Genetic Analysis of the Role of Pol II Holoenzyme Components in Repression by the Cyc8-Tup1 Corepressor in Yeast

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ABSTRACT

The Cyc8-Tup1 corepressor complex is targeted to promoters by pathway-specific DNA-binding repressors, thereby inhibiting the transcription of specific classes of genes. Genetic screens have identified mutations in a variety of Pol II holoenzyme components (Srb8, Srb9, Srb10, Srb11, Sin4, Rgr1, Rox3, and Hrs1) and in the N-terminal tails of histones H3 and H4 that weaken repression by Cyc8-Tup1. Here, we analyze the effect of individual and multiple mutations in many of these components on transcriptional repression of natural promoters that are regulated by Cyc8-Tup1. In all cases tested, individual mutations have a very modest effect on *SUC2* RNA levels and no detectable effect on levels of *ANB1*, *MFA2*, and *RNR2*. Furthermore, multiple mutations within the Srb components, between Srbs and Sin4, and between Srbs and histone tails affect Cyc8-Tup1 repression to the same modest extent as the individual mutations. These results argue that the weak effects of the various mutations on repression by Cyc8-Tup1 are not due to redundancy among components of the Pol II machinery, and they argue against a simple redundancy between the holoenzyme and chromatin pathways. In addition, phenotypic analysis indicates that, although Srbs8–11 are indistinguishable with respect to Cyc8-Tup1 repression, the individual Srbs are functionally distinct in other respects. Genetic interactions among *srb* mutations imply that a balance between the activities of Srb8 + Srb10 and Srb11 is important for normal cell growth.

THE yeast Cyc8-Tup1 corepressor complex is required for repressing diverse classes of genes that are expressed only under specific, but distinct, conditions of environmental challenge (DeRisi *et al.* 1997). Although Cyc8-Tup1 does not bind DNA, it is targeted to promoters by DNA-binding proteins that repress promoters in specific pathways: α2, cell type (Keleher *et al.* 1992; Komachi *et al.* 1994); Mig1 and Nrg1, glucose (Treitel and Carlson 1995; Tzamarias and Struhl 1995; Park *et al.* 1999); Rox1, oxygen (Deckert *et al.* 1995); Crt1, DNA damage (Huang *et al.* 1998); Acr1, osmolarity (Proft and Serrano 1999); and Rtg3, mitochondrial function (Conlan *et al.* 1999). Cyc8 and Tup1 are differentially important for recruitment by pathway-specific DNA-binding repressors; Cyc8 is important for recruitment by Mig1 and Rox1 (Tzamarias and Struhl 1994, 1995), whereas Tup1 is important for recruitment by α2 (Komachi *et al.* 1994). Tup1 is sufficient to mediate transcriptional repression in the absence of Cyc8, and short, nonoverlapping regions of Tup1 with minimal sequence similarity can independently mediate repression, suggesting that the Tup1 repression domain functions through protein-protein interactions (Tzamarias and Struhl 1994). Unlike the Sin3-Rpd3 corepressor complex, which possesses histone deacetylase activity (Rundlett *et al.* 1996; Kadosh and Struhl 1997, 1998), Cyc8-Tup1 has no known enzymatic function.

Two models, not mutually exclusive, have been proposed for the mechanism of repression by Cyc8-Tup1, one involving an effect on chromatin structure and the other involving direct action on the Pol II machinery. In support of the chromatin model, the Tup1 repression domain overlaps a region that interacts directly with histone H3 and H4 N-terminal tails *in vitro* (Edmondson *et al.* 1996), mutations in histone H3 and H4 tails can mildly reduce repression by α2 (Roth *et al.* 1992; Edmondson *et al.* 1996, 1998; Huang *et al.* 1997), and Cyc8-Tup1 can affect chromatin structure of some, but not all, repressed genes (Roth *et al.* 1990; Matallana *et al.* 1992; Cooper *et al.* 1994; Huang *et al.* 1997). However, the altered chromatin structure caused by Cyc8-Tup1 can be reversed by loss of Swi/Snf function, suggesting that ordering of nucleosomes at Cyc8-Tup1-repressed promoters occurs independently of the corepressor (Gavin and Simpson 1997). In support of a chromatin-independent model involving a direct effect on the Pol II machinery, Cyc8-Tup1 weakly represses transcription *in vitro* on purified DNA templates (Herschbach *et al.* 1994; Redd *et al.* 1997), and mutations in components of Pol II holoenzyme (Srb8, Srb9, Srb10, Srb11, Sin4, Rgr1, Rox3, and Hrs1) partially alleviate repression by Cyc8-Tup1 (Sakai *et al.* 1990; Chen *et al.* 1993; Balciunas and Ronne 1995; Kuchin *et al.*

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1995; Wahi and Johnson 1995; Song *et al.* 1996; Kadosh and Struhl 1997; Kuchin and Carlson 1998; Conlan *et al.* 1999; Papamichos-Chronakis *et al.* 2000). Sin4, Rox3, Hrs1, and Rgr1 are part of the same holoenzyme subcomplex (Li *et al.* 1995; Lee *et al.* 1999), whereas Srbs8–11 are present in a distinct subcomplex that is found in some holoenzyme preparations (Koleske and Young 1994; Liao *et al.* 1995), but not in others (Lee *et al.* 1997; Myers *et al.* 1998).

