Activator Gcn4p and Cyc8p/Tup1p Are Interdependent for Promoter Occupancy at ARG1 In Vivo‡

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The Cyc8p/Tup1p complex mediates repression of diverse genes in Saccharomyces cerevisiae and is recruited by DNA binding proteins specific for the different sets of repressed genes. By screening the yeast deletion library, we identified Cyc8p as a coactivator for Gcn4p, a transcriptional activator of amino acid biosynthetic genes. Deletion of CYC8 confers sensitivity to an inhibitor of isoleucine/valine biosynthesis and impairs activation of Gcn4p-dependent reporters and authentic amino acid biosynthetic target genes. Deletion of TUP1 produces similar but less severe activation defects in vivo. Although expression of Gcn4p is unaffected by deletion of CYC8, chromatin immunoprecipitation assays reveal a strong defect in binding of Gcn4p at the target genes ARG1 and ARG4 in cyc8Δ cells and to a lesser extent in tup1Δ cells. The defects in Gcn4p binding and transcriptional activation in cyc8Δ cells cannot be overcome by Gcn4p overexpression but are partially suppressed in tup1Δ cells. The impairment of Gcn4p binding in cyc8Δ and tup1Δ cells is severe enough to reduce recruitment of SAGA, Srb mediator, TATA binding protein, and RNA polymerase II to the ARG1 and ARG4 promoters, accounting for impaired transcriptional activation of these genes in both mutants. Cyc8p and Tup1p are recruited to the ARG1 and ARG4 promoters, consistent with a direct role for this complex in stimulating Gcn4p occupancy of the upstream activation sequence (UAS). Interestingly, Gcn4p also stimulates binding of Cyc8p/Tup1p at the 3’ ends of these genes, raising the possibility that Cyc8p/Tup1p influences transcription elongation. Our findings reveal a novel coactivator function for Cyc8p/Tup1p at the level of activator binding and suggest that Gcn4p may enhance its own binding to the UAS by recruiting Cyc8p/Tup1p.

Cyc8 and Tup1 are evolutionary conserved proteins that function in a complex to mediate repression of diverse sets of genes in Saccharomyces cerevisiae, including those expressed only in haploids or in MATα cells or in response to DNA damage, osmotic stress, or hypoxic (low-oxygen) conditions (42). The Cyc8p/Tup1p complex is targeted to promoters by DNA binding proteins specific for the different classes of repressed genes (42). Whereas Cyc8p is crucial for recruitment of Cyc8p/Tup1p by the Mig1 and Roxl repressors (45, 46), Tup1p makes contact with the α2 repressor (19). It is thought that Tup1p provides the main repressor functions of the complex, because tethering a LexA-Tup1 fusion protein to a promoter led to transcriptional repression in cyc8Δ cells (46), whereas repression by LexA-Cyc8p was dependent on Tup1p (4).

It has been proposed that Cyc8p/Tup1p can repress transcription by organizing nucleosomes into repressive chromatin structures (5, 12, 26). This function could be mediated by direct interaction of Tup1p with the N-terminal tails of histones H3 and H4 (11, 18) and nonhistone chromosomal protein Nhp6 (24). Other findings suggest that repression by Tup1p occurs by deacetylation of histone N-terminal tails at the promoter (1, 7, 8), which might involve direct recruitment of histone deacetylases by Cyc8p/Tup1p (6, 48, 49). A third repression mechanism was suggested by observations that Cyc8p/Tup1p-mediated repression is partially impaired by mutations in subunits of Srb mediator (henceforward referred to as mediator) (reference 25 and references therein). The mediator is a multisubunit cofactor that forms a holoenzyme complex with RNA polymerase II (Pol II) and other general transcription factors and is implicated in activation as well as repression (31). There is evidence that mediator subunits Srb7p, Srb10p, and Hrs1p/Med3p/Pgd1p are targets of Tup1 in the mediator. As mediator is required for optimal recruitment of TATA binding protein (TBP) and Pol II by various activators (23, 27, 39), interaction of Cyc8p/Tup1p with mediator subunits could interfere with assembly of the preinitiation complex (PIC).

It appears that none of these repression mechanisms alone can account for Tup1p-mediated repression. Mutations in H3, H4, and Nhp6p increase expression of only a subset of Cyc8p/Tup1p target genes, and repression of certain promoters in wild-type (WT) cells occurs in the absence of positioned nucleosomes (42). In addition, repression of ANB1 (30) and RNR3 (53) was unaffected by mutations in TUP1 and ISW2, respectively, that eliminate nucleosome positioning at these genes. In fact, nucleosome positioning, histone deacetylation, and mediator subunits make overlapping contributions to Tup1p-mediated repression of RNR3, as successive inactivation of each mechanism led to stepwise derepression of this gene (53).

