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Derepression of DNA damage-regulated genes requires yeast TAF_{II}s

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The general transcription factor TFIID and its individual subunits (TAF_{II}s) have been the focus of many studies, yet their functions *in vivo* are not well established. Here we characterize the requirement of yeast TAF_{II}s for the derepression of the ribonucleotide reductase (RNR) genes. Promoter mapping studies revealed that the upstream repressing sequences, the damage-responsive elements (DREs), rendered these genes dependent upon TAF_{II}s. DREs are the binding sites for the sequence-specific DNA binding-protein Crt1 that represses transcription by recruiting the Ssn6–Tup1 co-repressor complex to the promoter. We demonstrate that deletion of *SSN6*, *TUP1* or *CRT1* alleviated the TAF_{II} dependence of the RNR genes, indicating that TAF_{II} dependence requires the co-repressor complex. Furthermore, we provide evidence that Crt1 specifies the TAF_{II} dependence of these genes. Our studies show that TFIID interacts with the repression domain of Crt1, suggesting that the derepression mechanism involves an antagonism between TFIID and the co-repressor complex. Our results indicate that yeast TAF_{II}s have other functions in addition to core promoter selectivity, and describe a novel activity: the derepression of promoters.

Keywords: Crt1/repression/Ssn6–Tup1/TFIID/transcription

Introduction

The general transcription factor TFIID has been the focus of years of biochemical study because of its central role in transcription. Some of the proposed activities of this complex include co-activator, core promoter selectivity, kinase and histone modification (for review see Burley and Roeder, 1996; Verrijzer and Tjian, 1996; Lee and Young, 1998). The role of many of these activities in gene regulation has yet to be elucidated.

Initial analyses of yeast TAF_{II} mutants revealed that mutation or depletion of seven different TAF_{II}s resulted in a cessation of growth, but yet the transcription of many cellular genes was unaffected (Apone *et al*., 1996; Moqtaderi *et al*., 1996; Walker *et al*., 1996). These studies, and a recent genome-wide gene expression analysis using TAF_{II} mutants (Holstege *et al*., 1998), indicate that TAF_{II}s are not universally required for transcription *in vivo*. Likewise, conditional expression or mutation of metazoan TAF_{II}s resulted in similar phenotypes, indicating that a restricted transcriptional requirement is not unique to yeast TAF_{II}s (Wang and Tjian, 1994; Suzuki-Yagawa *et al*., 1997; Zhou *et al*., 1998; Martin *et al*., 1999; Metzger *et al*., 1999; Soldatov *et al*., 1999). The debate on the necessity for TAF_{II}s in transcription was revived by studies reporting broad transcriptional defects in a number of TAF_{II} mutants, some grouped by their sequence and structural similarities to the core histones (Apone *et al*., 1998; Holstege *et al*., 1998; Krishnamurthy *et al*., 1998; Michel *et al*., 1998; Moqtaderi *et al*., 1998; Komarnitsky *et al*., 1999; Sanders *et al*., 1999; Reese *et al*., 2000). However, the exact fraction of the genome affected by the inactivation of these mutants, and what accounts for their distinct phenotypes, is unclear and controversial (for reviews see Hahn, 1998; Green, 2000). Moreover, the interpretation of data obtained using certain TAF_{II} mutants is complicated by their presence in histone acetyltransferase (HAT) complexes (Grant *et al*., 1998a; Ogryzko *et al*., 1998; Wieczorek *et al*., 1998) and potential allele-specific defects (Michel *et al*., 1998).

The characterization of genes whose expression is sensitive to *TAF145* mutations revealed that the core promoter sequences, but not the upstream activating sequences, rendered them *TAF145* dependent (Shen *et al*., 1997; Walker *et al*., 1997; Tsukihashi *et al*., 2000). These findings indicate that TAF_{II}s are acting as promoter selectivity factors and not as co-activators for these genes, which is consistent with the core promoter selectivity functions of some metazoan TAF_{II}s (for reviews see Hoffmann *et al*., 1997; Verrijzer and Tjian, 1996).

