Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8–Tup1 corepressor complex to differentially regulated promoters

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The yeast Cyc8(Ssn6)–Tup1 complex is required for transcriptional repression of distinct sets of genes that are regulated by glucose, oxygen, cell type, and DNA damage. It has been proposed that the Cyc8–Tup1 complex is a corepressor that is recruited to promoters by interacting with pathway-specific DNA-binding proteins. Previously, we showed that a specific region of Tup1 mediates the general transcriptional repression function of the complex. Here, we define functional domains of Cyc8, a protein consisting primarily of 10 tandem copies of a TPR motif. Distinct combinations of TPR motifs are required specifically for direct interaction with Tup1, repression of oxygen-regulated genes, and repression of glucose-regulated genes. In contrast, the WD motifs of Tup1 are not essential for repression of genes regulated by glucose and oxygen, but they are required for those regulated by cell type and DNA damage. In addition, we show that the Cyc8–Tup1 complex functions both as a corepressor and an inhibitor of Mig1, a protein that binds to promoters of glucose-repressible genes. These observations suggest that different Cyc8 TPR motifs and the Tup1 WD domain mediate distinct protein–protein interactions that link the Cyc8–Tup1 corepressor to structurally dissimilar DNA-binding proteins required for pathway-specific regulation.

[*Key Words*: TPR motifs; Cyc8(Ssn6)–Tup1 complex; transcriptional repression; corepressor; eukaryotic gene regulation]

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Eukaryotic organisms have a variety of global repression mechanisms that negatively regulate the transcription of many apparently unrelated genes. One such mechanism, chromatin-based repression, involves histones (Grunstein, 1990) and nonhistone proteins such as Spt4–6 (Swanson and Winston 1992). A second global repression mechanism involves proteins that interfere directly with components of the basic transcription machinery. For example, human Dr1 (Inostroza et al. 1992) and yeast Mot1 (Auble et al. 1994) block the interaction of the TATA-binding protein with the TATA element, and the yeast NOT complex differentially affects TATA element utilization (Collart and Struhl 1994). Although chromatin and direct inhibitors of basic transcription factors should repress all genes, there is considerable variation in the extent to which individual genes are affected. In both cases, however, there is no clear pattern that distinguishes genes that are strongly repressed from those that are not affected. In contrast, there is a third mechanism, exemplified by the yeast Cyc8–Tup1 (see below) and the *Drosophila* polycomb (Paro 1990) complexes, in which specific but apparently unrelated classes of genes are repressed. This more specific form of global repression has the potential to coordinate the regulation of distinct biological pathways.

Cyc8(Ssn6) and Tup1 are physically associated proteins (Williams et al. 1991) that are required for repression of at least four unrelated classes of yeast genes. These include genes regulated by cell type (**a**-specific and haploid-specific) (Mukai et al. 1991; Keleher et al. 1992), glucose (Schultz and Carbon 1987; Trumbly 1992), oxygen (Zitomer and Lowry 1992), and DNA damage (Elledge et al. 1993). Although the Cyc8–Tup1 complex does not directly bind to promoter sequences, repression of these distinct classes of genes does require pathway-specific DNA-binding proteins. For example, Mig1 (Nehlin and Ronne 1990), Rox1 (Balasubramanian et al. 1993), and α2 in combination with Mcm1 (Keleher et al. 1988) or **a**1 (Goutte and Johnson 1988) bind, respectively, to the promoters of glucose, oxygen, and cell type-regulated genes. The Cyc8–Tup1 complex is clearly involved in α2-dependent repression (Keleher et al. 1992), whereas the evidence for its role in Mig1- and Rox1-dependent repression is only circumstantial.

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Several observations strongly suggest that the Cyc8–Tup1 complex is a corepressor that is recruited to promoters via pathway-specific DNA-binding proteins, whereupon it then represses transcription (Keleher et al. 1992). First, α2/Mcm1, the best characterized DNA-binding repressor, binds to its operator in vivo even in the absence of Cyc8–Tup1, but this binding is not sufficient for repression (Keleher et al. 1992). Second, artificial promoters bearing a1–α2 or α2–Mcm1 operators upstream of heterologous upstream activating sequences (UASs) are strongly repressed in a Cyc8–Tup1-dependent manner, indicating that the Cyc8–Tup1 complex does not inhibit the function of specific activators (Keleher et al. 1992). Third, α2 directly interacts with Tup1, and this interaction is required for repression of cell type-specific genes (Komachi et al. 1994). Fourth, although Cyc8 and Tup1 do not bind directly to DNA, both proteins strongly repress transcription when bound upstream of a functional promoter via the LexA DNA-binding domain (Keleher et al. 1992; Tzamarias and struhl 1994). Such negative regulation at a distance is characteristic of glucose and cell type repression (Johnson and Herskowitz 1985; Struhl 1985), and it suggests that the Cyc8–Tup1 complex inhibits transcription by an active mechanism rather than by steric hindrance.

Analysis of LexA hybrid proteins indicates that the transcriptional repression function of the Cyc8–Tup1 complex is mediated by a specific domain of Tup1 (Tzamarias and Struhl 1994). When tethered upstream of a promoter, this Tup1 repression domain does not require Cyc8 to inhibit transcription. Moreover, this domain is required for repression of genes regulated by glucose (*SUC2*) and oxygen (*ANB1*). Within this domain, short nonoverlapping regions with minimal sequence similarity can mediate the repression function independently. Although the nature of the repression mechanism is unknown, there is evidence both for effects on the chromatin template (Cooper et al. 1994) and on the basic transcription machinery (Herschbach et al. 1994; Tzamarias and Struhl 1994).

In addition to this repression domain, Tup1 contains a separable amino-terminal region (residues 1–72) that directly interacts with Cyc8 (Tzamarias and Struhl 1994) and a carboxy-terminal domain that contains six copies of a WD (also known as β-transducin) sequence motif (Williams and Trumbly 1990). WD repeats are present in many proteins that are involved in diverse cellular processes, and they have been suggested to mediate protein–protein interactions. The role of the Tup1 WD domain is complex. The Tup1 WD motifs interact directly with α2 and are required for repression of cell type genes (Komachi et al. 1994), and some mutations in the WD domain appear to abolish Tup1 function (Williams and Trumbly 1990; Tzamarias and Struhl 1994). However, a derivative that completely lacks the WD domain can repress *SUC2* and *ANB1* transcription and mediate other Tup1 activities (Tzamarias and Struhl 1994).

