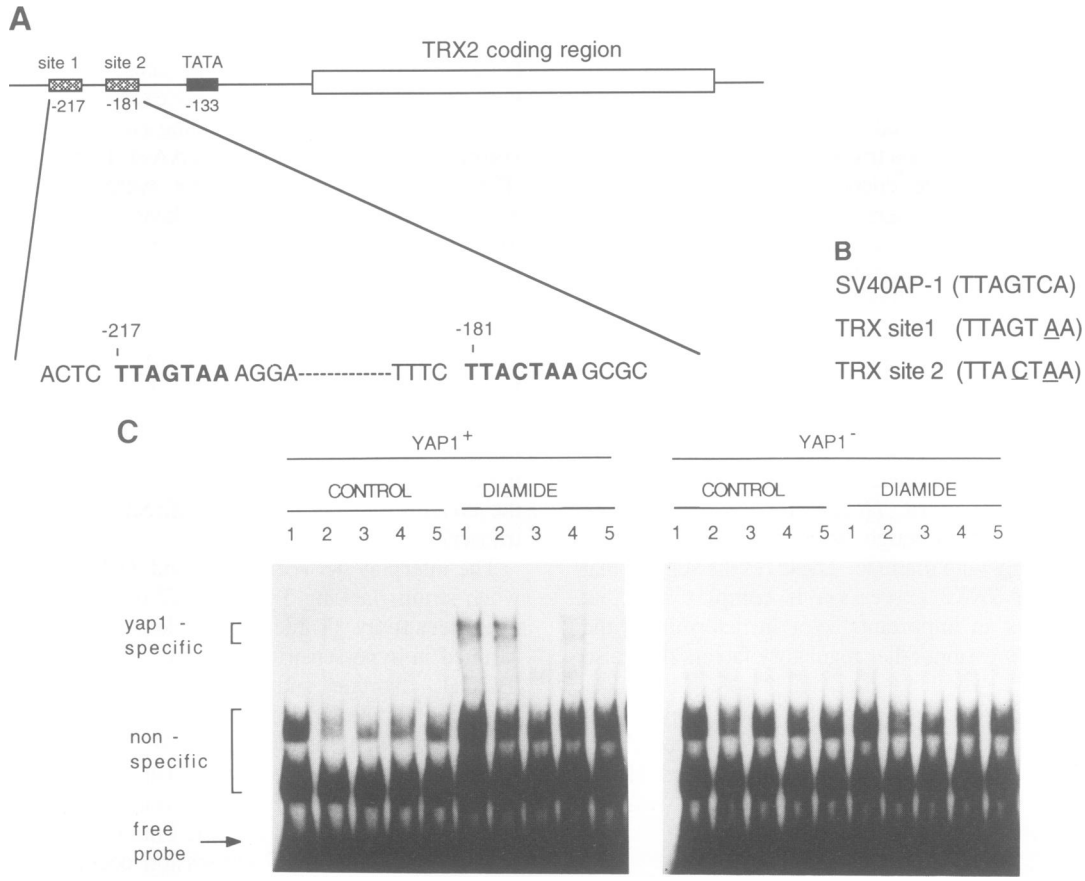

Fig. 5. The YAP1 protein can bind to sites within the *TRX2* promoter region. Inspection of the nucleotide sequences immediately upstream of the *TRX2* transcriptional start revealed the existence of two sequences resembling the SV40 AP-1 site. The sequence and position of these sites are indicated in (**A**), and a comparison with the SV40 site in (**B**). (**C**) Binding of YAP1 to these sites was assessed by a mobility shift assay using a 200 bp fragment of the TRX2 gene that contained both these sites as a probe. The assay was carried out as described in Materials and methods with either no competitor oligonucleotide present (lane 1) or a 50-fold excess of oligonucleotides containing a mutant SV40 AP-1 site (lane 2), wild-type SV40 AP-1 site (lane 3) or each of the two *TRX2* sites (site 1, lane 4; site 2, lane 5). The YAP1-specific complexes are indicated; they are missing in yap1$^-$ extracts.

the harmful effects of the particular conditions the cell is encountering. For example, the well characterized heat shock response which is universal among living organisms, involves the induction of a small set of proteins and is accomplished by an increase in the amount or activity of a particular transcription factor, the heat shock factor.