Most, if not all, promoters are under constant repression by nucleosomes (Roth, 1995) and transcriptional repressor proteins (Herschbach and Johnson, 1993; Lee and Young, 1998). The transcription machinery, including TFIID, must contend with these activities and factors. Unfortunately, the mechanism by which TFIID counteracts these two classes of transcriptional repressors is poorly understood. It has been proposed that the HAT activity of $yTAF_{II}145/dTAF_{II}250$ may play an important role in modifying histones near promoters of repressed target genes (Mizzen *et al*., 1996), although the exact function of this activity in gene regulation has not been determined. An important step in addressing these questions is to identify genes that are regulated predominantly by transcriptional repression and whose expression is dependent upon *TAF145*.

Here we report the characterization of TAF_{II}-dependent genes that are regulated by transcriptional repression and chromatin structure, the DNA damage-regulated ribonucleotide reductase (RNR) genes. The RNR genes are repressed in the absence of DNA damage by the gene-specific repressor Crt1 and the general co-repressor complex Ssn6–Tup1 (Zhou and Elledge, 1992; Huang

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et al., 1998), and we show that the TAF_{II} dependence of the RNR genes is mediated by these three factors. Our analysis defines a function for TAF_{II}s: the derepression of regulated promoters.

Results

Yeast TAF_{II}s are required for the transcription of the DNA damage-induced ribonucleotide reductase genes

We investigated the requirement of yeast TAF_{II}s for the regulation of DNA damage-induced genes using strains that conditionally express their gene products from the *GAL1* promoter (Walker *et al*., 1996). Cells containing the sole copy of *TAF145*, *TAF90*, *TAF68/61* or *TAF47* under the control of the *GAL1* promoter were transferred to dextrose-containing medium and then treated with the DNA-damaging agent methylmethane sulfonate (MMS) or the replication inhibitor hydroxyurea (HU). The effects of TAF_{II} depletion on the expression of the ribonucleotide reductase genes were examined by northern blotting. We focused on *RNR2*, *RNR3* and *RNR4* because they are the prototypical models to study DNA damage-induced transcription and they have been characterized extensively (Elledge and Davis, 1989, 1990; Yagle and McEntee, 1990; Elledge *et al*., 1992; Huang and Elledge, 1997). Depletion of yeast TAF_{II}145p, TAF_{II}90p or TAF_{II}68p strongly reduced, or eliminated, the DNA damage-induced expression of all three RNR genes (Figure 1A). In contrast, depletion of yeast TAF_{II}47p did not result in any significant changes in expression, indicating that RNR gene transcription is dependent upon specific TAF_{II}s and that depleting an essential gene product does not cause the defects observed in the other three conditional strains. Immunoblotting confirmed that within 8 h upon transfer from galactose- to dextrose-containing medium, each TAF_{II} was depleted (Figure 1B). These data also show that the levels of TATA box-binding protein (TBP) did not change significantly over the course of the experiment; thus, the loss of RNR transcription does not result from reduced TBP levels. Our results clearly demonstrate that the DNA damage-induced transcription of the RNR genes is dependent upon certain TAF_{II} genes.

We next analyzed the expression of the RNR genes in temperature-sensitive mutants of *TAF90*, *TAF145* and *TAF170/TSM1*, and in a strain containing a deletion of *TAF30/ANC1*. For the experiment shown in Figure 2A, cultures of TAF_{II} mutants were shifted to the restrictive temperature for 60 min and were then treated with MMS (0.015%) for 2 h. Analysis of the mRNA levels of *RNR2*, *RNR3* and *RNR4* revealed that all three were compromised, but to various degrees. Inactivation of *taf145-2* had the strongest effect on the expression of all three genes, but the inactivation of *taf90-1* showed weaker effects on *RNR2* and *RNR3* compared with the *TAF145* mutant (Figure 2A). Moreover, the expression of *RNR4* in the *TAF90* mutant seemed relatively unaltered. The weaker phenotype observed in the *TAF90* mutant may result from its incomplete inactivation prior to the addition of MMS because longer exposure of the temperature-sensitive mutant to the restrictive temperature prior to MMS addition (not shown), or depletion of its protein (Figure 1), had stronger effects on the expression of all three RNR genes. Inactivation of a *TSM1* mutant had little effect on the induced expression of all three RNR genes, once again indicating that their expression requires specific TAF_{II}s. The induction of these genes was unaffected whenever the mutant strains were grown at the permissive temperature, and results similar to those described above were also obtained using other DNA damage-inducing agents (not shown).