Interestingly, Tup1p/Cyc8p can participate in overcoming its own repressive function at certain target genes. Thus, phosphorylation of transcription factor Sko1p during osmotic stress overcomes the repressing function of the promoter-bound

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‡ Supplemental material for this article may be found at http://mcb.asm.org/.
Sko1p-Cyc8p/Tup1p complex, leading to Tup1p-dependent recruitment of SAGA and SWI/SNF and attendant transcription of Sko1p target genes. As deletion of TUP1 did not reduce transcription of these genes under inducing conditions, it appears that recruitment of SWI/SNF and SAGA is required primarily to overcome the repressor function of Cyc8p/Tup1p (38). Similarly, Cyc8p/Tup1p remains bound at the promoter under galactose-inducing conditions and Cyc8p recruits SAGA to the promoter via Ctk6p/Rxt1p (35). Again, deletion of CYC8 does not impair galactose induction of GAL1, suggesting that Ctk6p/Rxt1p-dependent recruitment of SAGA serves to counteract Cyc8p/Tup1p repression at GAL1. There is also evidence that Cyc8p can function as a conventional coactivator at CYC1 (52) and SUC2 (34), where gene induction is impaired in cyc8 mutant cells. Cyc8p/Tup1p plays a dual role at CIT2, with Tup1p mediating repression and Cyc8p supporting activation by Rtg3p. Indeed, Cyc8p/Tup1p was recruited by Rtg3p to the CIT2 promoter in vitro, and the Rtg3p activation domain can directly interact with Cyc8p (4). Cyc8p and, to a lesser extent, Tup1p are also required for activation of FRE2 by Aft1p. Cyc8p interacts directly with Aft1p in vitro and is recruited by Aft1p to the FRE2 promoter in vivo, where it mediates nucleosome remodeling of the promoter in conjunction with Nhp6p (13).

We have been analyzing the coactivator requirements for Gen4p, a transcriptional activator of amino acid biosynthetic genes in yeast. To this end, we have studied the coactivator requirements for the FRE2 promoter.

TABLE 1. Yeast strains used in this study

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a Strains purchased from Research Genetics.

b Strains isogenic to Research Genetics strains.

c HIS3* denotes the HIS3 allele from Saccharomyces kluyveri (28).

d NA, not applicable.

TABLE 2. Plasmids used in this study

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genes in yeast (33) that is induced at the translational level by starvation for any amino acid (16). The Gcn4p activation domain interacts specifically in vitro with mediator, SAGA, and the ATP-dependent chromatin remodeling complexes SWI/SNF and RSC (10, 32, 43, 47), and Gcn4p recruits all four coactivators to target genes in living cells (21, 22, 43, 44). Furthermore, mutations in various subunits of these coactivators confer sensitivity to inhibitors of amino acid biosynthetic enzymes (Gcn– phenotype) and reduce transcriptional activation of one or more Gcn4p-dependent target genes or reporters in vivo (39; reference 43 and references therein). Recent findings indicate that SAGA, SWI/SNF, and mediator arrive simultaneously at Gcn4p target promoters under inducing conditions but are highly interdependent for their recruitment by Gcn4p (15, 40). These coactivators stimulate both PIC assembly and one or more steps in transcription elongation at the Gcn4p target gene ARG1 (15, 39).

In this report, we show that Cyc8p/Tup1p is a coactivator for Gcn4p at multiple target genes in vivo, and we present evidence that Cyc8p/Tup1p functions to enhance Gcn4p binding to the upstream activation sequence (UAS) elements at ARG1 and ARG4. Interestingly, Gcn4p recruits Cyc8p/Tup1p to both
genes, which may provide a positive-feedback mechanism to maintain high-level Gcn4p binding at the UAS elements of these genes in vivo.

**MATERIALS AND METHODS**

**Yeast strains and plasmids.** All strains and plasmids used in this study are listed in Tables 1 and 2, respectively. The WT parent strains BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and deletion derivatives thereof were described previously (14) and purchased from Research Genetics. The presence of all reported deletion alleles was confirmed by PCR amplification or complementation of mutant phenotypes by plasmid-borne wild-type genes (43). myc-tagged strains were constructed as previously described (43). The presence of the myc-tagged alleles was verified by colony PCR and Western blot analysis using anti-Myc antibodies.

Plasmids p1208, p2382, pHYC2, pKN7, pSK1, pHQ1239, pHQ1240, and pHQ1303 were described previously (9, 17, 32, 43, 50), and the empty vector employed throughout was the URA3 CEN4 plasmid YCp50 (36). The BamHI-MluI fragment from pHQ1239 containing GCN4-myc13 was subcloned into the high-copy-number URA3 vector YEplac195 to produce pHQ1293. Plasmids pME2126 and pME2129 were described previously (37).