Although repression of cell type genes involves the direct interaction of α2 with the Tup1 WD motifs (Komachi et al. 1994), the mechanisms by which the Cyc8–Tup1 corepressor is recruited to other classes of promoters is unknown. In this regard, there are no obvious structural similarities among α2, Mig1, and Rox1. Previously, we suggested that Cyc8 might be involved in the recruitment process based on the facts that Cyc8 and the Cyc8–interaction domain are not required for repression by LexA–Tup1, but both are essential for repression of *SUC2* and *ANB1* (Tzamarias and Struhl 1994). The functionally important region of Cyc8 contains 10 copies of a 34-amino-acid TPR motif (specific motifs are defined herein as TPR1–10 starting from the amino-terminus) (Schultz et al. 1990). As is the case for WD motifs, TPR motifs are present in functionally diverse proteins that are often associated in protein complexes, and they have been proposed to mediate protein–protein interactions (Hirano et al. 1990; Sikorski et al. 1990; Goebl and Yanagida 1991; Lamb et al. 1994). However, there is no evidence that TPR motifs interact directly with other proteins, and their specific biochemical or physiological functions are unknown.

Here, we perform a functional analysis of Cyc8 by assaying a set of deletion mutations for interaction with Tup1 and for repression of natural and artificial promoters. We demonstrate that distinct combinations of TPR motifs are required specifically for direct interaction with Tup1, repression of oxygen-regulated genes, and repression of glucose-regulated genes. In addition, we show that Mig1 is a functional target of the Cyc8–Tup1 complex and that Cyc8–Tup1 functions both as a corepressor and as a transcriptional inhibitor of Mig1. We propose that different Cyc8 TPR motifs and the Tup1 WD domain mediate distinct protein–protein interactions whose cooperative function recruits the corepressor complex to pathway-specific promoters that are recognized by structurally dissimilar DNA-binding proteins.

Results

A specific region of the Cyc8 TPR domain associates directly with Tup1

The two-hybrid assay for protein–protein interactions was employed to map the region of Cyc8 that interacts with Tup1 (Table 1; Fig. 1). LexA–Cyc8 derivatives were introduced into a yeast strain that expresses a hybrid protein containing Tup1 residues 1–72 fused to the VP16 transcriptional activation domain. This region of Tup1 is necessary and sufficient for interacting with Cyc8, but it does not mediate transcriptional repression (Tzamarias and Struhl 1994). Interaction between Tup1 and Cyc8 regions in the hybrid proteins generates a transcriptional activator that stimulates a target promoter containing four LexA operators upstream of the *GAL1* TATA element.

As expected, the combination of Tup1N72–VP16 and LexA–Cyc8 confers 11-fold higher expression levels than either protein alone. Similar or even higher activation was observed with all derivatives that include TPR motifs 1–3 (N175, N300, N351, N597, and N816). In contrast, derivatives containing TPR1 (e.g., N98) or TPR2–7

Table 1. *Two-hybrid assays for Cyc8-Tup1 interaction*

LexA hybrid proteins	Fold activation (WT)	Fold activation (*cyc8*)	VP16 hybrid proteins (chromosomal *CYC8* allele) VP16 (WT)	Tup1 (WT)	Tup–VP16 (WT)	VP16 (*cyc8*)	Tup1 (*cyc8*)	Tup–VP16 (*cyc8*)
Cyc8	11	8.8	4	4	43	2	4	35
Cyc8–N816	24	15	6	4	95	3	4	61
Cyc8–N597	45	63	5	10	450	3	4	250
Cyc8–N351	36		7	10	360			
Cyc8–N300	40		7	6	240			
Cyc8–N175	22	32	4	6	130	4	3	95
Cyc8–N98	2.4	1.7	6	7	17	2	4	7
Cyc8–(79–300)	1.7	2.5	4	6	10	3	4	10
Cyc8–(113–300)	1.2		4	5	6			
LexA	1	1.7	6	5	5	3	3	5
Vector	1	2.5	6	5	5	3	2	5

β-Galactosidase activities (average of three independent transformants) of wild-type (WT) or *cyc8* deletion strains expressing the indicated proteins. The *lacZ* reporter plasmid contains four LexA operators upstream of the *GAL1* TATA element. Values are normalized to A_{600} of cells at the time of collecting and are accurate to ±30%. Fold activation represents the ratio of β-galactosidase activities in strains containing Tup1–VP16 vs. those containing Tup1. Nonrecorded entries indicate that the experiment was not performed.

(79–300) do not confer higher expression levels than that conferred by the LexA control. In all cases, transcriptional stimulation was specific for Tup1N72–VP16; it was not observed with Tup1N72 or the VP16 activation domain alone. Similar results were obtained in *cyc8* or *tup1* deletion strains, indicating that the observed interactions are not affected significantly by the the presence of wild-type Cyc8 or Tup1. These results demonstrate that the amino-terminal 175 residues of Cyc8 (TPR1–3) are necessary and sufficient for formation of the Cyc8–Tup1 complex in vivo.

To determine whether this two-hybrid interaction reflected direct contact between the proteins, we examined whether Cyc8–N175 could associate with Tup1 in vitro in the absence of additional yeast proteins (Fig. 2). Agarose beads containing glutathione *S*-transferase (GST) or GST–Tup1 were tested for interaction with in vitro-labeled Cyc8–N175 or Cyc8–(79–300). In accord with the results from the two-hybrid assay, GST–Tup1, but not GST, strongly interacts with Cyc8–N175, whereas no interaction is observed between GST–Tup1 and Cyc8–(79–300). Similar results were obtained using GST–Tup1–N72 (data not shown). These results indicate that there is a direct protein–protein interaction between the amino terminal 72 residues of Tup1 and the amino terminal residues of Cyc8 (TPR1–3).