Previous studies, including the isolation of drug resistance genes, suggest that the YAP1 transcription factor of *S.cerevisiae* plays a crucial role in the yeast cell's response to a number of adverse environmental conditions and, in particular, the cellular response to conditions of oxidative stress (Hertle *et al.*, 1991; Hussain and Lenard, 1991; Schnell and Entian, 1991). Active oxygen species are produced as by-products of aerobic metabolism which can be further enhanced by exposure to certain environment conditions or, in humans, as a result of certain medical disorders. Such active oxygens are extremely reactive and, if allowed to accumulate, result in widespread damage to cellular macromolecules including DNA. It is not surprising therefore, that all aerobic organisms have evolved complex defence and repair mechanisms to negate the harmful effects of such oxygen species. In this study, we used a number of model compounds to generate oxidative stress and to analyse the antioxidant capacity of *S.cerevisiae* cells that lacked the *YAP1* gene. Diamide and diethylmaleate treatment results in decreased levels of intracellular GSH either by direct oxidation of GSH to form a disulfide bond (GSSG) or by forming GSH conjugates through the action of glutathione *S*-transferase. The ability of yeast to survive treatment with these chemicals was strongly dependent upon the *YAP1* gene: deletion of *YAP1* resulting in hypersensitivity whereas overexpression of *YAP1* resulted in hyperresistance. The same was true for resistance to H_2O_2 and the alkyl hydroperoxide *t*BOOH. Importantly, these treatments led to a significant increase in the activity of the YAP1 factor although the degree of activation varied. The results clearly imply that YAP1 acts as an important sensor in the response to oxidative stress and as such is likely to regulate the expression of one or more genes whose products help to protect against the potential damaging effects of such stress conditions.

A number of interesting similarities exist between the role of YAP1 in yeast and the cellular role of the equivalent mammalian DNA binding activity AP-1. Both transcriptional complexes appear to play an integral role in the response of the cells to certain kinds of environmental stress. For example, just as we have shown in this report that YAP1 activity is induced following exposure to H_2O_2, so it has been shown in previous reports that AP-1 activity is rapidly induced following the addition of H_2O_2 to mammalian cells (Devary *et al.*, 1991; Nose *et al.*, 1991; Amstad *et al.*, 1992). Additionally, in both cases induction involves a