**FIG. 2.** Deletions of CYC8 and TUP1 impair induction of Gcn4p target genes. Total RNA was isolated from strains described in the legend for Fig. 1, grown under the same inducing (I) and noninducing (N) conditions described there and subjected to Northern analysis using probes for ARG1, ARG4 (A to C), HIS4 (C), TRP3 (C), or GCN4 (D) mRNAs. ACT1 mRNA and scR1 RNA were also probed as loading controls. The hybridization signals were quantified with a PhosphorImager (Molecular Dynamics), with ImageQuant 5.2 software, and normalized to the corresponding scR1 (A and B) and ACT1 (C and D) signals. The resulting ratios for the mutant strains were normalized to those measured for the WT strain, and the average normalized ratios measured for at least two independent cultures are shown in the histograms as percentages of the WT value under inducing conditions (A to C).
Biochemical methods. The reporter gene assays were performed as described previously (43). For Western analysis, whole-cell extracts (WCEs) were prepared as described previously (41) and analyzed using monoclonal anti-myc (Roche) and polyclonal anti-Gcd6p (3) antibodies. Northern analysis was carried out as described previously (43), with the following modification. QuikHyb hybridization solution (Stratagene) was used for prehybridization and hybridization as described by the vendor. After hybridization, the membranes were washed twice with 2× SSC buffer and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min and once with 0.1× SSC buffer-0.1% SDS at 60°C for 30 min. The washed membranes were subjected to phosphorimaging analysis for quantifying the signals. The chromatin immunoprecipitation (ChIP) experiments were conducted as described previously, using the same primers described there (39, 40, 43, 50). The coimmunoprecipitation and glutathione S-transferase (GST) pulldown assays shown in the supplemental material were conducted essentially as described previously (51).

RESULTS

Cyc8p is broadly required for wild-type activation of Gcn4p target genes. We screened the library of haploid deletion strains produced by the Saccharomyces Genome Deletion Project (14) for mutants with increased sensitivity to sulfometuron methyl (SM), an inhibitor of isoleucine and valine biosynthesis, to identify new mutants defective in transcriptional activation by Gcn4p. The cyc8Δ strain was found to be highly sensitive to SM (SMs), although it was less sensitive than the isogenic gcn4Δ mutant in the deletion library. The tup1Δ strain is also SMs but less so than the cyc8Δ mutant (Fig. 1A). We verified by PCR analysis of genomic DNA that these two mutants contain the reported deletions. We determined that the cyc8Δ/cyc8Δ and tup1Δ/tup1Δ mutants in the homozygous diploid deletion library exhibit SMs phenotypes comparable to those observed for the corresponding haploid mutants (data not shown). Finally, the SMs phenotype of the haploid cyc8Δ mutant was diminished by introducing a plasmid-borne CYC8 allele into this strain (data not shown). We conclude that inactivation of CYC8 or TUP1 confers sensitivity to SM.

FIG. 3. Deletion of CYC8 does not reduce myc-Gcn4p abundance, and the SMs phenotype of cyc8Δ cells is not overcome by overexpressing Gcn4p. Western analysis of myc-Gcn4p expression. (A) gen4Δ (249) and cyc8Δ gen4Δ (KS4) strains were transformed with s.c. (pSK1) or h.c. (pHQ1293) plasmids harboring the GCN4-myc allele or empty vector YCp50, as indicated. WCEs were prepared from two transformants of each strain cultured under the inducing conditions described in the legend for Fig. 1. Aliquots with equal amounts of total protein were separated by 8 to 16% SDS-polyacrylamide gel electrophoresis and probed with anti-myc and anti-Gcd6p antibodies, as indicated to the right of the blot. Probing with anti-Gcd6p antibodies provided a loading control. Samples from the two independent transformants of each strain were analyzed in successive lanes of the gel. (B) The strains described in the legend for Fig. 1 were transformed with s.c. plasmid p1208 carrying untagged GCN4, h.c. plasmid pHQ1303 harboring untagged GCN4, or empty vector (YCp50), as indicated. The spotting assay was performed as described in the legend for Fig. 1A.

Biochemical methods. The reporter gene assays were performed as described previously (43). For Western analysis, whole-cell extracts (WCEs) were prepared as described previously (41) and analyzed using monoclonal anti-myc (Roche) and polyclonal anti-Gcd6p (3) antibodies. Northern analysis was carried out as described previously (43), with the following modification. QuikHyb hybridization solution (Stratagene) was used for prehybridization and hybridization as described by the vendor. After hybridization, the membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min and once with 0.1× SSC buffer-0.1% SDS at 60°C for 30 min. The washed membranes were subjected to phosphorimaging analysis for quantifying the signals and also to autoradiography. The chromatin immunoprecipitation (ChIP) experiments were conducted as described previously, using the same primers described there (39, 40, 43, 50). The coimmunoprecipitation and glutathione S-transferase (GST) pulldown assays shown in the supplemental material were conducted essentially as described previously (51).