Yeast *TAF30/ANC1*, a non-essential gene, was isolated recently as a gene dosage suppressor of *Δrad53* and *Δmec1* lethality (Desany *et al*., 1998); therefore, the expression of the RNR genes was examined in a *TAF30/ANC1* null mutant. The results show that *TAF30/ANC1* is not required for the expression of the RNR genes (Figure 2B). This result suggests that *TAF30/ANC1* may affect the cell cycle arrest functions of *RAD53* and *MEC1*, rather than the DNA damage-regulated transcription pathway, which is consistent with the cell cycle regulatory functions of TAF_{II}s (for a review see Green, 2000).

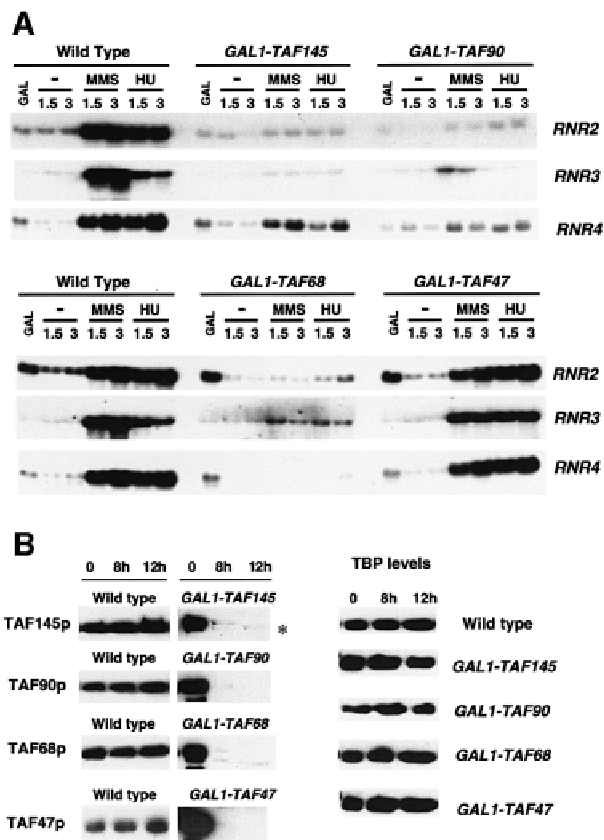


Fig. 1. Depletion of TAF_{II}s impairs DNA damage-dependent expression of the RNR genes. (**A**) Strains containing yeast *TAF145* (YSW94), *TAF90* (LYC-1), *TAF68* (YJR18) and *TAF47* (YJR11) under the control of the dextrose-repressible *GAL1* promoter were grown in galactose to an OD of 0.5–1.0 (GAL), collected, washed and resuspended in dextrose-containing medium. After 10 h in dextrose medium, the cultures were divided into three flasks, and MMS (0.03%) or HU (0.1 M) was added to separate cultures. After an additional 1.5 and 3 h induction period, cells were collected for RNA isolation. Note that the total amount of time in dextrose for these experiments was 11.5 and 13 h, respectively. (**B**) Western blot. The levels of TAF_{II} (left) and TBP (right) protein were analyzed after 0, 8 and 12 h in dextrose-containing medium. The asterisk on the left of the TAF_{II}145 blot indicates a cross-reacting protein migrating near TAF_{II}145.