Cyc8 represses transcription by recruiting Tup1

The various LexA–Cyc8 derivatives were analyzed for

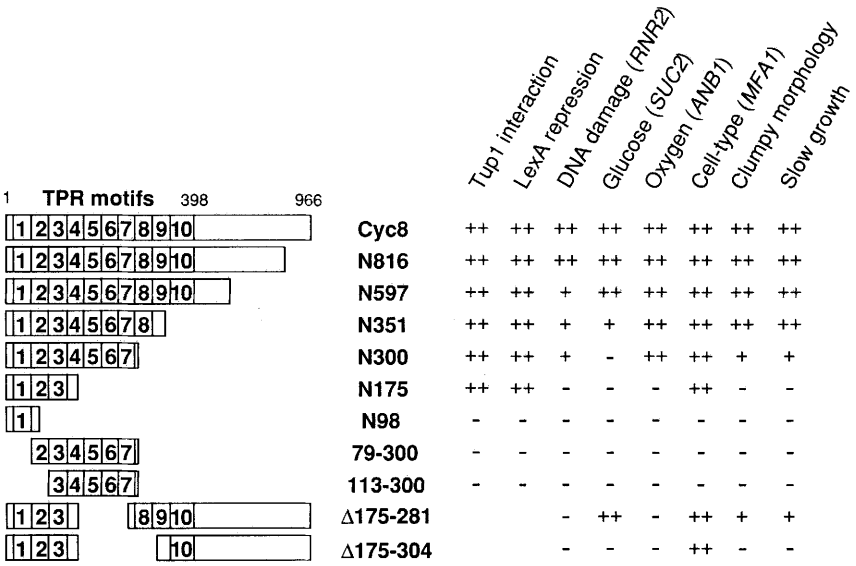

Figure 1. Structure and function of Cyc8 deletion derivatives. The structures of Cyc8 (966 amino acids, including 10 copies of a TPR motif numbered from the amino terminus) and deletion derivatives are indicated along with the intact TPR units that are present. For each derivative, the following phenotypic properties are indicated: Tup1–interaction (Table 1); LexA-dependent repression (Table 2); repression of genes regulated by DNA damage (*RNR2*), glucose (*SUC2*), oxygen (*ANB1*), and cell type (*MFA1*); and slow growth and clumpy colony morphology (determined by inspection). Phenotypes are defined as follows: (++) Functionally indistinguishable from wild-type allele (*CYC8*); (+) partial function; (−) functionally indistinguishable from *cyc8* deletion allele.

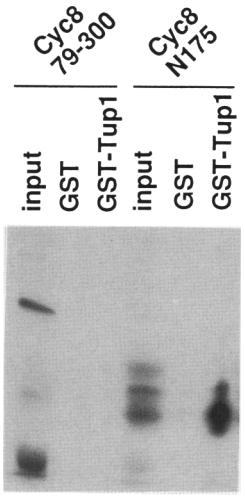

Figure 2. Cyc8–Tup1 interaction in vitro. ^{35}S-Labeled Cyc8–(79–300) or Cyc8–N175 stably bound to agarose beads containing either GST–Tup1 or GST alone. Lanes labeled input contain only 20% of the amount of protein that was incubated with the beads.

transcriptional repression of an artificial promoter that bears four LexA-binding sites upstream of the *CYC1* UAS and TATA elements (Table 2; Fig. 1). As reported previously (Keleher et al. 1992; Tzamarias and Struhl 1994), LexA–Cyc8 represses transcription 27-fold in a Tup1-dependent manner. All LexA–Cyc8 derivatives interacting with Tup1 (i.e., that contain ≥175 amino-terminal residues) also repress transcription, although some of them are slightly less efficient than LexA–Cyc8. However, LexA–Cyc8 derivatives that fail to interact with Tup1 (e.g., Cyc8 residues 1–98 or 79–300) do not confer repression. Similar results are obtained by testing these constructs in a *cyc8* deletion strain. In a *tup1* deletion strain, repression by the LexA–Cyc8 derivatives does not occur, and unexpectedly, several of the deleted derivatives actually activate transcription. Thus, when tethered upstream of a functional promoter, the Tup1–interaction domain of Cyc8 (TPR1–3) is necessary and sufficient for Tup1-dependent repression. This observation suggests that LexA–Cyc8 represses transcription simply by recruiting Tup1.

Distinct TPR motifs of Cyc8 are required for repression of pathway-specific genes

After removing the LexA domain, we tested the ability of the above Cyc8 deletion derivatives to repress transcription of genes representing the four regulatory pathways affected by Cyc8–Tup1: cell type (*MFA1*); glucose (*SUC2*); oxygen (*ANB1*); and DNA damage (*RNR2*). All of these genes are highly expressed in a *cyc8* or *tup1* deletion strain, but they are silent (or expressed at a low level in the case of *RNR2*) in a wild-type strain (Fig. 3). The 150 carboxy-terminal residues of Cyc8 do not appear to be functionally important because the N816 derivative behaves indistinguishably from the wild-type protein. In contrast, derivatives that fail to interact with Tup1 (N98, 79–300, 113–300) are unable to repress transcription of any of these genes.

The most interesting class of Cyc8 derivatives are those carboxy-terminal deletions that differentially affect the pathway-specific genes. Cyc8–N597, a derivative containing the entire TPR domain but lacking the carboxy-terminal 369 residues, efficiently represses *MFA1*, *ANB1*, and *SUC2* but only partially represses *RNR2*. Cyc8–N351, which contains TPR1–8 and most of TPR9, completely represses *MFA1* and *ANB1* but only partially represses *SUC2* and *RNR2*. Cyc8–N300, which contains TPR1–7, represses *MFA1* and *ANB1* but not *RNR2* and *SUC2*. Finally, Cyc8–N175, a derivative that

Table 2. *Transcriptional repression by LexA–Cyc8 derivatives*

LexA hybrid proteins	Fold repression			Promoter (strain background)					
	(WT)	(*cyc8*)	(*tup1*)	−Lop (WT)	4 Lop (WT)	−Lop (*cyc8*)	4 Lop (*cyc8*)	−Lop (*tup1*)	4 Lop (*tup1*)
Cyc8	27	29	2.4	60	2.3	58	2.0	29	12
Cyc8–N816	25	30	0.6	85	3.4	62	2.1	42	64
Cyc8–N597	21	16	0.7	92	4.3	55	3.5	47	65
Cyc8–N351	32	26	0.4	100	3.1	57	2.2	42	97
Cyc8–N300	30	13	0.4	85	2.8	51	4.0	39	85
Cyc8–N175	12	15	1.0	80	6.6	46	3.1	57	58
Cyc8–N98	1.8	1.9	0.8	84	46	48	25	61	74
Cyc8–(79–300)	0.9	0.9	0.9	64	70	49	52	60	68
Cyc8–(113–300)	1.0	1.0	0.8	72	71	55	54	55	69
LexA	1.2	1.1	1.3	100	80	55	50	90	70
Vector	1.2	1.0	1.3	100	85	55	55	90	70

β-Galactosidase activities (average of three independent transformants) of wild-type (WT), *cyc8*, or *tup1* strains containing the indicated promoters and expressing the indicated proteins. Values are normalized to A_{600} of cells at the time of collecting and are accurate to ±30%. Fold repression represents the ratio of β-galactosidase activities in strains containing plasmids that either lack (−Lop) or contain four LexA operators (4 Lop) upstream of the *CYC1* promoter fused to the *lacZ* structural gene.