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Consistent with its strong SMs phenotype, the cyc8Δ mutant showed a dramatic defect in activation of a Gcn4p-dependent lacZ reporter containing two Gcn4p binding sites upstream of the CYC1 core promoter (UAS<sub>GCRE</sub>-CYC1-lacZ) (Fig. 1B and C). This reporter is induced more than 10-fold in the WT strain when Gcn4p is induced by SM. Induction of the reporter was nearly eliminated in the cyc8Δ mutant, which exhibits only slightly higher reporter expression than does the gen4Δ mutant.
FIG. 4. Deletion of CYC8 and TUP1 impair binding of Gcn4p to the UAS elements at ARG1 and ARG4. Strains BY4741 (WT), 249 (gen4Δ), 7161 (cyc8Δ), and 7189 (tup1Δ) were transformed with empty vector (YCp50), s.c. GCN4-HA plasmid p2382, or h.c. GCN4-HA plasmid pHQ1239 (A, B, C, E, and F). Strains 249 (gen4Δ) and KS4 (cyc8Δ gen4Δ) were transformed with an s.c. (pSK1) or an h.c. (pHQ1293) GCN4-myc plasmid (D). Strains BY4741, 249, and 7189 were transformed with empty vector YCp50 (G). All strains were cultured in synthetic complete medium lacking Ile and Val at 30°C and treated with 1 μg/ml of SM for 2 h or for the indicated times (C). Cells were harvested, treated with formaldehyde, and broken by vortexing with glass beads, and the extracts were sonicated to produce chromatin fragments with average lengths of ~500 bp. Aliquots (5%) were immunoprecipitated with anti-Gcn4p antibodies, and DNA was extracted from the immunoprecipitate (IP) after reversing the
under inducing conditions (4% versus 1% of WT, respectively). By contrast, the cyc8Δ mutant showed >80% of WT UAS_{GCRE}/CYC1-lacZ expression under noninducing conditions, even though deleting GCN4 eliminates most of the reporter expression in nonstarved cells (Fig. 1B and C). Thus, Cyc8p is required primarily to support the high-level transcription of UAS_{GCRE}/CYC1-lacZ evoked by induced levels of Gcn4p produced under starvation conditions.

Cyc8p is also required for efficient activation of a HIS3-GUS reporter containing the intact HIS3 promoter, as induction of this reporter occurred at only ~30% of the WT level in cyc8Δ cells (Fig. 1D and E). Most of the HIS3-GUS expression under noninducing conditions is independent of Gcn4p. Interestingly, cyc8Δ led to an approximately sevenfold derepression of HIS3-GUS expression under noninducing conditions. These findings suggest that Cyc8p is required to repress basal HIS3 promoter function under noninducing conditions even though it is necessary for strong activation of this promoter by induced levels of Gcn4p.

Consistent with its weaker SM phenotype, the tup1Δ mutant showed only a modest defect in activation of the UAS_{GCRE}/CYC1-lacZ reporter and no defect in HIS3-GUS expression under inducing conditions. However, tup1Δ derepressed the UAS_{GCRE}/CYC1-lacZ and HIS3-GUS reporters by approximately threefold and fivefold, respectively, under noninducing conditions (Fig. 1B to E). It seems that Tup1p plays only a negative role in regulating the transcription of these reporter genes, being required to maintain low-level expression under noninducing conditions.

Somewhat different results were obtained when we compared the effects of cyc8Δ and tup1Δ mutations on mRNAs produced from the chromosomal GCN4 target genes _ARG1_ and _ARG4_. Northern analysis of the steady-state levels of these mRNAs relative to the Pol III transcript _scR1_ indicated that cyc8Δ impairs _ARG1_ and _ARG4_ expression under both inducing and noninducing conditions (Fig. 2A and B). Similar results were obtained for _ARG1_ and _ARG4_ and extended to include the Gcn4p targets _HIS4_ and _TRP3_ in cyc8Δ cells, using the Pol II transcript _ACT1_ as an internal control (Fig. 2C). Thus, cyc8Δ impairs transcriptional activation by Gcn4p at multiple authentic target genes. Interestingly, tup1Δ led to reductions in the induced levels of _ARG1_ and _ARG4_ mRNAs comparable to those observed for cyc8Δ (Fig. 2A and B), suggesting that Tup1p plays a positive role in activation of these chromosomal target genes even though it is dispensable for activation of the plasmid-borne reporters. Furthermore, tup1Δ did not elicit significant derepression of _ARG1_ or _ARG4_ mRNAs under noninducing conditions (Fig. 2A and B).

The results presented thus far suggest that Cyc8p is broadly required for wild-type activation by Gcn4p, whereas Tup1p contributes more selectively at specific Gcn4p-dependent promoters. Tup1p and Cyc8p also contribute to repression of plasmid-borne reporter genes under noninducing conditions. It is unclear whether the distinct effects of cyc8Δ and tup1Δ on expression of reporter genes versus authentic target genes reflect differences in the promoter sequences involved or, rather, an atypical chromatin structure of the plasmid-borne reporters.

