Interaction of a transcriptional repressor with the RNA polymerase II holoenzyme plays a crucial role in repression

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The yeast transcriptional repressor Tup1, tethered to DNA, represses to strikingly different degrees transcription elicited by members of two classes of activators. Repression in both cases is virtually eliminated by mutation of either member of the cyclin-kinase pair Srb10/11. In contrast, telomeric chromatin affects both classes of activators equally, and in neither case is that repression affected by mutation of Srb10/11. *In vitro*, Tup1 interacts with RNA polymerase II holoenzyme bearing Srb10 as well as with the separated Srb10. These and other findings indicate that at least one aspect of Tup1's action involves interaction with the RNA polymerase II holoenzyme.

The Tup1/Ssn6 repressing complex controls at least seven gene networks in yeast (1). These include genes regulated by glucose (2), osmotic shock (3), hypoxia (4), and DNA damage (5), as well as genes that determine the *a* mating type and, in diploids, haploid-specific genes (6–8). The Tup1/Ssn6 complex does not by itself bind DNA. Rather, it is recruited by specific DNA-binding proteins, and when it is thus localized, it counters the effects of activators on nearby promoters (9, 10). At promoters of the glucose-repressible genes *GAL1*, *GAL10*, *SUC2,* and *FBP1*, for example, the Tup1/Ssn6 complex is recruited by the glucose-responsive DNA-binding protein Mig1 (2, 11). And on promoters regulated by mating type status—for example, *BAR1*, *MFA1*, *MFA2*, *STE2,* and *STE6*—the Tup1/Ssn6 complex is recruited by the DNA-binding protein α2 in cells of mating type α (12). Mutations in either Tup1 or Ssn6 give rise to defects in repression, but the following two findings show that Tup1 plays the predominant role. First, defects in repression caused by deletion of both proteins can be overcome, at least in part, by overexpression of Tup1, but not by overexpression of Ssn6 (8). Second, LexA-Tup1 fusions (which bear the DNA-binding region of LexA fused to Tup1) repress, at least partially, transcription of synthetic promoters bearing LexA sites in the absence of Ssn6, whereas repression by LexA-Ssn6 fusions depends entirely on the presence of a functional Tup1 protein (13).

The telomere position effect is an example of a form of repression, referred to as "silencing", that does not involve Tup1 (14). In contrast to repression mediated by Tup1/Ssn6—which, as noted, typically requires recruitment of the complex to the gene—the telomere position effect requires that the gene be placed near a yeast telomere (14). Silencing also requires the product of various genes including *SIR2*, which are believed to establish a heterochromatin-like structure that reduces the accessibility of the DNA to the transcriptional machinery (15, 16). Silencing is most readily observed by measuring the basal level of expression of certain genes, and can be partially or even completely overcome by the binding of activators near the gene (17). Previous experiments have suggested that Tup1-mediated repression might also involve formation of silencing heterochromatin (18–20). These include reports that Tup1 binds histone tails along the entire length of the promoter and occludes the transcriptional machinery (18) and that Tup1 recruits a histone deacetylase activity (21). However, although the Tup1-histone tail interactions have been suggested to play a role in repression of certain promoters, others have reported that Tup1 represses significantly even where the relevant histone tails are presumably rendered nonfunctional by mutations (22, 23).

Srb10/11 constitute a kinase-cyclin pair that is found in the RNA polymerase II holoenzyme (24). Srb10 has been suggested to play two opposing roles in transcriptional regulation. On the one hand, by phosphorylating the activator Gal4, it can facilitate induction of the *GAL* genes by galactose (25), and, moreover, Srb10 has been identified as a target of activating regions (A.A., S.S.K., Z.Z., R.Y., and M.P., unpublished work). On the other hand, by phosphorylating the C-terminal domain of the largest subunit of RNA polymerase II, it can inactivate the holoenzyme, as assayed *in vitro*, and thus has been proposed to play a negative role in transcription (26). Deletion of Srb10/11 or Srb7 has been reported to decrease Tup1-mediated repression and TUP1 has recently been reported to interact with Srb7 (23, 27, 28).