It is important to note that all of the existing mutations in either the chromatin or holoenzyme pathways cause very modest effects on repression by Cyc8-Tup1. Similarly, Cyc8-Tup1 repression *in vitro* (typically 2- to 4-fold) is far less pronounced than repression of natural promoters *in vivo* (typically 15- to 50-fold). These observations suggest that Cyc8-Tup1 repression involves redundant functions, within and/or between the chromatin and holoenzyme pathways. In addition, repression by Cyc8-Tup1 might involve other, as yet undefined, molecular mechanisms. Here, we analyze Cyc8-Tup1 repression of natural yeast promoters in strains containing multiple mutations. Our results argue against functional redundancies among components of the Pol II machinery and against a simple redundancy between the holoenzyme and chromatin pathways. In addition, we provide evidence for distinct functions within the Srb8–11 module.

MATERIALS AND METHODS

Yeast strains: The initial SLY strains were kindly provided by the laboratory of Rick Young, and derivatives of these strains are described in Table 1. Several of the SLY strains had *srb* alleles containing a *hisG::URA3::hisG* cassette (Alani *et al.* 1987), and we removed *URA3* sequences by selecting for cells on medium containing 5-fluoroorotic acid (5-FOA). The histone N-terminal mutant strains were the generous gift of the laboratory of Michael Grunstein (Kayne *et al.* 1988; Mann and Grunstein 1992) and derivatives of these strains are also described in Table 1. Disruptions of the *SRB* genes and of *SIN4* were performed using standard protocols, with *srb9* and *srb11* alleles being generated by two-step gene replacement; the resultant deletion alleles are termed LC. It is extremely unlikely that the resulting strains have accumulated genetic modifiers that affect temperature sensitivity or growth on galactose, because all gene replacement events were performed on glucose medium at 30°, conditions where the strains grow well, and because the same phenotypes were observed in independent strains. The construct for disrupting *SRB9*, pJZ991 (kindly provided by Jianhua Zhang and Rick Young), contains the *SRB9* locus with a deletion of the entire open reading frame. The *SRB11* disruption construct (pML2042) deletes the N-terminal 129 amino acids of the coding region (between the translational start site and an internal SphI site), with no remaining in-frame start codons until the C-terminal 45 amino acids. The *srb10* and *sin4* alleles were generated by a one-step disruption construct in which the open reading frame of these genes was replaced with *LEU2* or *URA3*. *MAT*α strains were derived from *MAT***a** strains by transient expression of the *HO* endonuclease. Strains were transformed with a *URA3*-marked centromeric plasmid carrying the *HO* gene, and colonies were picked when visible to the naked eye and immediately streaked to FOA to cure the *HO* expression plasmid; the resulting cells were examined for mating type using *thr4* tester strains.

TABLE 1

Strains used in this study

Strain	Genotype
SLY3	*MAT***a** *his3Δ200 leu2-3,112 ura3-52*
LC3	SLY3 *MAT*α
LC4	SLY3 *tup1::LEU2*
LC5	LC3 *tup1::LEU2*
SLY40	SLY3 *srb8Δ1::hisG*
LC11	LC3 *srb8Δ1::hisG*
LC12	SLY3 *srb9ΔLC*
LC16	LC3 *srb9ΔLC*
SLY5	SLY3 *srb10Δ1::hisG*
SLY96	SLY3 *srb10-D290A*
LC13	LC3 *srb10Δ1::hisG*
SLY107	SLY3 *srb11Δ1::hisG*
LC21	SLY3 *srb8Δ1::hisG srb9ΔLC*
SLY42	SLY3 *srb8Δ1::HIS3 srb10Δ1::hisG*
LC23	SLY3 *srb8Δ1::hisG srb11ΔLC*
LC27	LC3 *srb8Δ1::hisG srb11ΔLC*
LC24	SLY3 *srb9ΔLC srb10Δ1::hisG*
LC25	SLY3 *srb9ΔLC srb11Δ1::hisG*
SLY72	SLY3 *srb10Δ1::HIS3 srb11Δ1::hisG*
LC26	LC3 *srb10Δ1::HIS3 srb11Δ1::hisG*
LC29	SLY3 *srb10-D290A srb11ΔLC*
LC31	SLY3 *srb8Δ1::HIS3 srb9ΔLC srb10Δ1::hisG*
LC32	SLY3 *srb8Δ1::hisG srb9ΔLC srb11ΔLC*
LC33	SLY3 *srb8Δ1::HIS3 srb10Δ1::hisG srb11ΔLC*
LC35	LC3 *srb8Δ1::HIS3 srb10Δ1::hisG srb11ΔLC*
LC34	SLY3 *srb9ΔLC srb10Δ1::HIS3 srb11Δ1::hisG*
LC41	SLY3 *srb8Δ1::HIS3 srb9ΔLC srb10Δ1::hisG srb11ΔLC*
LC100	SLY3 *sin4ΔLC*
LC101	SLY3 *sin4ΔLC srb10Δ1::hisG*
LC102	SLY3 *sin4ΔLC srb11Δ1::hisG*
PKY806	*MAT***a** *ade2-101 his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-2 thr- tyr- arg4-1 hhf1::HIS3 hhf2::LEU2* (*CEN4 ARS1 TRP1 hhf2Δ4-23*)
LC201	PKY806 *srb10ΔLC::URA3*
LC202	PKY806 *srb11ΔLC*
RMY430	*MAT***a** *ade2-101 his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 hht1,hhf1::LEU2 hht2, hhf2::HIS3* (*CEN4 ARS1 TRP1 hht2Δ4-30, HHF2*)
LC203	RMY430 *srb10ΔLC::URA3*
LC204	RMY430 *srb11ΔLC*