Cyc8p is required for efficient binding of Gcn4p to target genes in vivo. To determine whether the activation defects observed with cyc8Δ and tup1Δ mutants result from reduced binding of Gcn4p at its target genes, we first analyzed the effects of cyc8Δ on GCN4 expression. As shown in Fig. 2D, we observed no difference in _GCN4_ mRNA levels between WT and cyc8Δ cells under inducing or noninducing conditions. We next measured the steady-state expression of a myc-tagged form of Gcn4p by Western analysis. The _GCN4-myc_ allele is nearly indistinguishable from untagged _GCN4_ in complementing the SM phenotype of the _gcn4Δ_ mutant (data not shown). Western blot analysis revealed little difference in the induced levels of myc-Gcn4p between transformants of _gcn4Δ_ and _cyc8Δ_ _gcn4Δ_ strains harboring _GCN4-myc_ on single-copy (s.c.) or high-copy-number (h.c.) plasmids (Fig. 3A). It is noteworthy that the myc-Gcn4p level in _cyc8Δ_ cells containing h.c. _GCN4-myc_ is considerably higher than that in _gcn4Δ_ cells containing s.c. _GCN4-myc_ (Fig. 3A, cf. lanes 11 and 12 with lanes 5 and 6) yet overexpressing Gcn4p from an h.c. plasmid did not suppress the SM phenotype of the _cyc8Δ_ mutant. Thus, the activation defect in _cyc8Δ_ cells does not appear to result from reduced accumulation of Gcn4p in starved cells.

We next asked whether binding of Gcn4p to its target promoters is impaired by the _cyc8Δ_ mutation by conducting ChIP analysis. Remarkably, after 2 h of induction by SM, binding to the _ARG1_ UAS of native Gcn4p was greatly reduced in the _cyc8Δ_ mutant to a level only slightly above the nonspecific background signal measured for the _gcn4Δ_ strain (Fig. 4A and B, vector lanes). Moreover, overexpressing functional hemagglutinin (HA)-tagged Gcn4p increased binding at _ARG1_ by ~2.2-fold in WT cells but had no effect on Gcn4p binding in the _cyc8Δ_ mutant (Fig. 4A and B). Binding of Gcn4p at the _ARG1_ UAS increased between 30 min and 2 h of induction with SM in WT cells, and _cyc8Δ_ impaired Gcn4p binding at all three time points assayed in this time course (Fig. 4C). The _cyc8Δ_ mutation also impaired UAS binding of myc-tagged Gcn4p, whether expressed in _gcn4Δ_ cells from an s.c. or an h.c. plasmid (Fig. 4D). Finally, cyc8Δ impaired Gcn4p binding to the UAS at _ARG4_ to roughly the same extent observed at _ARG1_ in a manner unaffected by overexpressing HA-Gcn4p (Fig. 4E).

The _tup1Δ_ mutation had a somewhat different effect on Gcn4p binding at its target genes. Whereas _tup1Δ_ strongly
reduced Gcn4p binding at the ARG1 and ARG4 UAS elements (Fig. 4F and G), this defect was overcome by overexpressing HA-Gcn4p from an h.c. plasmid (Fig. 4F). Thus, Tup1p may be less critical than Cyc8p for efficient binding of Gcn4p at its target genes.

Recruitment of coactivators, TBP, and Pol II by Gcn4p is strongly impaired in cyc8Δ cells. We wished to determine whether the reduced UAS occupancy by Gcn4p in cyc8Δ cells observed with ChIP assays is sufficient to impair recruitment of SAGA, Srb mediator, TBP, and Pol II to the promoter after 2 h of induction. The ChIP analyses in Fig. 5 show that cyc8Δ reduces recruitment of myc-tagged subunits of SAGA (Spt7p) (Fig. 5A and B) and mediator (Srb6p) (Fig. 5C and D) to the UAS elements at ARG1 and ARG4 to nearly the same extent as does gcn4Δ, even in cells overexpressing HA-Gcn4p from a high-copy-number plasmid. The recruitment of myc-tagged