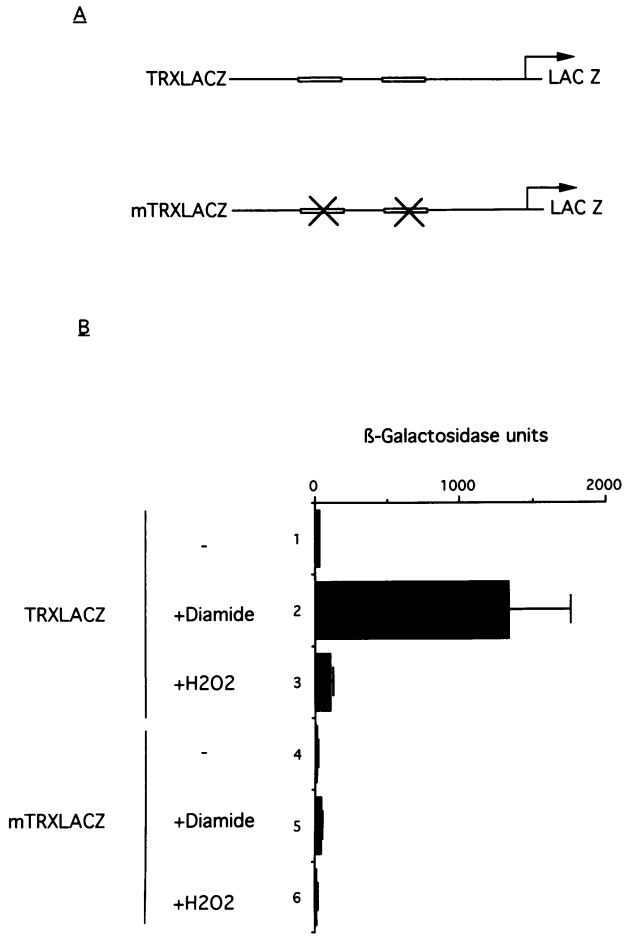

Fig. 6. The YAP1 binding sites are critical for *TRX2* promoter activity. (**A**) Schematic representation of two chimeric constructs that contain *TRX2* promoter sequences fused to the coding region of the *LACZ* gene. In mTRXLACZ there are single point mutations in each of the two YAP1 binding sites (see Materials and methods for details). (**B**) Activity of TRXLACZ and mTRXLACZ in the absence or presence of diamide or hydrogen peroxide. YAP1$^+$ yeast cells (W303B) transformed with the TRXLACZ or mTRXLACZ plasmids were cultured in SDA medium to an optical density of 1.0 at 600 nm, harvested and resuspended in the same medium or medium containing 1.5 mM diamide (lanes 2 and 5) or 1 mM H_2O_2. Incubation continued at 30°C for 1 h whereupon the cultures were tested for β-galactosidase activity as described in Materials and methods. Each point represents the mean +/− SEM of four experiments.

mechanism that does not require *de novo* protein synthesis but rather an increase in DNA binding activity of pre-existing protein. In both cases therefore, induced post-translational modification is likely to be involved. The c-Jun protein, a component of the AP-1 complex, is known to be regulated by phosphorylation. This regulation is complex with different phosphorylation events modulating either the DNA binding activity or the transcription activation activity of the protein (Binetruy *et al.*, 1991; Boyle *et al.*, 1991; Pulverer *et al.*, 1991; Lin *et al.*, 1992). We presume that *YAP1* is also regulated by phosphorylation, although a direct demonstration of this is currently lacking. A number of potential phosphorylation sites exist in *YAP1* but it is not known which, if any, are phosphorylated *in vivo*. However, preliminary analysis of a YAP1 mutant that lacks the C-terminal 11 amino acids suggests that like c-Jun, *YAP1* is regulated in a complex fashion (S.Kuge and N.Jones, unpublished observations). The C-terminal deletion resulted in a loss of transcription stimulation by H_2O_2 and diamide, although the response to diethylmaleate remained. With all three treatments, however, DNA binding activity was induced. Therefore, activation of *YAP1* involves more than just stimulation of DNA binding, suggesting the possibility that different post-translational modification events control different activities of the YAP1 protein.

The *TRX2* gene was found to be a direct target of the YAP1 factor. Sequences upstream of its transcription start site contain YAP1 binding sites and its pattern of expression mirrors the observed activity of YAP1. Thus expression is severely restricted in a yap1$^-$ strain and is highly induced in a YAP1$^+$ strain treated with H_2O_2, diamide or diethylmaleate. Additionally, studies with *TRX2* promoter−*LACZ* chimeric genes showed that the YAP1 binding sites are critical for basal and drug induced levels of expression. However, although different drugs induce *TRX2* expression, the consequence of such an increase to the cellular response is variable. Thus overexpression of *TRX2* can confer hyperresistance to H_2O_2 and *t*BOOH but not to diamide and diethylmaleate. Similarly, deletion of *TRX2* results in hypersensitivity to H_2O_2 and *t*BOOH. It is likely that YAP1 controls the expression of a number of target genes and that genes other than *TRX2* are critical for the cell's response to diamide and diethyl maleate. To date we have not iden-

Table III. Drug resistance phenotype of strains containing deletions or multiple copies of the *YAP1* and *TRX2* genes

	Half-drug concentrations of maximal inhibition (μM)[a]			
Genotypes[b] (plasmid)[c]	H_2O_2	*t*BOOH	Diamide	Diethylmaleate
WT (YEp13)	>1500	310	880	1750
trx2$^-$(YEp13)	630	310	880	1750
yap1$^-$(YEp13)	380	190	630	630
yap1$^-$ trx2$^-$(YEp13)	180	90	630	630
WT(pYAP1)	>1500	>500	>1500	1750
trx2$^-$(pYAP1)	880	310	>1500	1750
WT(pTRX2)	>1500	>500	880	1750
yap1$^-$(pTRX2)	380	190	630	630