Transcriptional activators may be divided into two broad classes called classical and nonclassical. Classical activators—e.g., Gal4, Hap4, and Gcn4—have acidic activating regions that are believed to contact an array of targets in the transcriptional machinery, an interaction that activates transcription by recruiting various components of the machinery to a nearby promoter (29). Vp16 is a mammalian viral acidic activator that also works in yeast in this fashion (30). Nonclassical activators, in contrast, typically comprise a DNA-binding (DBD) (e.g., gal11) domain fused to a component of the transcriptional machinery. When tethered to DNA near a promoter, these fusion proteins also activate transcription by recruiting that machinery to the promoter (29), but in this case the targets touched by the classical activators remain free.

Here we compare the abilities of two types of repression—Tup1-mediated repression and chromatin (telomere)-mediated silencing—to inhibit activation mediated by two classes of activators, classical and nonclassical. The asymmetry we observe implicates some mechanism other than, or in addition to, a chromatin-mediated silencing-like effect to explain the action of Tup1. Consistent with that finding we show that deletion of Srb10/11 eliminates Tup1-mediated repression of our reporters but has no effect on silencing induced by positioning those genes near a telomere. Moreover, Tup1 interacts *in vitro* with holoenzyme bearing Srb10, with Srb10/11 subcomplex, and with purified Srb10 itself. These and additional experiments indicate that the interaction of Tup1 with the RNA polymerase holoenzyme plays an important role in mediating repression.

Materials and Methods

Strains and Plasmids. The reporter gene was integrated in single copy form at the ura3–52 locus of yeast strain NLY2 to generate

Abbreviations: FOA, fluoroorotic acid; DBD, DNA-binding domain.

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the reporter strain. This strain was cotransformed with combinations of plasmids expressing activator and repressor proteins. From these transformants a sample of colonies was grown in selective media to midlog phase ($A_{595} \approx 0.3–0.7$), harvested, and assayed for β-galactosidase activity as described (30). Carbon source was 2% galactose and 2% raffinose. Yeast was NLY2 (MATα *ura3–52, his3Δ200, leu2–1, trp1Δ63, lys2–358,* Δ*gal4,* Δ*gal80*). Plasmids were reporter gene, plasmid pZZ41 constructed by inserting the *Xba*I repression cassette from plasmid pG_5L_2 (31) into plasmid pJP-50 template (gift from J. Pearlburg, Harvard University) at the *Xba*I site located upstream of the lacZ gene. All plasmids expressing Gal4 derivatives are *HIS3* ARS/CEN and contain Gal4 residues 1–100). Gal4-Tup1 consists of Gal4 (1–100) fused at the N terminus to full-length Tup1 (residues 1–713). LexA derivatives (all plasmids are *LEU2* ARS/CEN and contain lexA residues 1–202) fused N terminus in each case to the full activator protein minus the activator protein's DBD where appropriate. These fusion proteins bear full-length Srb2, Tbp, Srb11, and Gal11 (residues 141-1081), Gal4 (residues 74–881), Vp16 (residues 411–490), Gcn4 (1–142), and Hap4 (330–554), respectively. p201 has been described (32). The hisG::*URA3* method was used to knock out Srb10 and Srb11 proteins in the wild-type strain. The ΔSrb10 strain was generated by using plasmid pJZ991 and the ΔSrb11 strain was generated by using plasmid pJZ11–13 (gifts from C. Hengartner and J. Zhang, Whitehead Institute, MIT). The Srb10–3 mutant was generated by replacing the native Srb10 sequence with a PCR product containing the Srb10–3 sequence and replacing the native sequence as described (33).

Test for Telomere Position Effect. Yeast cells were grown overnight in selective media, suspended in H_2O, and equivalent aliquots of 10-fold serial dilutions were spotted onto synthetic medium with or without fluoroorotic acid (FOA). Plates were incubated for 4 days at 30°C before photography. Plates contained 2% (vol/vol) galactose and 2% (vol/vol) raffinose but lacked histidine to maintain plasmid selection. For yeast strains, the telomeric reporter strain was UCC420 (17) and the internal reporter strain was UCC419 (17). ΔSrb10 and ΔSrb11 strains were generated as derivatives of strain UCC420 in which the native chromosomal coding sequence of the Srb10 and Srb11 genes, respectively, was replaced by insertion of the *TRP1* gene.