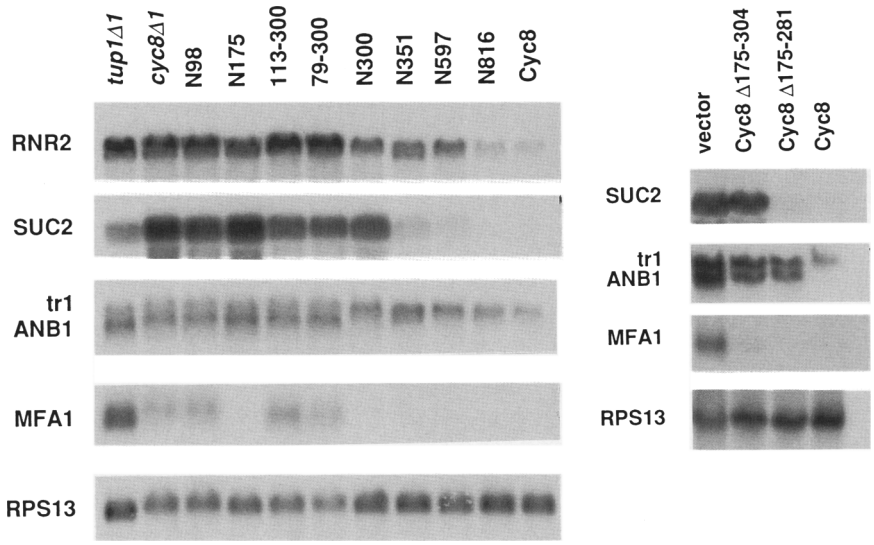

Figure 3. Transcriptional repression of *RNR2*, *SUC2*, *ANB1*, and *MFA1* genes by various *Cyc8* derivatives. RNA from *cyc8* deletion strains transformed by the indicated Cyc8 derivatives was fractionated in 1.4% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled probes specific for *RNR2*, *SUC2*, *ANB1*, *MFA1*, and *RPS13* (which serves as an internal control). Expression of *RNR2* is identical in the two internal deletions and the strain that contains only vector sequences (not shown). The *TR1* and *ANB1* transcripts are not related.

contains the minimal Tup1–interaction domain (TPR1–3), fully represses *MFA1* but does not affect expression of the other genes. Thus, specific repression functions of Cyc8 are progressively abolished as sequences are deleted from the carboxyl terminus.

The simplest explanation for these observations is that distinct subsets of TPR motifs are required for repression of the different classes of genes affected by the Cyc8–Tup1 corepressor. Alternatively, the different classes of genes might simply require quantitatively different amounts of a common TPR function. To distinguish between these possibilities, we analyzed two internal deletions, Δ175–281 and Δ175–304, which lack TPR4–7 and TPR4–8, respectively (Figs. 1 and 3). Both of these derivatives contain the Tup1–interaction domain, and as expected, they repress the transcription of *MFA1*. Neither derivative can repress *ANB1*, but interestingly, the Δ175–281 (but not the Δ175–304) derivative represses transcription of *SUC2* completely. Thus, with respect to repression of *ANB1* and *SUC2*, the Δ175–281 and N301 derivatives have opposite phenotypes.

These results suggest that besides Tup1 and the Tup1–interaction domain of Cyc8, discrete combinations of TPR motifs of Cyc8 are employed for repression of specific sets of promoters. TPR8, TPR9, and possibly TPR10, but not the region containing TPR4–7, are required for glucose repression. In contrast, TPR4–7, but not TPR8–10, are required for oxygen repression. Finally, repression of **a**-specific genes requires only the Tup1–interaction domain of Cyc8 (TPR1–3), whereas repression of DNA damage-regulated-genes appears to require the entire TPR domain and more carboxy-terminal sequences.

Functional interaction between Mig1 and the Cyc8–Tup1 corepressor

Mig1, a zinc finger repressor protein that binds to the promoters of *SUC2* and other glucose-repressible genes (Nehlin and Ronne 1990), has been proposed to be a target of the Cyc8–Tup1 corepressor complex (Keleher et al. 1992). To test this hypothesis, we examined whether the transcriptional repression by a LexA–Mig1 hybrid protein was affected by Cyc8 and Tup1 (Table 3). As shown previously for LexA–Cyc8 and LexA–Tup1 (Table 2; Tzamarias and Struhl 1994; Keleher et al. 1992), LexA–Mig1 represses transcription in a Cyc8- and Tup1-dependent manner from a promoter containing LexA operators up-

Table 3. *Transcriptional repression by LexA–Mig1*

LexA hybrid proteins	Fold repression			Promoter (strain)					
	(Glu)	(*cyc8*)	(*tup1*)	−Lop (Glu)	4 Lop (Glu)	−Lop (*cyc8*)	4 Lop (*cyc8*)	−Lop (*tup1*)	4 Lop (*tup1*)
LexA–Mig1	3.9	0.6	0.7	93	24	39	61	52	74
LexA	1.2	1.1	1.3	100	80	55	50	90	70
Vector	1.2	1.0	1.3	100	85	55	55	90	70

β-Galactosidase activities (average of three independent transformants) of wild-type, *cyc8*, or *tup1* strains grown in glucose medium that harbor the indicated promoters and proteins. Values are normalized to A_{600} of cells at the time of collecting and are accurate to ±30%. Fold repression represents the ratio of β-galactosidase activities in strains containing plasmids that either lack (−Lop) or contain four LexA operators (4 Lop) upstream of the *CYC1* promoter fused to the *lacZ* structural gene.

stream of the *cyc1* UAS and TATA elements. However, the repressive effect of LexA–Mig1 (4-fold) is considerably less than that of LexA–Cyc8 (27-fold) or LexA–Tup1 (15-fold) when compared on the same promoter. Unexpectedly, when tested on a minimal promoter consisting of four LexA operators upstream of a *GAL1* TATA element, LexA–Mig1 (but not LexA) activates transcription in *cyc8* and *tup1* deletion strains but not in an isogenic wild-type strain (Table 4). These results define a functional interaction between Mig1 and the Cyc8–Tup1 corepressor that is likely to be involved in repression of glucose-regulated genes. Furthermore, they suggest that Cyc8–Tup1 can function both as a corepressor and as an inhibitor of Mig1 activation. Similar conclusions concerning the relationship between Mig1 and Cyc8–Tup1 have been obtained by Treitel and Carlson.

The Cyc8-interaction and WD domains of Tup1 are differentially required for repression of specific promoters

We have shown previously that Tup1–N200, a derivative containing the Cyc8–interaction and transcriptional repression domains but lacking the region containing the six WD motifs, carries out many functions of the wild-type Tup1 protein (Tzamarias and Struhl 1994). In particular, Tup1–N200 partially represses *SUC2* and *ANB1* transcription, and it rescues the slow growth and clumpy phenotype of a *tup1* deletion strain. Tup1 derivatives lacking either the repression domain (e.g., N72) or the Cyc8–interaction domain (e.g., C565, C425) are unable to repress *SUC2* and *ANB1* transcription.