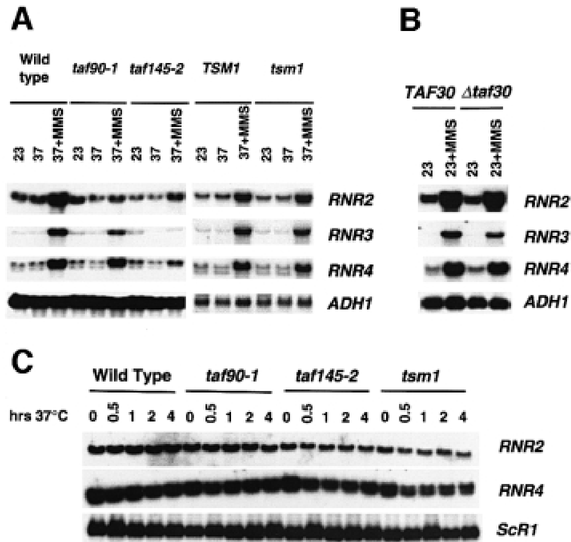


Fig. 2. Analysis of the expression of the ribonucleotide reductase genes in TAF_{II} mutants. (**A**) Wild type (SW87 and WCS131) and strains containing temperature-sensitive mutations in yeast *TAF90* (LY20), *taf145-2* (YSW93) and *tsm1* (WCS132) were grown at the permissive temperature of 23°C (23) and then shifted to 37°C for 1 h (37). Afterwards, MMS was added to 0.015% and the cells were collected after a 2 h induction period (37 + MMS). (**B**) Cells containing a deletion of *TAF30/ANC1* (DDY555) were grown at 23°C and treated with MMS for 2 h. (**C**) Analysis of the uninduced levels of *RNR2* and *RNR4* gene transcription. Strains were grown at 23°C and then shifted to 37°C. Aliquots were withdrawn prior to temperature shift (0) and at 30 min, 1, 2 and 4 h.

We often found that inactivation of *taf145-2* resulted in RNR gene mRNA levels that are equivalent to those observed in untreated cells (Figure 2A and data not shown), suggesting that TAF_{II}s are required specifically for their induced expression. We therefore examined the effects of TAF_{II} inactivation on the uninduced levels of RNR transcription. As expected, the results of Figure 1C show that TAF_{II}s are not required for the uninduced level of expression of the RNR genes.

TAF_{II} mutations directly affect the RNR promoters

Expression of the RNR genes requires a functional DNA damage signaling pathway that is activated by DNA damage (Elledge *et al*., 1992; Kiser and Weinert, 1996). Activation of this pathway causes the phosphorylation of Crt1, a transcriptional repressor, and the derepression of the RNR genes (Huang *et al*., 1998). The defects described above could result from a defective signaling pathway, rather than from a direct effect on the RNR promoters. To rule out this possibility, we examined the MMS-induced phosphorylation of Crt1 in the *TAF90* and *TAF145* mutants at the restrictive temperature. Inactivation of the *TAF90* or *TAF145* mutant did not affect the DNA damage-induced phosphorylation of Crt1 (Figure 3A), indicating that the kinase signaling pathway is intact throughout the course of our experiments.

To address this issue using another strategy, we compared the loss of *RNR3* and *RNR2* mRNA caused by the inactivation of TAF_{II} mutants with that of a TBP mutant (*tbp1-1*). The TBP is required for the transcription