[a]The drug resistance phenotype was determined as follows. The indicated strains were patched on SD agar plates and cultured at 30°C for 2 days. The patches were replica plated onto fresh plates containing the indicated drugs at various concentrations and cultured further, 2 days for H_2O_2, diamide or *t*BOOH and 3 days for diethylmaleate. The concentration indicated in the table represents 50% of the average between the maximum concentration tested upon which the yeast could grow and the lowest concentration tested which completely inhibited growth.
[b]DY was used for the YAP1$^+$ yeast (lines 1, 5 and 7). DWYU was used for the yap1$^-$ mutant (lines 3 and 8). DY trx was used for the trx2$^-$ mutant (line 2 and 6). DW trx2 was used for the trx2$^-$ yap1$^-$ double mutant (line 4).
[c]pYAP1 is GDR3 (see Table II), and pTRX2 is GDR19 (see Table II). YEp13 was used as a vector control.

tified other YAP1 targets. The clone GDR2, when present in high copy number, confers hyper-resistance to diamide. However, transcripts that hybridize to this clone were not sensitive to a deletion of *YAP1* indicating that it did not contain a target of this factor (our unpublished observations). Schnell *et al.* (1992) showed that the level of GSH as well as the activity of a number of important enzymes involved in the oxidative stress response such as G6PDH, glutathione reductase and superoxide dismutase were lower in a yap1$^-$ strain and increased in a strain carrying a multicopy plasmid containing *YAP1*. However, the question of whether any of the genes encoding these proteins were dependent upon YAP1 for expression was not addressed. There is a DNA sequence that resembles an SV40 AP-1 binding site ~300 nucleotides upstream of the G6PDH transcriptional start site. However, we found that the expression of this gene was not affected either by a *YAP1* gene disruption or by treatment with hydroperoxides, diamide or diethylmaleate (our unpublished observations).

How increased thioredoxin expression gives rise to H_2O_2 and *t*BOOH hyper-resistance is not clear. Thioredoxins are ubiquitous in both prokaryotes and eukaryotes and a number of activities have been ascribed to them. They can serve as hydrogen donors for ribonucleotide reductase (Laurent *et al.*, 1964) and methionine sulfoxide reductase (Gonzalez Porque *et al.*, 1970; Bort and Weissbach, 1992) as well as activators of a number of mammalian proteins such as the glucocorticoid receptor (Grippo *et al.*, 1985) and the IL-2 receptor (Tagaya *et al.*, 1989). Additionally, there are an increasing number of examples where the activity of DNA or RNA binding proteins is dependent on their redox status, for example AP-1 (Abate *et al.*, 1990; Frame *et al.*, 1991), NF-κB (Toledano and Leonard, 1991) and IRE-BP (Hentze *et al.*, 1989). Whether thioredoxin plays any *in vivo* role in maintaining the redox status of these factors remains to be seen. With respect to the cellular response to oxidative stress, however, two activities of thioredoxin are likely to be of utmost importance. Human thioredoxin has been shown to be able to reduce certain reactive oxygen species (Mitsui *et al.*, 1992). Thus it can help to protect cells from oxidative stress by scavenging some of the harmful oxygen species that accumulate. Secondly, thioredoxin could help repair some of the damage caused by oxidative stress by regenerating inactivated proteins. Thioredoxin can act catalytically as a protein oxidoreductase and thus regenerate enzymes damaged by oxidation of critical cysteine residues. Such regeneration has recently been shown in *in vitro* studies (Fernando *et al.*, 1992; Mitsui *et al.*, 1992). Interestingly, it has also been shown recently that in mammalian cells the level of a thioredoxin protein is induced on implementation of oxidative stress such as by the addition of H_2O_2. Once again, it suggests a degree of conservation in the response of cells to such conditions and that thioredoxin plays a major role in all eukaryotic cells in mediating this response.