We extended this analysis by assaying the same Tup1 derivatives for expression of *MFA1* and *RNR2* (Fig. 4). As expected, Tup1 derivatives containing only the Cyc8–interaction domain (e.g., N72) or the region containing the six WD motifs (C324) fail to repress any of the four genes. However, in contrast to its effects on *SUC2*, *ANB1*, and cell growth and morphology, Tup1–N200 fails to repress *MFA1* and *RNR2* transcription, indicating that additional regions of Tup1 are required for repression of genes regulated by cell type and DNA damage. In addition, Tup1 derivatives lacking the Cyc8–interaction domain (Tup1–C565 and Tup1–C425) partially repress the cell type-specific gene *MFA1* but do not affect transcription of *SUC2*, *ANB1*, or *RNR2*. The distinct roles of Cyc8 and Tup1 in repression of *MFA1* and *SUC2* are also observed in a comparison between *cyc8* and *tup1* deletion strains (Fig. 3). *SUC2* levels are notably higher in a *cyc8* strain as compared with a *tup1* strain, whereas the reverse is true for *MFA1*; levels of *ANB1* and *RNR2* are comparable in both strains. Our results on *MFA1* are in accord with recent observations that interaction of the Tup1 WD motifs with α2 is necessary for repression of **a**-specific genes (Komachi et al. 1994).

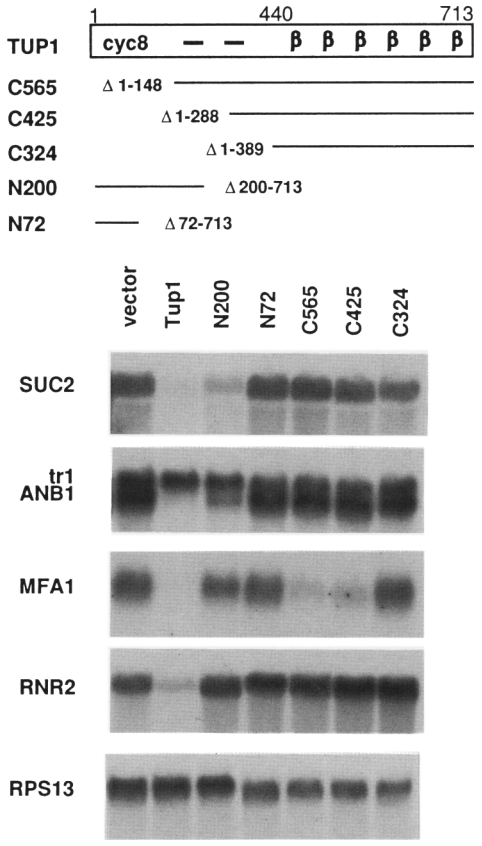

Figure 4. Repression of *SUC2*, *ANB1*, *RNR2*, and *MFA1* by various Tup1 derivatives. The three functional domains of Tup1, Cyc8–interaction domain (cyc8), independent repression domains (−), and WD motifs (β) are indicated along with the structure of the Tup1-deleted derivatives (named according to the amino- or carboxy-terminal sequence that it contains). RNA from *tup1* yeast strains expressing the indicating Tup1 derivatives was fractionated in 1.4% agarose–formaldehyde gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled probes specific for *SUC2*, *ANB1*, *MFA1*, *RNR2*, and the internal *RPS13* control.

Table 4. *Transcriptional activation by LexA-Mig1 in* cyc8 *and* tup1 *strains*

	Strains		
LexA proteins	WT	*cyc8*	*tup1*
LexA–Mig1	3	21	17
LexA	4	5	5

β-Galactosidase activities (average of three independent transformants) of wild-type (WT), *cyc8*, or *typ1* deletion strains expressing the indicated proteins. The *lacZ* reporter plasmid contains four LexA operators upstream of the *GAL1* TATA element. Values are normalized to A_{600} of cells at the time of collecting and are accurate to ±30%.

Tup1 forms multimers

In the course of testing LexA–Cyc8 derivatives for their ability to interact with Tup1–N72–VP16 in the two-hybrid assay, we examined the possibility that Tup1 might self-associate (Table 5). In combination with Tup1–N72–

Table 5. *Two-hybrid assays for Tup1–Tup1 interaction*

LexA hybrid proteins	Fold activation			VP16 hybrid proteins/strain background								
	(WT)	(*cyc8*)	(*tup1*)	V (WT)	T (WT)	TV (WT)	V (*cyc8*)	T (*cyc8*)	TV (*cyc8*)	V (*tup1*)	T (*tup1*)	TV (*tup1*)
Tup1	16	8.5	6.0	3	3	47	2	2	17	4	2	24
Tup1–C565	0.7	1.3	0.7	3	4	3	3	3	4	2	3	2
Tup1–N200	60	22	45	5	7	420	3	2	44	3	4	180
Tup1–N72	20	5.5	16	4	7	140	4	4	22	3	5	81
LexA	1.0	1.5	1.0	2	5	5	2	2	3	3	3	3

β-Galactosidase activities (average of three independent transformants) of wild-type (WT), *cyc8*, or *tup1* strains expressing the indicated proteins [(V) VP16; (T) Tup1; (TV) Tup1–VP16]. The *lacZ* reporter plasmid contains four LexA operators upstream of the *GAL1* TATA element. Values are normalized to A_{600} of cells at the time of collecting and are accurate to ± 30%. Fold activation represents the ratio of β-galactosidase activities in strains containing Tup1–VP16 vs. those containing Tup1.

VP16, LexA–Tup1 stimulates transcription 16-fold, and smaller amino-terminal derivatives (LexA–Tup1–N200 and LexA–Tup1–N72) are equally or more efficient. These two-hybrid interactions occur in *cyc8* and *tup1* deletion strains, although to a slightly lesser extent in some cases, indicating that they do not require the chromosomal copies of *TUP1* and *CYC8*. In contrast, carboxy-terminal LexA–Tup1 derivatives that lack 148 or more amino-terminal amino acids do not stimulate transcription above the background level. Thus, the 72 amino-terminal residues of Tup1 are necessary and sufficient for homomultimerization in vivo. Interestingly, the same region of Tup1 interacts directly with Cyc8 (Tzamarias and Struhl 1994).

To test whether Tup1 multimerization occurs in the absence of additional yeast proteins, we incubated ^{35}S-labeled Tup1–N253 protein with agarose beads containing GST–Tup1–N250, GST–Tup1–C324, and GST alone. As seen in Figure 5, Tup1–N253 strongly associates with GST–Tup1–N250 but not with GST alone or GST–Tup1–C324. These results indicate that Tup1 forms multimers through the Cyc8–association domain (amino acids 1–72) but in a manner independently from Cyc8, suggesting the possibility that Cyc8 associates with a dimeric (or higher order) form of Tup1.