FIG. 5. Recruitment of SAGA (myc-Spt7p), Srb mediator (myc-Srb6p), TBP (myc-TBP1), and Pol II (myc-Rpb3p) by Gcn4p is strongly dependent on Cyc8p and moderately dependent on Tup1p. ChIP analysis was conducted on the following strains harboring empty vector (Ycp50) or h.c. GCN4-HA plasmid pHQ1239, as indicated, as described in the legend for Fig. 4 except using anti-myc antibodies. (A and B) SPT7-myc strains HQY453 (WT), KS8 (cyc8Δ), KS112 (tup1Δ), and HQY457 (gcn4Δ); (C and D) SRB6-myc strains HQY464 (WT), KS12 (cyc8Δ), KS108 (tup1Δ), and HQY470 (gcn4Δ); (E) TBP1-myc strains HQY366 (WT), KS15 (cyc8Δ), KS111 (tup1Δ), and HQY382 (gcn4Δ); (F) RPB3-myc strains HQY403 (WT), KS17 (cyc8Δ), KS110 (tup1Δ), and HQY422 (gcn4Δ). Occupancy of ARG1UAS was measured for myc-Spt7p (A) or myc-Srb6p (C), occupancy of ARG1UAS was measured for myc-Spt7p (B) or myc-Srb6p (D), occupancy of ARG1TATA was measured for myc-TBP (E), and occupancy of ARG1TATA was measured for myc-Rpb3p (F). IP, immunoprecipitate.
forms of TBP and the Rpb3p subunit of Pol II to the ARG1 promoter also was impaired by cyc8Δ (Fig. 5E and F). The reduction of TBP recruitment to the promoter and of Pol II occupancy in the ARG1 open reading frame (ORF) was evident in the cyc8Δ mutant immediately on induction of Gcn4p in parallel with decreased UAS binding of Gcn4p at each time point analyzed (Fig. 6A to C). We showed previously that the myc-tagged alleles of SPT7, SRB6, SPT15/TBP1, and RPB3 are functional for transcriptional activation by Gcn4p (39, 43), and we verified here that cyc8Δ and tup1Δ do not reduce steadystate expression of myc-Spt7p, myc-Srb6p, myc-TBP, or myc-Rpb3p (see Fig. S1 in the supplemental material). Thus, the reduced UAS occupancy of Gcn4p in cyc8Δ cells is associated with substantial defects in coactivator recruitment and PIC assembly by Gcn4p.

We also observed impaired recruitment of coactivators and PIC assembly at Gcn4p target genes in the tup1Δ mutant. Recruitment of myc-Spt7p and myc-Srb6p to the UAS elements at ARG1 and ARG4 and of myc-TBP and myc-Rpb3p binding to the ARG1 promoter were all reduced in tup1Δ cells. With the possible exception of myc-Rpb3p, these recruitment defects were suppressed by overexpressing HA-Gcn4p, such that the levels of factor recruitment in tup1Δ cells expressing h.c. GCN4-HA were comparable to the levels in TUP1 cells expressing Gcn4p at the native level (Fig. 5A to F). These results are consistent with the fact that overexpressing Gcn4p greatly reduced the SM phenotype of the tup1Δ mutant (Fig. 3B) and restored WT Gcn4p binding to the ARG1 UAS in tup1Δ cells (Fig. 4F).

Gcn4p recruits Cyc8p and Tup1p to target genes in vivo. In an effort to determine whether Cyc8p and Tup1p function in a direct manner to promote Gcn4p binding to the UAS, we asked whether Cyc8p and Tup1p are present at ARG1 and ARG4 under inducing conditions by conducting ChIP analysis of strains expressing myc-tagged forms of these proteins. We verified that the CYC8-myc and TUP1-myc alleles conferred wild-type growth on SM plates (data not shown) and thus appeared to be functional in supporting transcriptional activation by Gcn4p. The ChIP results shown in Fig. 7B and C reveal Gcn4p-dependent binding of myc-Cyc8p and Tup1p to the ARG1 UAS after 30 min or 1 h of induction, in parallel with the increased binding of Gcn4p at the UAS observed at these time points (Fig. 7A). In the strain containing native levels of Gcn4p (GCN4/vector), binding of both myc-Cyc8p and Tup1p to the UAS under inducing conditions was only twofold or less above the background level observed for the gcn4Δ strain. However, much higher levels of myc-Cyc8p and Tup1p recruitment to the UAS occurred in strains overexpressing HA-Gcn4p (GCN4-hc. GCN4-HA). Similar levels of Gcn4p-dependent binding of myc-Cyc8p and myc-Tup1p were observed for the ARG4 UAS (data not shown). Western analysis showed that gcn4Δ does not reduce the steady-state levels of myc-Cyc8p or myc-Tup1p (see Fig. S1 in the supplemental material). Hence, we conclude that Gcn4p recruits Tup1p and Cyc8p to the ARG1 and ARG4 UAS elements under inducing conditions, consistent with the idea that these proteins function directly as coactivators at these Gcn4p target genes.

Surprisingly, we observed relatively high levels of myc-Cyc8p binding at the 3′ ends of the ARG1 and ARG4 ORFs in both the WT strain and the strain overexpressing HA-Gcn4p (Fig.
Thus, there appears to be greater Gcn4p-dependent Cyc8p binding to the ORF than to the UAS (cf. Fig. 7B and D). By contrast, Tup1p association with the ARG1 ORF was more similar in magnitude to that observed at the UAS (cf. Fig. 7C and E), and comparable findings were obtained for Tup1p at ARG4 (Fig. 7G and data not shown). These results raise the possibility that Cyc8p and Tup1p influence transcription elongation in the ORF in addition to promoting Gcn4p binding to the UAS elements at these genes.