The YAP1 protein is not only involved in the oxidative stress response but also in the response to a varied range of toxic conditions. The same may be true of the homologous protein from *Schizosaccharomyces pombe*, pap1, which has a very similar binding specificity to YAP1 (Toda *et al.*, 1991). Pap1 was isolated as a gene that, when present in high copy number, could confer resistance to a toxic kinase inhibitor staurosporin. Thus in *S.cerevisiae, S.pombe* and in mammalian cells, transcription complexes with similar DNA binding specificity are involved in important host defence mechanisms. It will be of considerable interest to identify additional targets of these proteins.

Materials and methods

Yeast strains, media and drugs

Saccharomyces cerevisiae strains used in this study are listed in Table IV. The strains were grown either on YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) supplemented with 55 μg/ml of adenine, or minimal SD medium (0.67% yeast nitrogen base, 2% glucose) supplemented as described previously (Ausubel *et al.*, 1987) with either 0.5% casamino acids (SDA) or individual amino acids as required. Plates contained the same media together with 2% agar. Hydrogen peroxide, *t*BOOH, diamide and diethylmaleate were purchased from Sigma.

Determination of drug resistance

For the determination of half-drug concentration of maximal inhibition, yeast strains were grown on YPD or SD agar plates and cultured at 30°C for 2 days. The yeast patches were replica plated onto plates containing various concentrations of drugs, and cultured further: 2 days in the case of H_2O_2, diamide or *t*BOOH and 3 days in the case of diethylmaleate. For the determination of IC_{50} values, log phase cultures of yeast strains were harvested and inoculated into medium containing different drugs at various concentrations. Following incubation at 30°C for 15 h, growth was assessed by OD measurement. The IC_{50} value was calculated from the probit curve.

Construction of yeast strains containing the LacZ gene

A derivative of the plasmid pLG559Δ-312 (Guarente and Mason, 1983) was constructed and contained three SV40 AP-1 binding sites upstream of the *LacZ* coding region. The *SmaI*−*SphI* fragment of this plasmid which contains the CYC1 wild-type UAS elements was replaced with an oligonucleotide linker of the following sequence: 5′-GGGTCTAGAGTC-

Table IV. *S.cerevisiae* strains used in this study

Strain	Genotype	Reference/source
W303B	*MATα his3 can1-100 ade2 leu2 trp1 ura3*	R.Rothstein
WYU	*MATα his3 can1-100 ade2 leu2 trp1 ura3 yap1::URA3*	a derivative of W303B containing a URA3 disrupted *YAP1* gene
DY	*MATα his3 can1-100 ade2 leu2 trp1 ura3*::(3xSV40AP1-LAcZ)	a derivative of W303B containing *LacZ* inserted into the *URA3* gene
DWYU	*MATα his3 can1-100 ade2 leu2 trp1 ura3*::(3xSV40AP1-LacZ) *yap1::URA3*	a derivative of WYU containing *LacZ* inserted into the *URA3* gene
WYT	*MATα his3 can1-100 ade2 leu2 trp1 ura3 yap1::TRP1*	a derivative of W303B containing a *TRP1* disrupted *YAP1* gene
DYtrx2	*MATα his3 can1-100 ade2 leu2 trp1 ura3*::(3xSV40AP1-LacZ) *trx2::HIS3*	a derivative of DY containing a *HIS3* disrupted *TRX2* gene
DWtrx2	*MATα his3 can1-100 ade2 leu2 trp1 ura3*::(3xSV40AP1-LacZ) *yap::URA3 trx2::HIS3*	a derivative of DW containing a *HIS3* disrupted *TRX2* gene

GACCCATCTCAATTAGTCAGCAAGCCATCTCAATTAGTCAGCA-AGCCATCTCAATTAGTCAGCAGCATG-3'.

The consensus SV40 AP1 binding sites are underlined. An *Sma*I−*Nco*I fragment from this plasmid containing the *LacZ* coding region, CYC1 basal promoter and the SV40 AP-1 binding sites was inserted into the *Stu*I site in the middle of the *URA3* gene. Strain W303B (see Table IV) was transformed with a fragment containing the disrupted *URA3* gene together with a plasmid containing the *LEU3* marker. *LacZ*$^+$ transformants were selected from a *Leu*$^+$ transformant pool by a β-galactosidase filter assay (Breeden and Nasmyth, 1987; Dalton and Treisman, 1992).