***In Vitro* Interaction Assays.** Flag epitope-tagged Tup1, Gal4-Tup1, Srb10/11, and Srb10–3/11 were purified as described (26, 34). For Tup1 interaction with holoenzyme, 300 μg of precleared nuclear extracts from isogenic strains differing only in the presence of Srb10 was incubated with 1 μg of Gal4-Tup1 in 200 μl of *in vitro* transcription reaction compatible MTB buffer [20 mM Hepes (pH 7.5)/100 mM K-glutamate/15 mM Mg-Acetate/0.5 mM EGTA/0.1% Nonidet P-40/10% (vol/vol) glycerol]. The holoenzyme and proteins bound to it were precipitated by using 5 μl of the anti-Srb5 polyclonal antibody along with protein A Sepharose. The pellets were washed five times with MTB buffer. Coimmunoprecipitation experiments of Tup1 with Srb proteins and subsequent immunoblot analyses were performed as described (35). Rpb1 and Tup1 were detected by using monoclonal antibodies, 8WG16 and anti-flag, respectively. Srb5, Srb9, Srb10, Srb11, Tbp, and ovalbumin were detected with polyclonal antibodies raised against each of those proteins. Horseradish peroxidase-conjugated anti-mouse (Pierce) and anti-rabbit (Amersham Pharmacia) secondary antibodies were used. Immunoblots were visualized by using enhanced chemiluminescence according to the manufacturer's directions (Amersham Pharmacia).

Chromatin Immunoprecipitation Assays and RNA Analysis. Total RNA was isolated from MATa (strain Yag2) and MATα (strain JPY9) cells grown to mid log phase in yeast media containing 2% (vol/vol) glucose and subjected to quantitative reverse transcription–PCR analysis as described. Isogenic strains of either mating type were used. Chromatin immunoprecipitation studies were carried out as follows. Mata and Matα strains were grown as indicated above and subjected to chromatin immunoprecipitation analysis as described (36–38, 40). Real-time detection and quantitative PCR analysis were done by using the GeneAmp Sequence Detection System (Perkin–Elmer) according to the manufacturer's instructions. PCR conditions were determined empirically for each primer pair. Typically, a PCR cycle consisted of a 94°C 30-sec denaturing step, a 50°C 45-sec annealing step, and extension at 72°C for 1 min. Primer sequences were selected to give an approximately 250-bp PCR product centered at the core promoter region of each gene. Primer sequences are available on request.

Results

Differential Tup1-Mediated Repression of Two Classes of Activator and Srb10-Dependence. The effects of DNA-tethered Tup1 on transcription stimulated by classical and nonclassical activators as assayed with a reporter integrated away from the telomere are shown in Fig. 1. The figure shows that activation by five nonclassical activators, working over a wide array of efficiencies, is repressed much more markedly by DNA-tethered Tup1 than is transcription elicited by four classical activators. The former repressive effect ranges from 11-fold to 30-fold, the latter ranges from 3-fold to 5-fold. Included in the category of nonclassical activators is p201, a novel nonacidic activator that is believed to contact a target or targets different from those contacted by classical, acidic activators (32). As shown in Table 1, the Tup1-mediated repression we observed is virtually eliminated for both classes of activator by deletion of Srb10 or Srb11, or by substituting for wild type the Srb10–3 point mutant, a derivative that lacks kinase activity (24).

The experiment of Fig. 1, as indicated, used a *GAL1-lacZ* reporter bearing five Gal4 DNA-binding sites (for binding the repressor Gal4-Tup1) upstream of two LexA DNA-binding sites (for binding activators bearing the LexA DBD). The experiment was repeated using an otherwise identical reporter except that the *GAL1-lacZ* fusion was replaced by *URA3-lacZ*. Results essentially identical to those of Fig. 1 were obtained (data not shown).

Equal Telomeric Repression of Two Classes of Activator and Srb10 Independence. In the experiment of Fig. 2 we placed a *URA3* gene, bearing a single upstream Gal4 site, near a telomere (*A*), and compared its expression with that of an identical reporter positioned internally, away from the telomere (*B*). The cells produced either the inert Gal4 DBD, the classical activator Gal4 (DBD-Gal4), or the nonclassical activator DBD-Gal11, but there is no Gal4-Tup1 in these cells. The assay depends on the fact that *URA3* expression renders cells sensitive to FOA, a sensitivity that provides a convenient assay for telomeric repression (14). Thus, a *URA3* gene located at an internal chromosomal locus, even in the absence of an activator, is expressed at sufficiently high levels to prevent growth in the presence of FOA. In contrast, when placed near the telomere, the gene is silenced and cells grow in the presence of FOA (compare Fig. 2*A* rows 1 and 7 with *B* row 13).