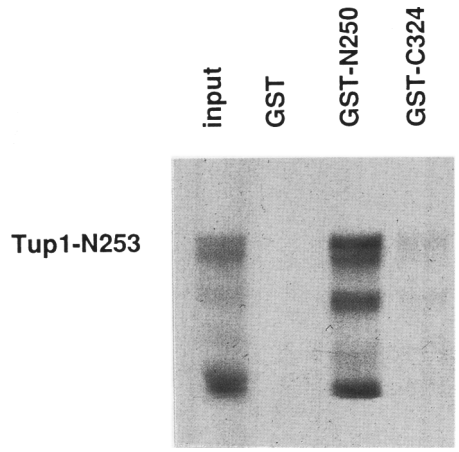

Figure 5. Tup1–Tup1 interaction in vitro. ^{35}S-Labeled Tup1–N253 stably bound to agarose beads containing GST, GST–Tup1–N250, and GST–Tup1–C324. The lane labeled input contains only 20% of the amount of the protein that was incubated with the beads.

Discussion

A specific set of Cyc8 TPR motifs interact directly with Tup1

TPR motifs have been found in a wide variety of eukaryotic proteins, and they have been proposed to form amphipathic α-helices that mediate protein–protein interactions (Hirano et al. 1990; Goebl and Yanagida 1991). The biochemical functions of TPR motifs are generally unknown. In the best-studied case, the TPR-containing proteins Cdc16, Cdc23, and Cdc27 form a complex essential for mitosis (Lamb et al. 1994). Mutations in the most conserved TPR motif of Cdc27 reduce the interaction (direct or indirect) with Cdc23 but not with Cdc16 or Cdc27 itself. Although this observation could be interpreted in terms of a specific role for this TPR motif in the formation of complexes containing Cdc27 and Cdc23, such complexes might simply require more TPR motifs than complexes containing Cdc27 and Cdc16 or Cdc27 multimers. Furthermore, it is unknown whether TPRs in Cdc27, Cdc23, and Cdc16 directly interact with each other or with other proteins.

In this paper we demonstrate that TPR motifs can directly mediate protein–protein interactions. Specifically, a region of the Cyc8 TPR domain interacts with Tup1 in vitro in the absence of yeast proteins (Fig. 2). In contrast to previous expectations (Hirano et al. 1990; Sikorski et al. 1990; Goebl and Yanagida 1991), the region of Tup1 that interacts with the Cyc8 TPR motifs does not contain TPR or WD motifs. However, the sequence of this Tup1 region (residues 1–72) is compatible with α-helix formation, suggesting the possibility that the Cyc8–Tup1 interaction is mediated by interacting α-helices. Given that Tup1 residues 1–72 also self-associate in vitro (Fig. 5), the Cyc8–Tup1 complex might involve a three (or more)-stranded α-helical coiled coil (Harbury et al. 1994).

The Cyc8–Tup1 interaction is mediated by a specific combination of TPR motifs. TPR1–3 interact efficiently with Tup1, whereas derivatives with more TPR motifs (e.g. TPR2–7) do not. Thus, despite the primary sequence similarity between individual repeats, the TPR motifs of Cyc8 are not functionally redundant. Consistent with this idea, TPR4–7 and TPR8–10 are functionally distinct with respect to glucose and oxygen repression. It is likely that TPR4–7 and TPR8–10 interact with distinct, although as yet unidentified, proteins. Taken together, our results suggest that TPR motifs represent a basic structural scaffold that accommodates a variety of protein surfaces that specifically interact with other proteins. In this view the highly conserved residues in TPR motifs are likely to be involved in the basic structure, with less conserved residues being important in determining specificity.

Differential recruitment of the Cyc8–Tup1 complex to pathway-specific promoters

The Tup1 repression domain mediates the transcriptional inhibitory function of the Cyc8–Tup1 corepressor and, hence, is required for all known functions of the complex (Fig. 4; Tzamarias and Struhl 1994). As a consequence, the Tup1–interaction domain of Cyc8 (TPR1–3) is essential for all known Cyc8 functions. Cyc8 derivatives that lack this domain fail to repress genes regulated by cell type, glucose, oxygen, and DNA damage (Fig. 3), and they do not rescue any of the physiological defects of a *cyc8* deletion strain such as slow growth, temperature-sensitive lethality, and aberrant colony morphology. Furthermore, these Cyc8 derivatives fail to repress the transcription when they are bound to DNA via the LexA DNA-binding domain.

Although the Tup1 repression domain inhibits transcription when artifically tethered upstream of a promoter, repression of natural promoters requires recruitment of the Cyc8–Tup1 complex through other protein–protein interactions. We provide strong evidence that Cyc8 plays an important role in differential recruitment of Cyc8–Tup1 to pathway-specific promoters. Two specific combinations of TPRs, along with but distinct from the Tup1–interaction region, are required for repression of *SUC2* and *ANB1*. TPR8–10 are specifically required for glucose repression of *SUC2*, whereas TPR4–7 are required specifically for oxygen repression of *ANB1*. However, these TPR motifs are dispensable, and the Tup1–interaction domain (TPR1–3) is sufficient, for Tup1-dependent repression by LexA–Cyc8.

From these observations, we propose that TPR4–7 and TPR8–10 mediate distinct protein–protein interactions that recruit the co-repressor complex to oxygen- and glucose-repressible promoters, respectively (Fig. 6). In the simplest model for this recruitment, the relevant TPR motifs of Cyc8 would directly interact with pathway-specific, DNA-binding proteins such as Mig1 and probably Rox1. Alternatively, the Cyc8 TPR motifs might interact with intermediary proteins that interact with Mig1 or Rox1. Affinity chromatography experiments of the type used to characterize the Cyc8–Tup1 interaction have revealed only a tenuous Cyc8–Rox1 interaction and no detectable Cyc8–Mig1 interaction even in the pres-