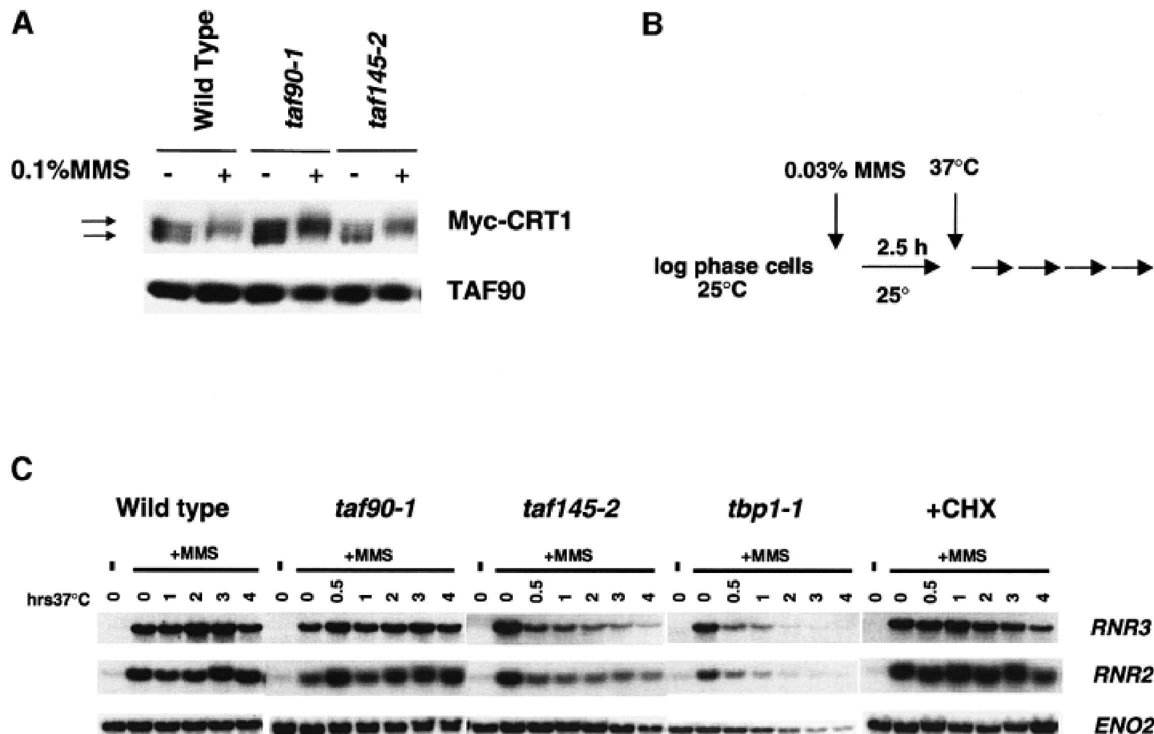


Fig. 3. TAF_{II}s directly regulate the RNR genes. (**A**) Analysis of the MMS-induced phosphorylation of Crt1. Wild-type (SW87), *taf90-1* (LY20) and *taf145-2* (YSW93) cells transformed with pMH190 (GAL-3MYC-CRT1, ARS/CEN, URA3) were grown in SC-URA plus raffinose, and Crt1 was induced for 2 h by the addition of galactose (Huang *et al*., 1998). Cultures were then incubated for 1 h at 37°C, followed by a 2 h treatment with MMS (0.1%). Crt1 was detected by western blotting using anti-myc monoclonal antibodies. TAF_{II}90p served as a loading control. (**B**) Effects of the inactivation of TAF_{II} mutants on active RNR genes. Cells were treated with 0.03% MMS for 2.5 h at 23°C and then shifted to 37°C. Aliquots of cells were withdrawn at the times indicated in the figure. A separate culture of wild-type cells was treated with 100 μg/ml cycloheximide at the time of temperature shift (+CHX). (**C**) Northern blot of *RNR2* and *RNR3* mRNA.

of all genes; thus, the loss of transcription in this mutant is indicative of a direct effect on the promoter (Cormack and Struhl, 1992). Since TAF_{II}s are required for the induced expression of these genes, we treated the cells with MMS at the permissive temperature to activate the genes prior to shifting the cultures to 37°C (Figure 3B). Transferring wild-type cells to 37°C after MMS treatment resulted in a small, transient increase in *RNR* transcription. However, in both the *taf145-2* and *tbp1-1* strains, the level of *RNR2* and *RNR3* mRNA declined sharply within 30 min (Figure 3C). This rapid loss of message is consistent with a direct effect on the promoter, since the time required to produce most secondary effects involves a substantial reduction in both a transcript and its translation product. Nonetheless, since the half-lives of the regulatory proteins in the DNA damage pathway are not known, we simulated a secondary effect using the protein synthesis inhibitor cycloheximide. Blocking protein synthesis provides an estimation of the time required to deplete essential regulatory factors, and this strategy has been used previously to distinguish a primary from a secondary effect (Cormack and Struhl, 1992). In a separate experiment, we verified that cycloheximide treatment did not affect the half-life of the *RNR2* or *RNR3* mRNA (not shown). Inhibition of protein synthesis did not affect the level of *RNR2* or *RNR3* mRNA until after 3 h (Figure 3C), a time significantly longer than the initial effects observed upon the inactivation of the *taf145-2* or *tbp1-1* mutant.