Transcriptional analyses: RNA levels for Cyc8-Tup1-regulated and control genes were assayed by Northern blotting. In general, strains were grown in complete casamino acid medium containing 2% glucose and harvested at an OD_{600} below 0.5. For induction of *SUC2*, cells were grown as above, harvested by centrifugation, washed in an equal volume of complete casamino acid medium lacking glucose, and resuspended in the same medium containing 0.1% glucose for 1 hr. Total RNA from cells was prepared by hot acid phenol extraction (Iyer and Struhl 1996), quantitated by OD_{260}, and tested for integrity by agarose gel electrophoresis and ethidium bromide staining. The ^{32}P-labeled probes were generated by random hexamer labeling of the following DNAs: a 1.2-kb *Hin*dIII *SUC2* fragment; a 1.5-kb *Sma*I-*Bam*HI frag-

ment containing *ANB1* (which also hybridizes with the tr1 transcript); a 1-kb *Hin*dIII fragment from *MFA2*; and a 250-bp *Pst*I-*Eco*RI fragment of *TUB2*. β-Galactosidase assays for transcriptional repression by LexA hybrid proteins (Tzamarias and Struhl 1994) and for Gal4-dependent activation (Singer *et al.* 1990) were carried out as described previously.

RESULTS

Very modest role of Srbs8–11 in transcriptional repression of natural promoters by Cyc8-Tup1: Strains containing individual or multiple mutations in *SRB8*, *SRB9*, *SRB10*, and *SRB11* were analyzed for transcriptional repression of natural promoters representing four regulatory pathways regulated by Cyc8-Tup1: cell type (*MFA1*, *MFA2*), glucose (*SUC2*), oxygen (*ANB1*), and DNA damage (*RNR2*). Deletion of any combination of these *SRB* genes, including the quadruple mutant, exhibits an identical effect on the expression of all genes tested (Figure 1). Unexpectedly, *SUC2* is the only message that is affected, with *srb* mutant strains showing 3-fold higher levels of expression than the wild-type strain. This effect is modest in comparison to a *tup1* deletion strain, which shows a 50-fold increase. The low level of *SUC2* transcription in *srb* mutant strains is not due to a concurrent defect in transcriptional activation, because all *srb* mutant strains are fully competent for *SUC2* induction in response to conditions of low glucose. Furthermore, deletion of *tup1* in the background of the *srb* quadruple mutant results in levels of *SUC2* transcription that are indistinguishable from that observed in a *tup1* mutant strain (data not shown).

Transcription of genes representing the other three pathways repressed by Cyc8-Tup1 are not detectably affected by mutations in *srbs8–11*. Our inability to see an effect on *MFA2* RNA levels is in apparent contrast to the observation that *srb8* and *srb10* mutant strains can result in increased expression of an integrated *MFA2-LacZ* reporter (Wahi and Johnson 1995). This apparent discrepancy might be due to an inability to detect very low RNA levels, although there are increasing numbers of examples in which *LacZ* reporter assays and RNA measurements give different results. However, even in the case of the experiments involving the *MFA2-LacZ* fusions, *srb8* and *srb10* strains show robust, although somewhat weakened, repression. In any event, the combined results indicate that Srbs8–11 have a minimal or very modest effect on Cyc8-Tup1 repression of natural promoters.