**DISCUSSION**

The results of this study show that Cyc8p and, to a lesser extent, Tup1p are required for high-level activation of Gcn4p target genes in vivo. Deletion of CYC8 confers sensitivity to SM and impairs transcriptional induction of two Gcn4p-dependent reporters (UAS_GCRE_CYC1-lacZ and HIS3-GUS) and authentic Gcn4p target genes (ARG1, ARG4, HIS4, and TRP3). ChIP analysis revealed that cyc8Δ cells exhibited diminished recruitment of SAGA and Srb mediator to the UAS, reduced promoter occupancy of TBP and Pol II, and decreased Pol II association with the 3' ends of the ORFs at ARG1 and ARG4. The tup1Δ mutant showed similar defects in coactivator recruitment, PIC assembly, and transcriptional activation of authentic target genes but was less sensitive to SM and did not significantly impair induction of the reporter genes. Thus, it appears that Cyc8p is more broadly required than Tup1p for transcriptional activation by Gcn4p.

It is possible that all of the defects in transcriptional activation of Gcn4p-dependent promoters observed with cyc8Δ and tup1Δ cells can be attributed to the decreased Gcn4p binding to the UAS elements at its target genes in these mutants. This decrease in UAS occupancy by Gcn4p cannot be attributed to reduced expression of GCN4 mRNA or decreased steady-state levels of Gcn4p protein in cyc8Δ or tup1Δ cells. No other mutations in subunits of SAGA, SWI/SNF, Srb mediator, or RSC that impair transcriptional activation by Gcn4p have been found to reduce Gcn4p binding at ARG1 or ARG4 (15, 39). Thus, reduced Gcn4p promoter occupancy in cells containing WT levels of Gcn4p is a unique phenotype of cyc8Δ and tup1Δ mutants.

One way to account for the role of Cyc8p and Tup1p in Gcn4p binding to UAS elements is to propose that they are...
required for efficient nuclear import of Gcn4p. For example, expression of one or more factors involved in nuclear import of Gcn4p could be reduced in *cyc8Δ* and *tup1Δ* cells. It was shown previously that a functional green fluorescent protein (GFP)-Gcn4 fusion protein is localized primarily in the nucleus independently of amino acid abundance (37). Thus, nuclear localization of Gcn4p is probably not regulated by amino acid levels. We have compared levels of localization of this GFP-Gcn4p fusion for *cyc8Δ* versus WT cells and found only a small decrease in the percentage of cells (from 95% to 79%) containing GFP-Gcn4p that was localized exclusively in the nucleus in *cyc8Δ* cells (see Fig. S2 in the supplemental material).

Thus, we believe that Cyc8p/Tup1p has no significant role in nuclear localization of Gcn4p.

A direct role for Cyc8p/Tup1p in promoting UAS binding by Gcn4p is suggested by our finding that both proteins are recruited by Gcn4p to its target genes in vivo. This raises the possibility that the Cyc8p/Tup1p complex modifies the chromatin structure of the UAS to increase accessibility of Gcn4p to its binding sites, perhaps by positioning nucleosomes in an organized array (5, 12, 26, 53) or by recruiting histone deacetylases to reduce histone acetylation (1, 6–8, 48, 49). Another possibility is that Cyc8p/Tup1p impedes degradation of UAS-bound Gcn4p. This idea is prompted by the finding that Srb10p, a component of the mediator, phosphorylates the Gcn4p activation domain and targets the protein for rapid degradation by the proteosome (2, 20, 29). Hence, recruitment of the mediator by Gcn4p could accelerate the degradation of UAS-bound Gcn4p without affecting the turnover of unbound Gcn4p (2). According to this last model, Cyc8p/Tup1p recruited to the UAS by Gcn4p would decrease the rate of Srb10p-mediated degradation of UAS-bound Gcn4p. To account for the fact that total steady-state levels of Gcn4p are not reduced by *cyc8Δ*, it would be necessary to stipulate that UAS-bound Gcn4p represents only a small fraction of the total cellular pool of the protein and that Gcn4p degraded at the UAS in *cyc8Δ* cells is quickly replenished by new synthesis.

Whatever the mechanism involved, it seems that Cyc8p plays a more important role than Tup1p in promoting Gcn4p binding to UAS elements. Thus, in addition to the less severe activation defects in *tup1Δ* cells versus *cyc8Δ* cells, we found that overexpressing Gcn4p can alleviate the Gcn4p binding defect in *tup1Δ* cells but not in *cyc8Δ* cells. Presumably, Gcn4p binding to the UAS can be driven to nearly WT levels in *tup1Δ* cells by increasing the cellular concentration of Gcn4p, but the impediment to UAS binding is too great to overcome by mass action in *cyc8Δ* cells.