Two results of Fig. 2 are important in the current context. First, telomere-mediated silencing is overcome partially, and about equally well, by a classical and a nonclassical activator (Fig. 2*A*, compare rows 2 and 3 with row 1, and rows 8 and 9 with row 7). Put another way, unlike the effect of Tup1, telomeric heterochromatin silences activation elicited by the classical and the nonclassical activator equally. The addition of three more Gal4 binding sites allows both activators to completely overcome

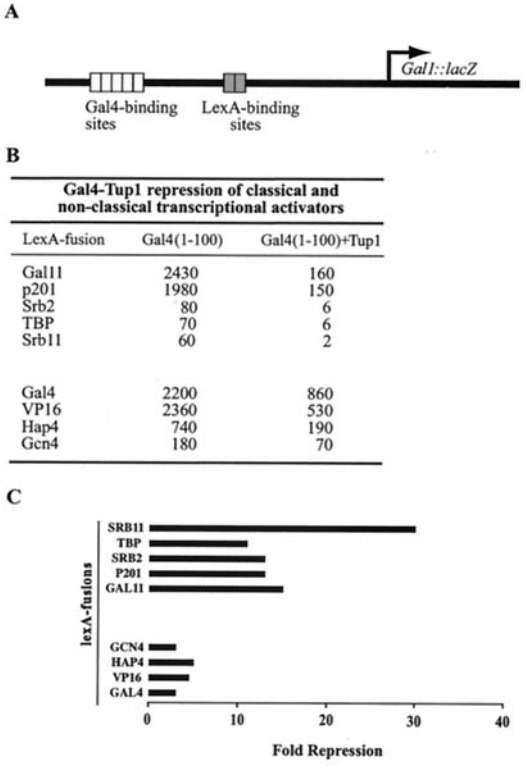

Fig. 1. Tup1 represses transcription mediated by nonclassical activators particularly efficiently. (*A*) The reporter used in the repression assays contains five Gal4 DNA-binding sites upstream of two lexA sites which are, in turn, positioned 50 bp upstream from the natural *GAL1* TATA box. (*B* and *C*) Cells harboring the reporter gene were cotransformed with plasmids expressing Gal4-Tup1 and a variety of LexA-fusion activators. In each case, LexA-fusion activators consisted of full-length protein with lexA replacing the respective DNA-binding domain where applicable. Gal4-Tup1 consisted of Gal4 (residues 1–100) fused to full-length Tup1. Cells were assayed for *GAL1-lacZ* expression by β-galactosidase activity (*B*). The numbers represent the average of assays on three independent colonies performed in duplicate. The standard errors were typically 10–15%. The fold repression, shown in *C*, was calculated as the ratio of values obtained with and without the repressor function of Tup1.