We also examined potential redundancy among Srbs8–11 under conditions where the Cyc8-Tup1 complex is artificially recruited to promoters via a LexA DNA-binding domain (Table 2). Specifically, LexA-Cyc8 and LexA-Tup1 fusion proteins were analyzed on β-galactosidase reporters driven by promoters that do or do not contain four LexA-binding sites upstream of the *CYC1* UAS and TATA element. In accord with previous results (Kadosh and Struhl 1997; Kuchin and Carlson 1998), repression by either of these LexA

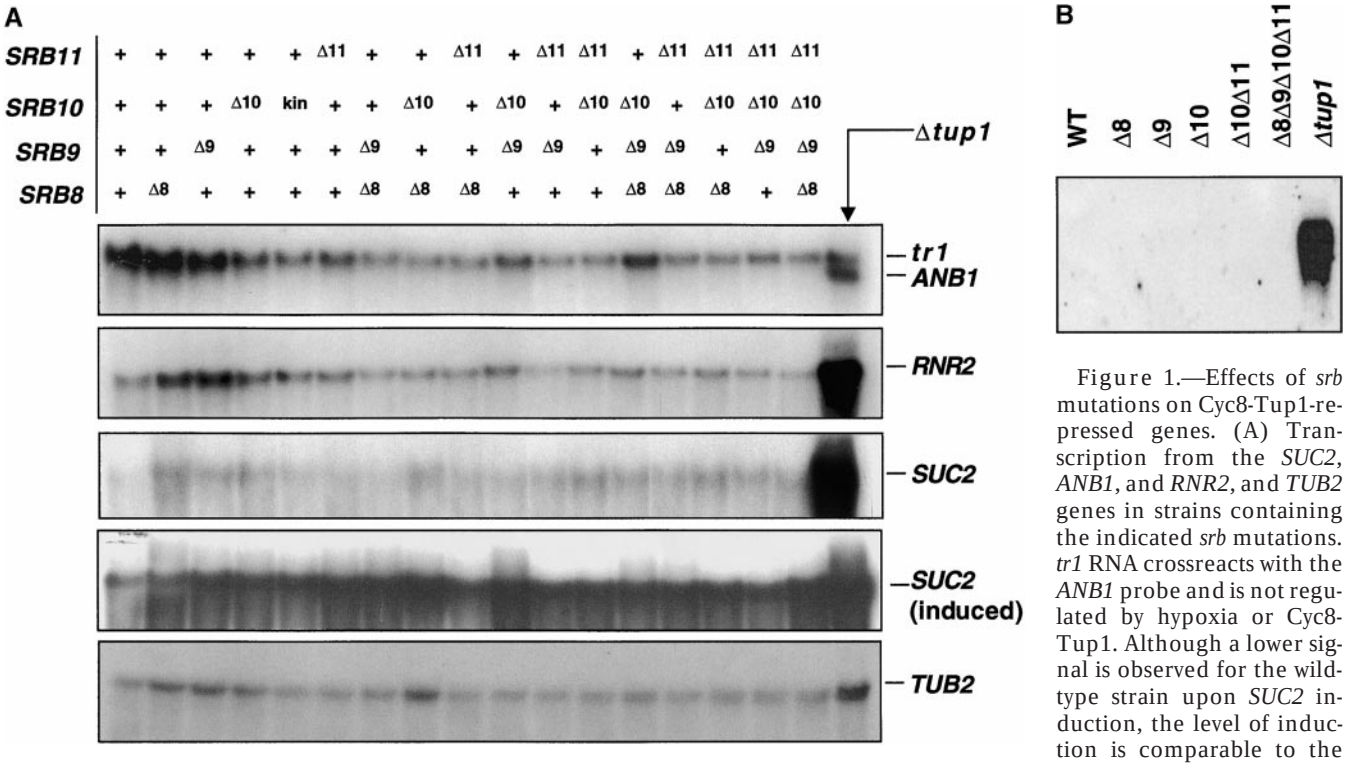

Figure 1.—Effects of *srb* mutations on Cyc8-Tup1-repressed genes. (A) Transcription from the *SUC2*, *ANB1*, and *RNR2*, and *TUB2* genes in strains containing the indicated *srb* mutations. *tr1* RNA crossreacts with the *ANB1* probe and is not regulated by hypoxia or Cyc8-Tup1. Although a lower signal is observed for the wild-type strain upon *SUC2* induction, the level of induction is comparable to the other strains when normalized to the *TUB2* control in the same experiment (data not shown). (B) Effect on *MFA2* repression in *MAT*α strains of the indicated genotypes.

TABLE 2

Effects of *srb* mutations on repression by LexA-Cyc8 and LexA-Tup1

Strain	Fold repression	
	LexA-Cyc8	LexA-Tup1
SRB+	8.0	7.9
srb8	3.3	2.5
srb9	1.9	2.7
srb10	1.7	3.0
srb10-D290A	1.2	2.5
srb11Δ	2.0	1.7
srb10 srb11	1.8	1.4
srb9 srb10 srb11	2.7	3.0
srb8 srb9 srb10 srb11	3.4	4.1

fusion proteins is reduced three- to fivefold in *srb10* and *srb11* strains. As noted previously (Kuchin and Carlson 1998), repression by artificial recruitment of Cyc8-Tup1 is quite modest (two- to threefold) in *srb* mutant strains, which is in contrast to the situation with natural promoters where Cyc8-Tup1 repression is robust. A similar effect on repression by LexA-Cyc8 and LexA-Tup1 is observed in *srb8* and *srb9* strains as well as strains lacking all four proteins. Taken together, these observations indicate that Srbs8–11 play indistinguishable roles in repression by Cyc8-Tup1 and that the minimal or modest effects of Srbs8–11 on natural promoters are not due to redundant functions within this module.