It is interesting that high-level UAS occupancy of Gcn4p is dependent on Cyc8p and Tup1p, while at the same time, Gcn4p recruits Cyc8p and Tup1p. Thus, Gcn4p and Cyc8p/Tup1p are interdependent for high-level binding at *ARG1* and *ARG4*. Although the reduction in UAS occupancy by Gcn4p in *cyc8Δ* cells is substantial, it is not complete (e.g., Fig. 4C). Indeed, the SM sensitivity and reductions in reporter and target gene transcription are less severe in *cyc8Δ* cells than in *gcn4Δ* cells. Hence, it seems that Gcn4p can bind to a UAS element lacking associated Cyc8p, albeit with reduced efficiency. By subsequently recruiting Cyc8p/Tup1p, Gcn4p may achieve a higher level of UAS occupancy, thereby creating a positive-feedback loop (Fig. 8). We cannot rule out the possibility, however, that Cyc8p/Tup1p binds at *ARG1* independently of Gcn4p at a level below the detection limit of the ChIP assay and then promotes binding of Gcn4p to the unoccupied UAS. Once bound to the UAS, Gcn4p would then recruit Cyc8p/Tup1p to the higher levels observed under inducing conditions with our ChIP assays.

We have done several experiments to determine whether Gcn4p recruits Cyc8p/Tup1p to target genes by direct binding to these cofactors but have uncovered no evidence to support this possibility. First, in cells where myc-tagged Gcn4p was induced by SM, we observed no communoprecipitation of Tup1p with myc-Gcn4p from cell extracts, whereas Tup1p communoprecipitated with myc-Cyc8p under the same conditions (see Fig. S3 in the supplemental material). We also failed to observe a specific interaction of GST-Gcn4p with myc-Tup1p or myc-Cyc8p in yeast WCEs under conditions where robust binding of GST-Gcn4p to the mediator was readily detected (see Fig. S3 in the supplemental material). We presume that Gcn4p recruits Cyc8p/Tup1p to the UAS and ORF of its target genes in a manner dependent on other coactivators or histone modifications, and future experiments will address the mechanism of this indirect recruitment, particularly as it applies to the coding sequences.

Most previous studies of Cyc8p and Tup1p have underscored their functions as corepressors (42), but the complex can also function in activation. As noted above, Cyc8p/Tup1p remains bound at promoters regulated by Sko1p (38) and at *GAL1* (35) under inducing conditions and participates in recruitment of coactivators to overcome its own repressing functions. In the Cyc8p-dependent induction of *CIT2* by Rtg3p (4), it is unknown whether Cyc8p stimulates UAS occupancy by Rtg3p or provides a coactivator function for UAS-bound Rtg3p. The exact step in activation of *CYC1* by Hap1p that is dependent on Tup1p/Cyc8p is also unknown (52). Regarding Cyc8p-dependent activation of *FRE2* by Aft1p, Cyc8p binds in vitro to the DNA binding domain of Aft1p, and Aft1p binding at *FRE2* was apparently reduced by a small amount in *cyc8Δ* cells. Nevertheless, activation by a LexA-Aft1p fusion from LexA

![FIG. 8. Model for interdependent binding of activator Gcn4p and Cyc8p/Tup1p at *ARG1* and *ARG4* in vivo. Cyc8p/Tup1p functions to stimulate Gcn4p binding to the UAS elements at *ARG1* and *ARG4*. Interestingly, Gcn4p recruits Cyc8p/Tup1p to the promoter and ORF regions of both genes, which may provide a positive-feedback mechanism to maintain high-level Gcn4p binding at the UAS elements under inducing conditions. It is possible that Cyc8p/Tup1p binds to these genes at low levels independently of Gcn4p under noninducing conditions and then promotes binding of Gcn4p to an unoccupied UAS. Gcn4p would then recruit Cyc8p/Tup1p to the higher levels observed under inducing conditions. We do not mean to imply a direct interaction of Cyc8p/Tup1p with DNA; rather, interaction with nucleosomes is predicted (see text for further details).]
binding sites was strongly Cyc8p dependent, pointing to a role for Cys8p in the activation function of Aft1p rather than in Aft1p promoter binding (13). Thus, the prominent role of Cys8p in stimulating Gcn4p binding to the UAS elements at ARG1 and ARG4 described here appears to be unique among the yeast activators studied thus far.

We obtained one indication that Cyc8p/Tup1p also enhances the ability of Gcn4p to recruit coactivators. In strains overexpressing Gcn4p from the h.c. GCN4-PA plasmid, there was essentially the same level of Gcn4p binding to the ARG1 UAS in WT and tup1Δ cells (Fig. 4F); however, the tup1Δ cells showed a relatively lower level of SAGA and mediator recruitment to the UASs and also less myc-Rpb3p recruitment to the promoter (Fig. 5A, C, and F). Hence, Cyc8p/Tup1p may play a dual role at Gcn4p target genes, increasing the efficiency of coactivator recruitment by Gcn4p in addition to stimulating Gcn4p binding to the UAS.

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