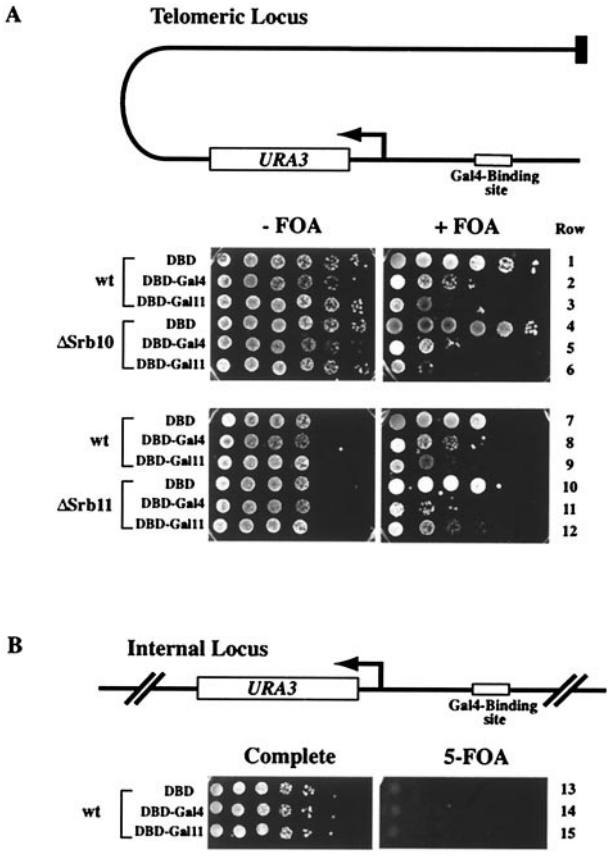

Fig. 2. Classical and nonclassical activators equally overcome telomeric repression, and that repression does not require Srb10. Yeast strains harboring a *URA3*-based reporter gene located at a telomeric (*A*) or internal (*B*) chromosomal locus were assayed for *URA3* expression in the context of coexpressed classical (Gal4) and nonclassical (Gal11) activator molecules. Serial dilution spot assays on cell viability were performed by using plates with (+FOA) or without (−FOA) FOA. Decreased viability on +FOA plates indicates *URA3* gene expression, whereas growth indicates *URA3* repression by telomere position effect. (*A*) Promoter bound Gal4 and Gal11 can overcome telomeric repression equally well in both wild-type and ΔSrb10 or ΔSrb11 cells. The *URA3* reporter contains binding sites for Gal4. Activators were tethered to the promoter via fusions to the DBD of Gal4 (residues 1–100). (*B*) When placed at an internal chromosomal locus, the *URA3* reporter gene is expressed at sufficient levels even in the absence of an activator to render cells sensitive to FOA.

telomeric silencing (39). Second, also in contrast to Tup1-mediated repression, telomeric repression is unaffected by deletion of either Srb10 or Srb11 (Fig. 2*A*, compare rows 5 and 6 with rows 2 and 3, and rows 11 and 12 with rows 8 and 9).

Tup1 Interacts with Holoenzyme and SRB10. The following experiments performed *in vitro* are consistent with the idea that Tup1 interacts with the holoenzyme, and suggest in particular that Srb10 is a target of Tup1. The results in Fig. 3*A* show that purified Gal4-Tup1 bound more effectively to the holoenzyme found in nuclear extracts of wild-type cells than to the holoenzyme found in nuclear extracts of an isogenic strain deleted for Srb10. The experiment was performed by immunoprecipitation

Table 1. Repression of classical and nonclassical activation is Srb10/Srb11 dependent

	LexA-Gal4			LexA-Gal11		
	DBD	DBD-Tup1	Fold repression	DBD	DBD-Tup1	Fold repression
Wild type	2,200	750	3.0	2,400	150	16.0
ΔSrb10	720	710	1.0	1,190	1,010	1.2
ΔSrb11	890	850	1.0	1,250	1,100	1.1
Srb10-3	230	220	1.0	250	240	1.0

Repression of LexA-Gal4 and LexA-Gal11 was assayed as in Fig. 1 using wild type, Srb10 deleted (ΔSrb10), Srb11 deleted (ΔSrb11), and Srb10-3 (an SRB10 point mutant) strains carrying the reporter gene shown in Fig. 1. DBD represents the DNA binding domain of Gal4, residues 1–100. DBD-Tup1 represents Gal4 residues 1–100 fused to the N-terminal region of Tup1 residues 1–713. The numbers represent the average of assays on three independent colonies performed in duplicate. The standard errors were typically 15–20%.