Functional distinctions among components of the Srb8–11 module: Although the entire panel of *srb8–11* disruption strains behaves indistinguishably with respect to repression by Cyc8-Tup1, we found that certain *srb* mutant strains are unable to grow at 37° (Figure 2). Among the single mutants, only the *srb11* strain exhibited temperature-sensitive (ts) growth. However, strains deleted for either *srb8* or *srb10* in combination with the *srb11* deletion were viable at 37° as was the *srb8*, *srb10*, *srb11* triple mutant. Deletion of *srb9* did not alter the ts phenotype of any of these strains. Given that Srb10 and Srb11 encode a kinase/cyclin pair (Kuchin *et al.* 1995; Liao *et al.* 1995), we introduced an *srb11* deletion allele into a strain carrying *srb10*-D290A, which encodes a version of Srb10 with a point mutation in the kinase active site (Liao *et al.* 1995). The resulting strain was phenotypically identical to the *srb10Δ*, *srb11Δ* strain, indicating the involvement of the kinase activity in this genetic interaction. One interpretation of these results is that Srb11 might regulate the activity of Srb8 and Srb10 and that the ts phenotype is due to hyperphosphorylation by Srb10. However, the *srb8*, *srb10* double mutant exhibits a ts phenotype that is suppressed by an *srb11* deletion, indicating that the ts phenotype is not simply related to Srb10 kinase function.

A related, though nonidentical, distinction between

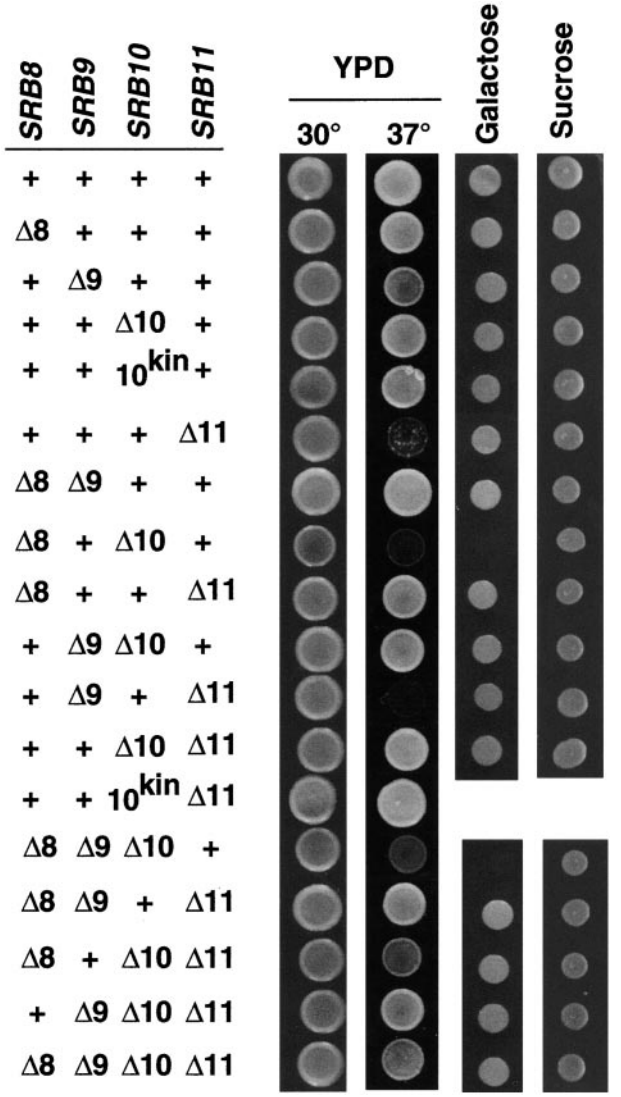

Figure 2.—Genetic interactions among the *srb* mutations. Approximately 10^5 cells of the indicated genotypes were spotted on plates containing YPD (30°, 37°) or synthetic minimal medium containing 2% galactose.

components of the Srb8–11 module is observed when strains are grown on galactose. It has been observed previously that *srb10* mutants are significantly defective for Gal4-dependent activation (Liao *et al.* 1995; Kuchin and Carlson 1998), which is likely due to Gal4 being a substrate for Srb10 kinase (Hirst *et al.* 1999). Although the *srb10* and other single mutant *srb* strains grow on synthetic minimal medium containing galactose as the sole carbon source, a strain deleted for both *srb8* and *srb10* fails to grow. This Gal^- phenotype is unaffected by an *srb9* deletion, but it is suppressed by an *srb11* deletion. In accord with these growth phenotypes, *srb8*, *srb10* strains are unable to activate a Gal4-responsive β-galactosidase reporter, whereas single mutant strains and the *srb8*, *srb10*, *srb11* triple mutant strain show only mildly reduced levels of activity (Figure 3). Taken together, these genetic interactions imply that a