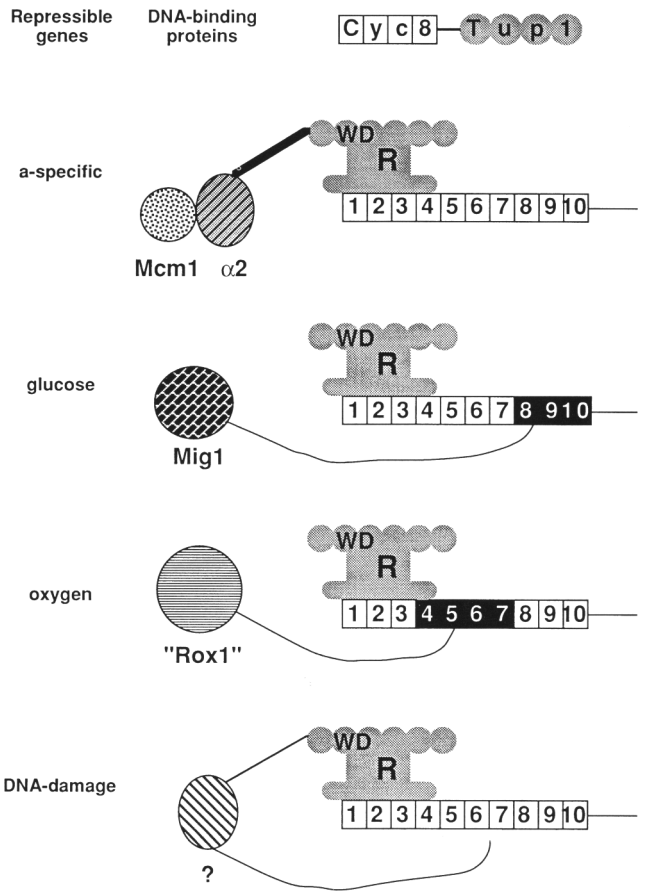

Figure 6. Model for differential recuitment of Cyc8–Tup1 to pathway-specific promoters. For each class of repressible gene, interactions between the pathway-specific, DNA-binding proteins and the Cyc8–Tup1 complex are indicated. Repression of a-specific genes involves a direct interaction (thick line) between α2 and the WD domain (six copies indicated by small, shaded circles) of Tup1 (Komachi, 1994). Repression of glucose-regulated genes involves a functional interaction (thin line) between Mig1 and Cyc8 TPR8–10 (blackened). Repression of oxygen-regulated genes involves a functional interaction (thin line) between Rox1 and Cyc8 TPR4–7 (blackened); the evidence that Rox1 is the DNA-binding protein that responds to Cyc8–Tup1 is suggestive but not conclusive (indicated by quotation marks around Rox1). Repression of DNA damage-regulated genes involves functional interactions (thin lines) between an unknown DNA-binding protein and both the WD domain of Tup1 and unspecified regions of Cyc8. Each of the above functional interactions might reflect direct protein–protein contact and/or interactions with other proteins. Other interactions besides those indicated may also be important for recruitment. Formation of the Cyc8–Tup1 complex requires an amino terminal region of Tup1 (thin gray oval) and TPR1–3 of Cyc8; it is likely that the complex contains multiple Tup1 molecules. In all cases, transcriptional repression is mediated by a specific region of Tup1 (shaded box labeled R). (See text for details.)

ence of Tup1 (D. Tzamarias and K. Struhl, unpubl.). Finally, whereas our results clearly implicate TPR4–6 and TPR8–10 in recruitment of the Cyc8–Tup1 complex to these two sets of promoters, they do not exclude possible contributions of Tup1 and/or the Tup1–interaction domain of Cyc8.

In contrast to repression of glucose- and oxygen-regulated genes, recent results indicate that recruitment of the Cyc8–Tup1 corepressor to cell type specific genes is mediated by a direct interaction between α2 and the WD motifs of Tup1 (Komachi et al. 1994). Moreover, several results indicate that cell type-regulated genes can be partially repressed in the absence of Cyc8. First, the derepression level of **a**-specific genes such as *MFA1* (Fig. 3) and *STE6* (Cooper et al. 1994) is significantly lower in a *cyc8* deletion strain than in an isogenic *tup1* deletion strain. Second, Tup1 derivatives lacking the Cyc8–interaction domain but containing the WD and repression domain partially repress *MFA1* transcription (Fig. 4). Third, the only region of Cyc8 necessary for full repression of *MFA1* is the Tup1–interaction domain (TPR1–3), which is not sufficient for repression of any other pathway-specific gene tested (Fig. 3). Thus, Cyc8 plays only an auxiliary role in repression of cell type genes, unlike its essential role in repression of glucose- and oxygen-regulated genes. In this auxiliary role, TPR1–3 of Cyc8 might stabilize a multimeric form of Tup1 or mediate additional interactions that strengthen the association of the Tup1 WD motifs with α2.

Repression of *RNR2*, a gene induced by DNA damage, is distinct from repression of *SUC2*, *ANB1*, and *MFA1* in that it requires the entire TPR domain of Cyc8. Moreover, unlike the case for *SUC2* and *ANB1*, repression of *RNR2* is abolished in Tup1 derivatives lacking the WD domain. Thus, recruitment of the corepressor complex to DNA damage-inducible promoters may be mediated by the cooperative function of both the TPR domain of Cyc8 and the WD domain of Tup1.

Taken together, our results and those of Komachi et al. (1994) indicate that the Cyc8–Tup1 corepressor complex is differentially recruited to pathway-specific promoters (Fig. 6). We suggest that the underlying mechanism of this differential recruitment is that specific combinations of Cyc8 TPR motifs and the Tup1 WD domain possess distinct protein–protein interaction specificities. In this view different surfaces of the Cyc8–Tup1 complex interact (directly or indirectly) with the pathway-specific repressors, whose DNA-binding domains are structurally dissimilar (α2 contains a homeo domain, Mig1 contains a zinc finger, and Rox1 contains an HMG motif). Although the Cyc8–Tup1 complex appears to be differentially recruited to promoters regulated by glucose, oxygen, cell type, and DNA damage, we presume that transcriptional inhibition is mediated by the Tup1 repression domain in all cases.

Cyc8–Tup1 can also inhibit the function of specific activation domains

As proposed initially (Keleher et al. 1992), the pathway-specific, DNA-binding proteins play a passive role in the repression process, serving merely to recruit Cyc8–Tup1 to the relevant promoters. This view was based on the following observations on repression of cell type-specific genes. First, comparable expression levels are observed in strains lacking functional operators (α2–Mcm1 or **a**1–α2), DNA-binding proteins (α2, **a**1), or the Cyc8–Tup1 complex. Second, when tested upstream of a heterologous TATA element, the α2–Mcm1 operator behaves as a very weak activator (because of Mcm1, which can bind the operator in the absence of α2) that is unaffected by loss of Cyc8 function (Keleher et al. 1992). Whereas α2 plays a passive role in repression, it is unknown whether this is the case for other DNA-binding proteins that are targets of the Cyc8–Tup1 corepressor.

The fact that transcriptional activity of LexA–Mig1 depends on Cyc8–Tup1 function demonstrates that Mig1 is a target of the Cyc8–Tup1 corepressor complex. Furthermore, LexA–Mig1 represses transcription when bound upstream of an intact promoter (Table 3; Treitel and Carlson 1995), suggesting that recruitment of Cyc8–Tup1 plays a role in Mig1–dependent repression. However, LexA–Mig1 behaves as a transcriptional activator in *cyc8* and *tup1* strains (Table 4; Treitel and Carlson 1995), suggesting that Cyc8–Tup1 can also function as an inhibitor of Mig1 transcriptional activity, possibly by interacting with and, hence, masking an activation domain in Mig1. Thus, in addition to acting as a general repressor of transcription that is recruited to particular classes of promoters, Cyc8–Tup1 can inhibit the function of specific activator proteins.