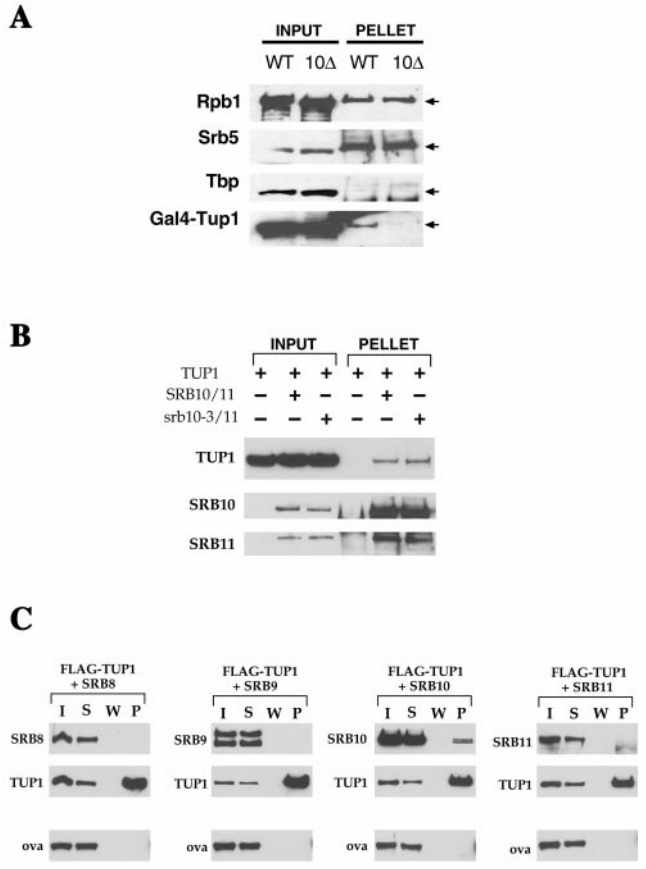

Fig. 3. Tup1 interacts with the holoenzyme, at least in part, through its interactions with Srb10. (*A*) Tup1 immunoprecipitates with the holoenzyme found in wild-type extracts but much less so with holoenzyme found in extracts from a strain deleted for Srb10. Purified Tup1 was incubated with equivalent amounts of nuclear extracts of either the wild-type strain or an isogenic strain lacking Srb10 (Input). Each extract contained equivalent levels of Rpb1 (largest subunit of the RNA polymerase II), Srb5 (an integral component of the holoenzyme), and Tbp (the TATA binding protein which does not stably associate with the RNA pol II holoenzyme). Holoenzyme and proteins bound to it were precipitated with affinity-purified antibodies against-Srb5 (Pellet). As expected, Tbp did not immunoprecipitate well, whereas both Srb5 and Rpb1 did immunoprecipitate efficiently. (*B*) Tup1 interacts with Srb10/11 complex. Purified Tup1 was incubated with purified Srb10/11 complex (Input) and then immunoprecipitated by using affinity-purified anti-Srb10 antibodies (Pellet). Srb10–3 has a point mutation in the kinase domain that eliminates the catalytic function of the enzyme without altering its ability to interact with its cyclin partner or the holoenzyme. (*C*) Tup1 interacts with Srb10. Purified, flag epitope-tagged Tup1 was incubated with four components of the repression subcomplex of the holoenzyme. Anti-flag monoclonal antibody was used to immunoprecipitate the tagged Tup1 incubated individually with insect-cell extracts containing overexpressed Srb8, 9, 10, and 11. The input (I), the supernatant (S), the wash (W), and the precipitated (P) levels of each protein in the reaction are shown. Ovalbumin was used as a control for nonspecific aggregation. In lane P of the Srb11 subpanel, the faster migrating band is not a degradation product of Srb11 but a chain of the flag monoclonal antibody that is detected by the secondary antibody used in the immunoblot.

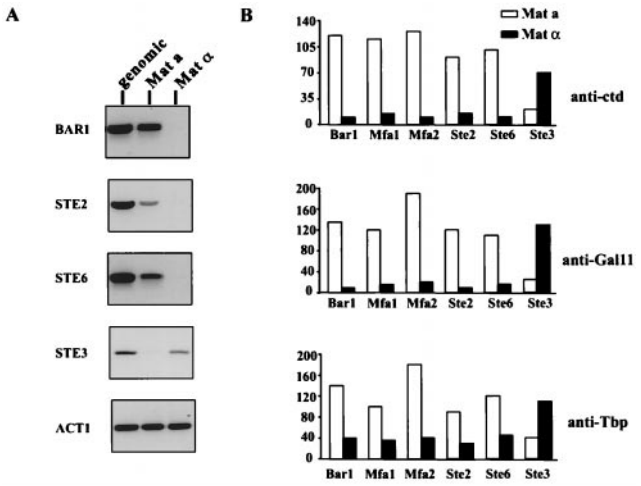

Fig. 4. Tup1 repression occludes the holoenzyme and Tbp from the promoter. (*A*) Tup1 tightly regulates mating-specific gene expression. To determine the relative expression levels of a subset of mating type-specific genes we performed quantitative reverse transcription–PCR analysis on RNA isolated from the isogenic cells of opposite mating types. A constant amount of chromosomal DNA was used in the analysis of each gene to control for PCR efficiency of each primer pair. Actin gene expression was monitored to control for total RNA recovery. (*B*) Tup1 recruited to α2 repressed genes occludes the holoenzyme and Tbp from a-specific gene promoters. Chromosomal immunoprecipitation assays were performed on yeast cells of opposite mating type. Antibodies specific for PolII (anti-ctd), Gal11 (anti-Gal11) and Tbp (anti-Tbp) were used to immunoprecipitate *in vivo* formaldehyde cross-linked chromatin. Promoter DNA coupled to complexes containing PolII, Gal11, and Tbp were analyzed by quantitative PCR studies. Primer pairs encompassed the core promoter region of each gene. The histograms indicate DNA immunoprecipitated relative to the Actin promoter region for each gene. Plotted values correspond to the mean values obtained with three independent experiments. SEM = 15–25%.