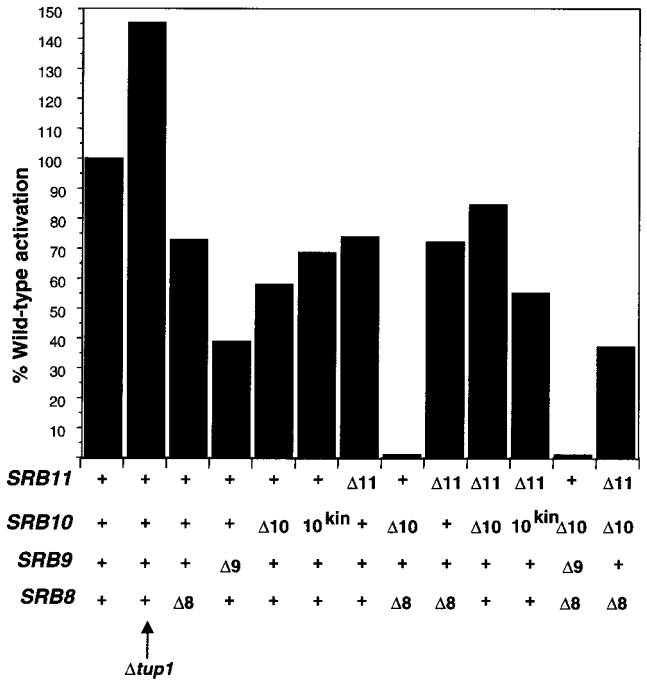

Figure 3.—Gal4-dependent transcriptional activation in *srb* mutant strains. β-Galactosidase activities in the indicated *srb* mutant strains containing Ycp86-Sc3801 (Singer *et al.* 1990), a Gal4-dependent *LacZ* reporter that was initially grown in casamino acid medium with 2% raffinose (noninducing condition) and then induced for 12 hr by addition of 2% galactose.

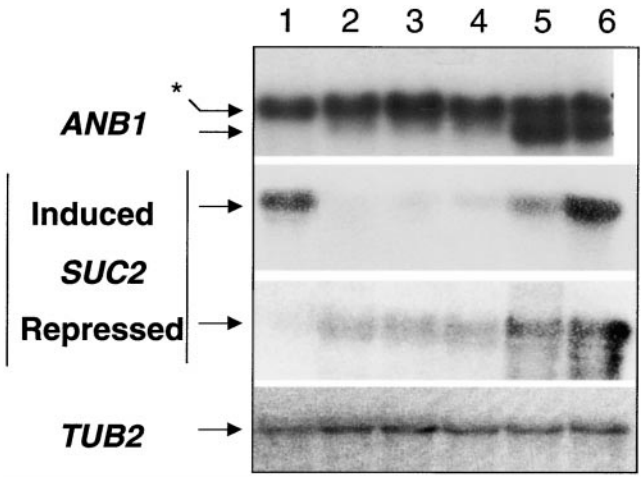

Figure 4.—Expression of Cyc8-Tup1-repressed genes in strains containing *sin4* and *srb10* or *srb11* mutations on Cyc8-Tup1-repressed genes. Northern blots of *ANB1*, *SUC2* (induced and repressed), and *TUB2* mRNA levels in wild-type (lane 1), *sin4* deletion (lane 2), *sin4*, *srb10* double deletion (lane 3), *sin4*, *srb11* double deletion (lane 4), *sin4*, *tup1* double deletion (lane 5), and *tup1* deletion (lane 6) strains. For the *SUC2* experiments, the panel for induced expression is exposed for a shorter time than the panel for repressed expression, and lanes 5 and 6 of the panel for repressed expression are underexposed in comparison to lanes 1–4. The faint band seen below the tr1 band in lanes 2–4 is of a different size than the band for *ANB1* and was not reproducible in repeated trials.

balance between the activities of Srb8 + Srb10 and Srb11 is important for normal growth of the cell, and that individual components of the Srb8–11 module have different functions.

Srb8–11 does not have redundant functions with Sin4 or histones H3/H4 with respect to Cyc8-Tup1 repression: Having demonstrated the absence of redundancy between Srbs8–11, we tested for redundancy between these Srbs and Sin4. As mentioned in the Introduction, although Sin4 and Srbs8–11 have been implicated in Cyc8-Tup1 function, Sin4 appears to be an integral component of the Pol II holoenzyme, while Srbs8–11 appear to be in a distinct and more loosely associated subcomplex. If distinct subcomplexes with Pol II holoenzyme represent redundant targets for Cyc8-Tup1 action, simultaneous loss of Sin4 and Srbs8–11 might be expected to cause a dramatic loss of Cyc8-Tup1 repression. Moreover, Pol II holoenzyme in *sin4* deletion strains also lacks Hrs1, Med2, Gall1, and perhaps other components (Li *et al.* 1995; Myers *et al.* 1999). However, under repressing conditions, a strain lacking both Sin4 (and associated holoenzyme components) and Srbs10 or 11 shows only a weak increase in *SUC2* expression that is comparable to that observed in strains lacking either Sin4 or Srbs8–11 (Figure 4). It should be noted that the *SUC2* induction in low glucose medium is significantly compromised in *sin4* deletion strains (Figure 4, lanes 2–4). However, a *sin4*, *tup1* double deletion strain (Figure 4, lane 5) displays levels of *ANB1* and *SUC2* that are comparable to *tup1* strain (Figure 4, lane 6). For this reason, it appears unlikely that the absence of synergistic loss of repression in the *sin4*, *srb* double deletion strains can be attributed to an activation defect caused by the *sin4* mutation. Instead, we conclude that Srbs8–11 and Sin4 are not redundant for Cyc8-Tup1 repression.