Surprisingly, we found that activated *RNR3* and *RNR2* genes are insensitive to *TAF90* mutations. Furthermore, while the level of *RNR* transcription decreased dramatically in the *TAF145* mutant, it remained significantly above the uninduced level (compare lane 14 with lanes 19 and 20 of Figure 3C). This is in contrast to the results obtained when *taf145-2* is inactivated prior to MMS addition: under these conditions, we observe promoter activity similar to the uninduced level (see Figure 2A). A possible explanation for these results is that TAF_{II}s are required for overcoming a rate-limiting step in the derepression process. Given the results of Figure 3, we conclude that TAF_{II} mutations directly affect the RNR promoters.

TAF_{II} dependence maps to the damage-responsive elements (DREs)

We next sought to identify the region(s) of the *RNR3* promoter that renders it sensitive to TAF_{II} mutations. We focused on the *RNR3* gene because it is the best characterized of the RNR gene family, and its promoter elements were identified by previous mapping studies (Endo-Ichikawa *et al*., 1996; Huang *et al*., 1998). As an initial step, we verified that the TAF_{II}-dependent transcription that was observed on the chromosomal copy of the *RNR3* gene can be recapitulated on a plasmid-borne copy. The promoter region of *RNR3* (sequences from –420 to +5, *RNR3*^{FL}-*lacZ*) was fused to the *lacZ* gene and introduced into yeast on a low-copy number plasmid. The results show that, like the chromosomal copy of *RNR3*, transcription from the RNR3^{FL}-LacZ transgene required functional *TAF90* and *TAF145*, but not *TSM1* (Figure 4A). Confident in this strategy, we constructed chimeric promoters using sequences from *RNR3* and *ADH1*. The expression of *ADH1* is not affected by TAF_{II} mutations (Apone *et al*., 1996; Walker *et al*., 1996; Shen and Green, 1997). The sequences used in the construction of these promoters were chosen based on previous promoter mapping studies of *ADH1* (Shen and Green, 1997) and *RNR3* (Endo-Ichikawa *et al*., 1996; Huang *et al*., 1998). We first asked whether the core promoter alone conferred TAF_{II}-dependent transcription on *RNR3* by analyzing the expression of a *lacZ* reporter gene driven by the *RNR3* core promoter (*RNR3*^{core}-*lacZ*; Figure 4A). Consistent with our results of Figure 1C, demonstrating that the uninduced levels of RNR gene transcription are not unaffected by TAF_{II} mutations, inactivation of *taf90-1*, *taf145-2* or *tsm1* alleles had no effect on the expression of *RNR3*^{core}-*lacZ* (Figure 4A). Next, we analyzed a promoter composed of the upstream regulatory sequences from *RNR3* and the core promoter from the TAF_{II}-independent *ADH1* gene (*RNR3*^{US}-*ADH1*^{core}; Figure 4A). The regulatory region of *RNR3* used in this construct has three DREs (X-boxes), which were shown to confer DNA damage-dependent transcription on the *CYC1* promoter in previous studies (Endo-Ichikawa *et al*., 1996; Huang *et al*., 1998). The expression from the *RNR3*^{US}-*ADH1*^{core} chimeric promoter was dependent upon MMS treatment in the wild-type cells, and required functional *TAF90* and *TAF145* (Figure 4A).