Materials and methods

Expression of Cyc8 and Tup1 derivatives in yeast

The *TRP1* centromeric vector YCp91, which was used for expression of all Cyc8, Tup1, and LexA derivatives, contains the *ADH1* promoter and 5′-untranslated sequence (including the ATG start codon), following by sequences encoding the SV40 nuclear localization signal and the HA1 epitope from the influenza virus (NLS–Flu), a polylinker, three stop codons (in all three frames), and a 410-bp fragment containing the *CYC8* termination region (Tzamarias and Struhl 1994). The entire *CYC8*-coding sequences from an artificial *Bam*HI site inserted 9 bp upstream of the ATG initiation codon to the *Ase*I located 20 bp downstream of the stop codon were cloned between *Bam*HI and *Asp*718 sites. Cyc8 deletion derivatives containing 100, 175, 301, 353, 597, and 816 amino-terminal residues were constructed in the same manner by inserting *Bam*HI–*Pst*I, *Bam*HI–*Bst*XI, *Bam*HI–*Asp*718, *Bam*HI–*Mlu*I, *Bam*HI–*Pvu*II, and *Bam*HI–*Alw*NI, respectively. To generate molecules containing Cyc8 residues 79–300 and 113–300, *Sma*I sites were introduced at positions 79 and 113 by PCR, and the resulting *Sma*I–*Asp*718 fragments were cloned into the YCp91 expression vectors. Internally, deletions (Δ175–281 and Δ175–304) were constructed by using *Bal* 31 to remove the amino-terminal 281 and 304 residues and then ligating the resulting molecules in-frame with a DNA fragment encoding amino acid sequence 1–174 (*Bam*HI–*Bst*XI fragment).

YCp91 molecules expressing Tup1 and LexA–Tup1 derivatives have been described previously (Tzamarias and Struhl 1994). The hybrid Tup1N72–VP16, which was used in the two-

hybrid experiments, contains a *Sma*I–*Mlu*I fragment of Tup1 (amino acids 1–72) fused in-frame to a *Bgl*II–*Bam*HI fragment containing the activation domain of VP16 (amino acids 414–553); control plasmids contain either the Tup1–N72 or the VP16 fragment. All three fragments were inserted in the YEp92, which contains the expression casette of YCp91 in the *LEU2* multicopy plasmid YEpLac181 (Tzamarias and Struhl 1994). To generate a plasmid expressing LexA–Mig1, a *Bam*HI–*Asp*718 DNA fragment containing the entire Mig1-coding region from −6 to +1514 was cloned in YCp91.

Transcriptional repression and two-hybrid assays in vivo

Yeast strains FT5 (*MAT*α *ura3–52 trp1-Δ63 his3-Δ200 leu2::PET56*) and derivatives containing the *tup1Δ::HIS3* (remove Tup1 codons −2 to 672) and *cyc8-Δ9::HIS3* (removes Cyc8 codons 99–862) alleles have been described previously (Tzamarias and Struhl 1994). For two-hybrid assays, strains contained the *URA3* multicopy plasmid JK103, in which the *lacZ* gene is driven by a promoter with four LexA operators upstream from the *GAL1* TATA element (Kamens et al. 1990). The *lacZ* reporter constructs used in the LexA-dependent repression assay were derived from the plasmids pLGΔ312S and JK1621 and integrated at the *URA3* locus (Tzamarias and Struhl 1994). pLGΔ312S contains a *CYC1* fragment (−324 to +141), including the two UASs and TATA region (Guarente and Mason 1983). JK1621 is a derivative pLGΔ312S with an insertion of four LexA-binding sites at a *Sma*I site upstream of the two UASs (Keleher et al. 1992).

β-Galactosidase assays were performed on yeast cells that were harvested in early log phase (A_{600}<1.0) and then washed in 20 mM Tris (pH 7.5), 10 mM EDTA to disperse the clumpy *cyc8* and *tup1* cells. Cells were grown in glucose medium containing casamino acids and uracil. The numbers in the tables are normalized to A_{600} and represent the average of at least three independent transformants; they are accurate to ±30%.

Repression of natural yeast genes was analyzed by RNA blotting. Total RNAs from appropriate strains grown in glucose medium containing casamino acids and uracil were fractionated in a 1.4% agarose gel containing 5.5% formaldehyde, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes that were generated by nick translation or 5′phosphorylation of the following DNAs: 1.2-kb *Hin*dIII fragment from *SUC2*; 1.5-kb *Sma*I–*Bam*HI fragment containing the entire *ANB1* gene (which also hybridizes with a second transcript that is indicated tr1); 1-kb internal *Hin*dIII fragment from *RNR2*, oligonucleotide complementary to the *MFA1*-coding strand (codons 21–35); and 450-bp *Hin*dIII–*Eco*RI fragment from *RPS13*, which encodes a ribosomal protein and serves as an internal control.

In vitro interaction assay

GST hybrid proteins from 500 ml of exponentially growing *Escherichia coli* (strain DH5α) cells with the appropriate plasmids that were induced with 0.25 mM IPTG for 3 hr at 30°C, harvested by centrifugation, and frozen immediately. The bacterial pellets were resuspended in 5 ml of a buffer containing 100 mM NaCl, 20 mM HEPES (pH 7.5), 1 mM DTT, 1 mM EDTA, 1% Triton, 20% glycerol, 0.5% BSA, and protease inhibitors (1 mM PMSF, 5 mg/ml of leupeptin, and 5 mg/ml of pepstatin) and sonicated (six strokes, 10 sec each) at 4°C. Cell debris was removed by centrifugation at 10,000g for 15 min, and the supernatant was incubated with an equal volume (0.5–2.0 ml, depending on protein yield) of glutathionine–agarose beads (activated according to instructions given by Sigma) for 1 hr at 4°C with rocking. The protein-containing beads were washed four times with 10 volumes of the same buffer (without BSA) and stored at 4°C.

^{35}S-labeled proteins were synthesized in vitro, using T3 or T7 RNA polymerase and rabbit reticulocyte lysate in a 40 μl reaction according to the manufacturer (Promega). Five to 10 μl of ^{35}S-labeled protein was incubated with 1–2 μg of agarose bead-bound GST hybrid protein in 400 μl reaction containing 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.1% NP-40, and 0.25% BSA (plus protease inhibitors) at 4°C for 2 hr with rocking. The agarose beads were washed three times with 1.5 ml of interaction buffer and one more time with the same buffer lacking BSA. The bound proteins were eluted in buffer containing SDS and then fractionated by SDS-PAGE.

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