Because mutation of the N-terminal tails of histones H3 and H4 can partially interfere with Cyc8-Tup1 repression (Edmondson *et al.* 1996, 1998), we examined potential redundancy between holoenzyme and chromatin mechanisms. In apparent contrast to a previous report involving *RNR2*- and α2-dependent *LacZ* reporter constructs (Edmondson *et al.* 1996), we did not observe any effect of the histone tail mutations on the *ANB1* promoter (Figure 5). This apparent discrepancy might

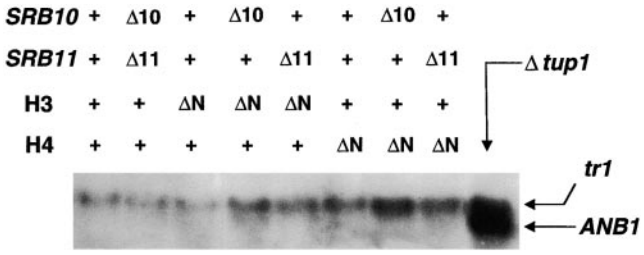

Figure 5.—Expression of *ANB1* in strains containing *srb10* or *srb11* and histone H3 and H4 tail mutations.

reflect differences in sensitivities of the assays and/or differences between *LacZ* reporters and natural promoters, although the reported effects of the histone tail mutations appear to be roughly comparable to the modest effects of the *srb* mutations. In any event, strains containing mutations in the histone H3 or H4 tails as well as Srbs8–11 do not result in a synergistic loss of Cyc8-Tup1 repression; indeed, repression of the *ANB1* promoter is virtually unaffected (Figure 5). This result suggests that the mechanisms involving histone tails and Srbs8–11 do not represent redundant pathways for repression by Cyc8-Tup1.

DISCUSSION

Genetic analysis from many laboratories has identified a number of proteins that appear to have some involvement in repression by Cyc8-Tup1 (Sakai *et al.* 1990; Chen *et al.* 1993; Balciunas and Ronne 1995; Kuchin *et al.* 1995; Wahi and Johnson 1995; Song *et al.* 1996; Kuchin and Carlson 1998; Papamichos-Chronakis *et al.* 2000). These include components of the Srb8–11 and Sin4-Rgr1-Rox3 subcomplexes of Pol II holoenzyme (Koleske and Young 1994; Li *et al.* 1995; Liao *et al.* 1995; Lee *et al.* 1997 #2530, 1999; Myers *et al.* 1998; Hampsey and Reinberg 1999), Ctk1, which phosphorylates the C-terminal tail of Pol II in a manner distinct from Srb10 (Hengartner *et al.* 1998), and the N-terminal tails of histone H3 and H4 (Edmondson *et al.* 1996, 1998). Importantly, all of the existing mutations in either the chromatin or holoenzyme pathways cause modest effects on repression by Cyc8-Tup1. Indeed, when RNA levels of natural genes are assayed, the effects of many of these mutations are modest on the *SUC2* gene and undetectable on the *ANB1*, *RNR2*, and *MFA2* genes.

There are several possible explanations, not mutually exclusive, for why the above mutations have a modest effect on Cyc8-Tup1 repression. First, the mutations might not completely abolish the functions of the proteins or complexes. This is unlikely to be the case for the individual proteins, as the mutations analyzed are drastic disruptions or complete deletions, but it might account for the modest effects of the histone tail mutations. It is not possible to analyze yeast strains lacking all histone tails, as such strains are inviable. Second, Cyc8-Tup1 might function through multiple targets with the Pol II machinery and/or distinct holoenzyme and chromatin pathways defined by the mutations. In the face of such functional redundancy, complete loss of Cyc8-Tup1 function would require inactivating all of these independent targets or pathways. Third, Cyc8-Tup1 repression might involve a novel function that has yet to be revealed through mutational analysis.

Here, we provide evidence that various forms of functional redundancy do not account for the modest effects of the mutations on Cyc8-Tup1 repression. Specifically, we demonstrate that multiple mutations within Srbs8–11, between Srbs8–11 and Sin4, and between Srbs8–11 and histone tails affect Cyc8-Tup1 repression to the same modest extent as the individual mutations. We were unable to examine the effects of more complex combinations of mutations, because the resulting strains were extremely sick or nonviable. Nevertheless, the failure to observe any additional defect in the multiply mutated strains tested here is noteworthy, because eliminating redundant functions should lead to increased loss of Cyc8-Tup1 repression even if some redundant functions remain. In this regard, the CTD kinases Srb10 and Ctk1 can independently affect Cyc8-Tup1 repression, although the double mutant strain still retains considerable Cyc8-Tup1 function (Kuchin and Carlson 1998). Taken together, our results argue that components of Pol II holoenzyme make only a minor contribution to Cyc8-Tup1 repression of natural promoters.