Detailed analyses of the *RNR3* gene revealed that it is regulated primarily by a transcriptional repression mechanism mediated by three DREs (Zhou and Elledge, 1992; Endo-Ichikawa *et al*., 1996; Huang *et al*., 1998); therefore, it is likely that the requirement for TAF_{II}s maps to these regulatory sequences. Nonetheless, the region of *RNR3* used in our initial mapping studies is quite large, and it is possible that TAF_{II}s are functioning as co-activators for an unidentified transcription factor that binds to this region. Therefore, to map definitely the TAF_{II} dependence to the DREs, we constructed a promoter containing a tandem array of consensus DREs (X-boxes) positioned upstream of the *ADH1* core promoter (DRE-*ADH1*^{core}; Figure 4B). The results of Figure 4B show that the expression of the DRE-*ADH1*^{core} promoter in wild-type cells was dependent upon the addition of MMS to the culture medium and, more importantly, they also show that its expression was *TAF145* and *TAF90* dependent. On the basis of these results, we conclude that the TAF_{II} dependence of the RNR genes is mediated by the DREs.

The Ssn6–Tup1 complex is required for the TAF_{II} dependence of the RNR genes

Mapping the TAF_{II}-dependent region to the DREs indicates that the transcription factors that function at these sequences confer TAF_{II} dependence to the RNR genes. In the absence of DNA damage-induced signals, the RNR genes are repressed by the Ssn6–Tup1 co-repressor complex, which is recruited to the promoter by the sequence-specific DNA-binding protein Crt1 (Huang *et al*., 1998). It is feasible that TAF_{II}s control RNR gene transcription by antagonizing Ssn6–Tup1-mediated repression and, if this is so, deleting *SSN6* or *TUP1* should alleviate the TAF_{II} dependence. Double TAF_{II}^{ts-}/*Δssn6* and TAF_{II}^{ts-}/*Δtup1* mutants were shifted to 37°C to inactivate the TAF_{II} mutants, and the levels of *RNR2* and *RNR4* mRNA were measured by northern blotting. Deletion of *SSN6* (Figure 5A) or *TUP1* (not shown) resulted in the derepression of the RNR genes in the wild-type and the mutant strains at the permissive temperature.