Construction of TRX−LACZ fusions

Wild-type and mutant *TRX2* promoter fragments were generated by the polymerase chain reaction (PCR) and cloned into a vector containing the coding region of *LACZ*. The four primers used for the PCR reactions had the following sequence:

Primer 1 5'-GTCACCCGGGATCCAGACTTTTACGGGTGGCA-3'
Primer 2 5'-GTCAGGATCCAGTGACCATTATTGATGTGTTA-3'
Primer 3 5'-TGTAGGGAGCATCCTTTCCTAAGAGTATAA-3'
Primer 4 5'-GCTCCCTACAAGGTGGCTCTTTTCTTCCTAAGCGCG-3'

The wild-type *TRX2* promoter fragment containing sequences from −497 to +9 (+1 representing the start of translation) was generated by PCR using primers 1 and 2 with the *TRX2* gene as the template. The primers were designed to introduce *Sma*I and *Bam*HI restriction sites at the 5' and 3' ends of the fragment, respectively, to allow efficient cloning into *SmaI*/*Bam*HI digested K7 vector giving the plasmid TRXLACZ. This vector (to be described in detail elsewhere, S.Kuge, in preparation) contains the *URA3* marker and CEN-ARS control sequences as well as the *LacZ* coding region. Insertion into the *Sma*I−*Bam*HI sites results in a fusion of the promoter to the *LacZ* sequence. A mutant promoter fragment containing single point mutations in each of the two YAP1 binding sites was generated by first synthesizing subfragments of the promoter using primers 1 and 3 and primers 2 and 4. Primer 3 contains a substitution of A to C at position −213 within YAP1 binding site 1 and primer 4 contains a substitution of A for C at position −179 within YAP1 binding site 2. The PCR reactions generated two fragments of 302 bp (−497 to −196) and 214 bp (−170 to +9) which were purified, mixed and used as templates for a PCR reaction primed with primers 1 and 2. A 506 bp fragment was generated containing base substitutions at −213 and −179. This was cloned into the *SmaI*/*Bam*HI restricted K7 vector to generate mTRXLACZ.

Gene disruption and yeast transformation

To make a yap$^-$ disruption mutant, the region encoding the DNA binding domain of *YAP1* was replaced by either the *URA3* or the *TRP1* gene. An *Sph*I−*Eco*RI 2.5 kb genomic DNA fragment containing *YAP1* was subcloned into pUC19 [pUC-YAP1(S-E)], and a 1.2 kb *Hin*dIII fragment containing the *URA3* gene or a 0.95 kb *Aat*II−*Bgl*II fragment containing the *Trp1* gene was inserted between the *Bam*HI and *Hpa*I sites located in the DNA binding domain of *YAP1* (Moye-Rowley *et al.*, 1989). W303B or DY cells (see Table IV) were transformed with a *Hin*dII−*Eco*RI fragment containing the disrupted *YAP1* gene and stable Ura$^+$ or Trp$^+$ transformants were selected. The *HIS3* marker gene was used to disrupt the *TRX2* gene. A 1.7 kb *Bam*HI fragment containing the *HIS3* gene was inserted into the *TRX2* gene between positions −27 and +517. A 4 kb genomic fragment containing the disrupted *TRX2* gene was used to transform DY or DWYU cells, and His$^+$ transformants were selected. All these stable transformants were subjected to genomic Southern hybridization analysis to verify correct disruption.

Yeast spheroplast transformation was carried out as previously described (Burgess and Perceval, 1991).