with antibodies to Srb5, a holoenzyme component (41, 42). The residual weak interaction seen with the holoenzyme lacking Srb10 (Fig. 3*A*) might be accounted for by interactions with other holoenzyme components (28, 43). The interaction between Tup1 and the holoenzyme or Srb10 is not as robust as that observed for activators with the holoenzyme (not shown). The experiment of Fig. 3*B* shows that antibodies to Srb10 immunoprecipitated purified Tup1 along with Srb11, and that this interaction was also observed with the Srb10–3 mutant. In this case, the Srb proteins were purified to homogeneity from a baculovirus system (34). The converse coimmunoprecipitation experiment of Fig. 3*C* shows that the antibodies to Flag-tagged, purified Tup1 coprecipitated Srb10 but not Srb8, 9, or 11. In this case, each of the Srb proteins was expressed in a baculovirus system, and the experiment was performed in the insect cell extracts.

Absence of the Transcriptional Machinery at Tup1 Repressed Promoters. The chromatin immunoprecipitation experiment of Fig. 4 shows that at five native genes repressed by Tup1 in Matα cells, no bound holoenzyme (as assayed by using two antibody probes; one directed at the RNA polymerase II C-terminal domain, the other against Gal11) was detected at their respective promoter regions (Fig. 4*B*). We extended our study to Tbp and found that, in agreement with a previous finding (44), Tbp is absent from these promoters. At the same genes in Mata cells, where these genes are highly expressed, the holoenzyme was readily detected by using all three antibodies (Fig. 4*B*). The opposite result was obtained for a gene that is transcribed in Matα cells but repressed in Mata cells (i.e., Ste3); in all cases, the presence of the bound holoenzyme correlated with mRNA expression (Fig. 4*A*).

Discussion

Our genetic experiments indicate that Tup1-mediated repression involves interaction with some component of the transcriptional machinery. Thus, first, DNA-tethered Tup1 represses activation elicited by nonclassical activators much more efficiently than it represses equivalent activation elicited by classical activators. This differential repression is unlikely to be accounted for by a hypothetical Tup1-induced heterochromatic structure similar to that found at the telomere. That surmise follows from the

observation that positioning the reporters near a telomere, and therefore within repressive heterochromatin (and in the absence of Tup1), silences both to a modest but equivalent degree. Second, mutation of either of the holoenzyme components Srb10 or Srb11 virtually eliminates repression by Tup1, but has no effect on telomere-induced silencing. The simplest explanation for these results would be that Tup1 repression requires, at least in part, interaction of Tup1 with some part of the transcriptional machinery. Our biochemical experiments are consistent with this idea: Tup1 binds specifically to holoenzyme bearing Srb10, to the Srb10/11 complex, and to purified Srb10. The dependence of Tup1-mediated repression on the kinase function of Srb10 suggests that the latter's role in destabilizing the holoenzyme (26) could, at least in part, mediate repression by Tup1. Tup1 might, for example, activate the kinase and/or help position it next to its target in the holoenzyme.

A model consistent with the findings of Watson *et al.* (21), as well as with results reported here, would be as follows. DNA-tethered Tup1 (or some other component of the Tup1 complex) recruits a histone deacetylase activity that, by deacetylating histones, increases the affinity of those histones for Tup1 (20, 45, 46). Tup1 would then interact more efficiently with the holoenzyme and repress in an SRB10/11-dependent fashion. We might then imagine three reasons why classical activators are less sensitive to repression by Tup1 than are nonclassical activators. (*i*) According to the targeting studies of Utley *et al.* (47), classical activators, in addition to interacting with the transcribing machinery itself, also recruit histone acetyltransferase complexes, and the acetylating activities of those complexes would diminish Tup1 accessibility. Nonclassical activators, which presumably recruit such complexes less efficiently if at all, would therefore lack this anti-Tup1 effect. (*ii*) The classical activators could compete for binding of Tup1 to the holoenzyme. This idea is consistent with evidence indicating that Srb10 also serves as a target for Gal4's activating region (A.Z.A., S.S.K., Z.Z., R.Y., and M.P., unpublished data) as well as for Tup1, as assayed here. (*iii*) By virtue of manifold contacts with the transcriptional machinery, classical activators could more readily overcome the negative effect of SRB10's action destabilizing the transcribing machinery.

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