Our suggestion that Pol II holoenzyme plays a minor role in Cyc8-Tup1 repression is consistent with the observation that Cyc8-Tup1 blocks the association of TATA-binding protein (TBP) with natural promoters *in vivo* (Kuras and Struhl 1999). As numerous biochemical experiments indicate that TBP is required for the association of the remainder of the Pol II machinery, this observation suggests that Cyc8-Tup1 repression occurs under conditions where the entire Pol II machinery is not associated with promoters. Thus, we disfavor models in which Cyc8-Tup1 interacts with holoenzyme components associated at the promoter and blocks transcriptional activity at a later step such as phosphorylation of the C-terminal tail of Pol II. Indeed, blocking phosphorylation of the Pol II tail by inactivating the Kin28 kinase subunit of TFIIH does not affect TBP occupancy, even though it eliminates transcription (Kuras and Struhl 1999). More generally, it is difficult to explain how direct interaction of Cyc8-Tup1 with targets in the Pol II machinery makes a major contribution to repression given that Cyc8-Tup1 blocks association of the machinery with promoters.

If the Pol II holoenzyme plays a minor role in repression of natural promoters by Cyc8-Tup1, what is the predominant mechanism? One possibility is that Cyc8-Tup1 functions predominantly through a chromatin mechanism and that the current experiments have adequately addressed the issue of redundancy. In this regard, if interactions of Cyc8-Tup1 with histone tails are crucial (Edmondson *et al.* 1996), it would be difficult to completely remove all potential "targets" without killing the cell. Alternatively, repression of natural promoters might involve a distinct function of Cyc8-Tup1 that has not been revealed by mutations. For example, a great deal of Cyc8-Tup1 repression of natural promoters might be due simply to steric hindrance. Binding of LexA to operators located between the enhancer and TATA elements can inhibit transcription by a factor of 5–10 (Brent and Ptashne 1984), and such a blocking

effect is likely to be more significant if a large complex such as Cyc8-Tup1 is located at a similar position. The complex, consisting of one molecule of Cyc8 and three to four molecules of Tup1 (Varanasi *et al.* 1996; Redd *et al.* 1997), is ~600 kD, and multiple complexes are likely to be recruited to promoters given the multimeric nature of the DNA-binding proteins.

The effects of the *srb* mutations vary among the different promoters regulated by Cyc8-Tup1. As shown here and elsewhere (Kuchin and Carlson 1998), *srb* mutant strains affect repression of artificial promoters by LexA-Cyc8 and LexA-Tup1 more significantly than repression of natural promoters. Steric interference by Cyc8-Tup1 might account for this observation, particularly because the LexA-binding sites in the artificial promoters are located ~100 bp upstream of the enhancer regions, a position that should minimize potential steric effects. However, we cannot exclude the possibility that LexA-Cyc8 and LexA-Tup1 are partially compromised for repression function such that the effects of the *srb* mutations are exaggerated. Our results also indicate that repression of *SUC2* is more sensitive to the various mutations tested than repression of *ANB1*, *MFA2*, and *RNR2*. Although we do not understand the basis for this observation, the apparent specificity for *SUC2* might be related to Sfl1, a protein that binds the *SUC2* promoter and associates with the Srbs and other holoenzyme components (Song and Carlson 1998).

Although the functions of Srbs8–11 appear indistinguishable with respect to repression by Cyc8-Tup1, our results indicate that these proteins are not functionally equivalent in other respects. For example, loss of both Srb8 and Srb10 causes a failure to grow at high temperature and on galactose medium, and these effects are reversed by loss of Srb11. In addition, loss of Srb11 causes a ts phenotype that is reversed by loss of either Srb8 or Srb10. These observations suggest that Srb11 cyclin can negatively regulate the activity of Srb10, its associated kinase, and that Srb8 can both positively regulate the function of Srb10 and also contribute functions independent of the kinase. While we do not understand the molecular or biological bases for the distinct phenotypes and suppression properties conferred by the various *srb* mutations, it is clear that the Srb8–11 module is more complicated than previously expected. One explanation for these genetic results is that Srbs8–11 might be present in distinct versions of Pol II holoenzyme and/or other complexes lacking Pol II. In this regard, the mammalian NAT and SMCC complexes contain homologues of Srb8 and Srb10 but not the other Srbs associated with mediator, and these complexes can negatively regulate transcription *in vitro* (Sun *et al.* 1998; Gu *et al.* 1999). It is becoming increasingly clear that eukaryotic transcriptional regulatory proteins are often present in multiple complexes, and it is almost certain that this contributes to the complex patterns of gene expression.

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