Northern blot analysis

Yeast strains were cultured to log phase (A_{600} = 0.5−0.8) in SD medium, and cells precipitated. The yeasts were resuspended in the same volume of fresh medium and aliquoted. Various drugs were added to each aliquot and incubated for a further 1 h at 30°C. Each aliquot normally consisted of 20 ml of culture. Total RNAs were prepared from each aliquot as described previously (Ausubel *et al.*, 1987). RNA samples (5 μg) were denatured and separated on 1.2% agarose gels containing 2.2 M formaldehyde (Ausubel *et al.*, 1987). The RNA was transferred to Hybond-N (Amersham) filters and hybridization was carried out in 50% formamide, 5 × Denhardt's, 5 × SSC, 0.1% SDS, 125 μg/ml denatured salmon testis DNA at 42°C for 12−16 h. The filters were washed four times in 0.5 × SSC, 0.1% SDS at room temperature followed by one wash at 50°C for 30 min.

β-galactosidase assays

Relative β-galactosidase levels were measured as described previously (Harshman *et al.*, 1988) and expressed as units where one unit = 1000 $A_{420}(CVt)^{-1}$ with A_{420} being the absorbance at 420 nm, C being the density of cell suspension (mg/ml) determined by a protein assay using BSA as a standard, V being the volume of cell suspension (ml) and t being the total incubation time of the assay.

Cloning of genes that can confer hyper-resistance to the drugs

DY (YAP1$^+$), was transformed with an *S.cerevisiae* genomic library constructed in YEp13 (provided by P.Russell). Leu2$^+$ transformants were pooled and replated onto SD plates containing diamide (1.5 or 2.0 mM) or *t*BOOH (0.3 or 0.5 mM) and incubated at 30°C for 6 days. Plasmid DNA was recovered from viable colonies and analysed by restriction enzyme analysis.

Characterization of GDR19

The plasmid GDR19 was found to confer hyper-resistance to *t*BOOH in the YAP1$^+$ DY strain. It contained a 3.5 kb *Bam*HI−*Eco*RI genomic fragment. This fragment was subcloned into the plasmid pUC19. A series of deletions were generated by exonuclease III followed by exonuclease VII treatment. The end points of the deletions were determined by restriction endonuclease digestion and direct DNA sequencing and the deleted inserts transferred into YEp24. The resulting derivatives were transformed into DY cells in order to assay their ability to confer hyper-resistance.

Preparation of yeast extracts and electrophoresis mobility shift assays

Saccharomyces cerevisiae total extracts were prepared by glass bead disruption as described previously (Ausubel *et al.*, 1987), except for the use of a modified breakage buffer containing 200 mM Tris−HCl, pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 500 mM TPCK and 25 mM TLCK. Normally extracts were prepared from 25−50 ml of yeast culture that was treated with various drugs as described above for the isolation of RNA samples. The total extracts were used for gel electrophoresis mobility shift assays (EMSA) without further purification. The following oligonucleotides were used as probes or competitors for the EMSA.

mSV40AP-1 5'-GATCCATCTCAATTAGGAAGCAAG -3'
3'- GTAGAGTTAATCCTTCGTTCCTAG-5'

TRX2 site 1 5'-GATCCATACTCTTAGTAAAGGATG -3'
3' GTATGAGAATCATTTCCTACCTAG-5'

TRX2 site 2 5'-GATCCTCTTTTCTTACTAAGCGCG -3'
3' GAGAAAAGAATGATTCGCGCCTAG-5'

The wild-type SV40 AP-1 oligonucleotide was as described previously (Jones *et al.*, 1988), as was the E4ATF oligonucleotide (Jones and Jones, 1989). In some assays, a 200 bp fragment from the *TRX2* promoter was used. Probes were 3' end labelled with [α-^{32}P]dATP and [α-^{32}P]dCTP by reverse transcriptase. Each binding assay contained 1 ng of probe (~10 ng for the 200 bp *TRX2* promoter fragment), 2 μg of poly(dI)·poly(dC) non-specific competitor and 30 μg of the extract in a buffer containing 20 mM Tris−HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM PMSF and 10% glycerol. Specific competitors (50 ng) were added to the mixture when required. The binding assay was started by the addition of the probe and binding continued at room temperature for 15 min. The reaction mixtures were subjected to electrophoresis in 6% polyacrylamide gels (11 parts acrylamide:0.8 parts *bis*-acrylamide) prepared in 0.5 × TBE buffer.

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