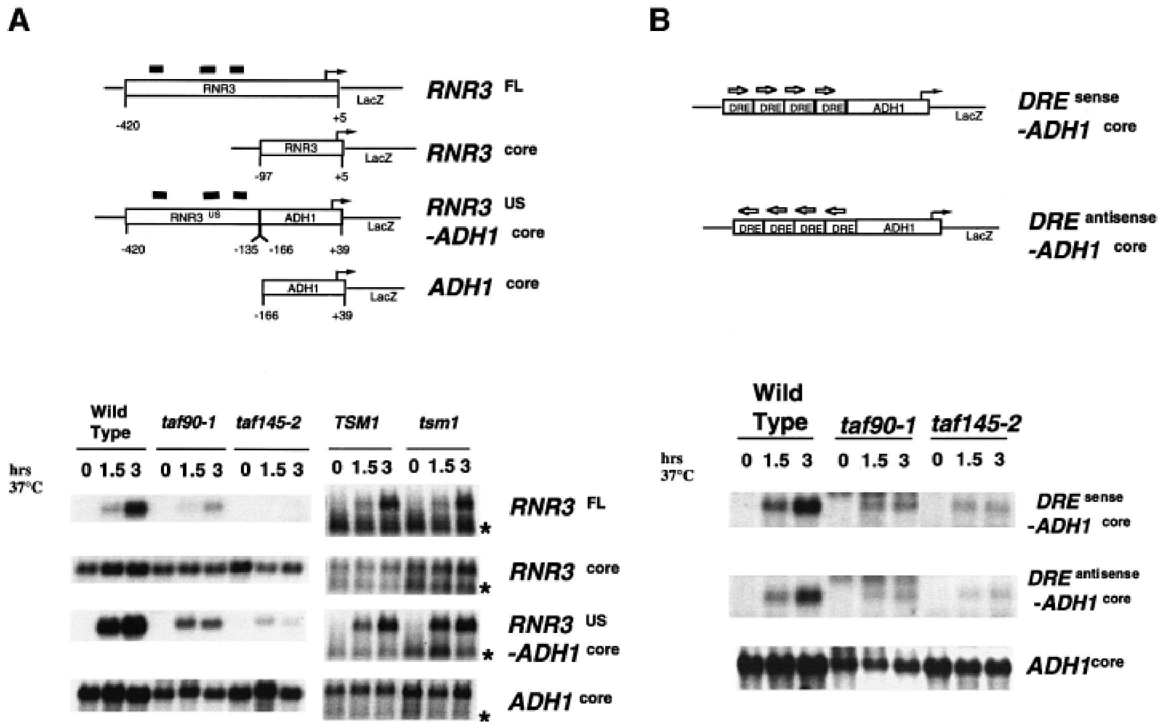


Fig. 4. The TAF_{II}-dependent region of *RNR3* maps to the damage-responsive elements (DREs). (**A**) Schematic representations of the chimeric promoters (above). The positions of the DREs are indicated by the black bars above the *RNR3* promoter and are located at –213, –261 and –323 relative to the major transcription start site. The TATA boxes are located at *RNR3*, –75, *ADH1*, –91. Yeast strains transformed with the plasmids indicated on the right hand side of the panel were grown at 23°C to midlog (0), transferred to 37°C for 45 min, and MMS was added to a final concentration of 0.015% to cells containing $RNR3^{FL}$-LacZ, $RNR3^{core}$-LacZ and $RNR3^{US}$-$ADH1^{core}$LacZ. Cells were withdrawn for RNA isolation after 1.5 and 3 h. *LacZ* RNA was detected by northern blotting. Note that the *lacZ* transcript marked with an asterisk originates from the *TSM1* expression plasmid, pRS313-*TSM1*. (**B**) The same as (A), but using a construct containing a synthetic tandem array of four DREs (Xs) from the *RNR2* promoter positioned upstream of the *ADH1* core promoter.

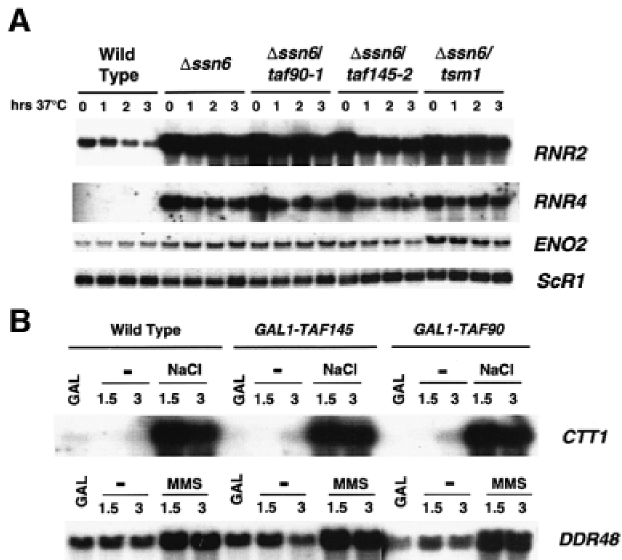


Fig. 5. TAF_{II}-dependent transcription of the RNR genes requires the Ssn6–Tup1 co-repressor complex. (**A**) Analysis of RNR transcription in *Δssn6* and double TAF^{ts-}/*Δssn6* mutants. Strains were grown at 23°C and then transferred to 37°C. Aliquots of cells were withdrawn for RNA isolation at 1, 2 and 3 h following temperature shift. (**B**) Analysis of Ssn6–Tup1-regulated stress-responsive genes in conditional expression strains. Strains were grown in galactose (GAL), collected, washed, transferred into dextrose-containing medium for 10 h and then separated into three flasks. At that time, MMS (0.03%) or NaCl (1 M) was added to individual flasks. Aliquots of cells were removed after 1.5 and 3 h and used to isolate RNA.

More importantly, shifting the TAF_{II}^{ts-}/*Δssn6* mutants to the restrictive temperature did not significantly affect the levels of *RNR2* and *RNR4* mRNA, when compared with the *Δssn6* strain (Figure 5A). A slight (~2-fold) heat shock-induced reduction was noted in all cells. This experiment shows that deletion of *SSN6* or *TUP1* alleviates the requirement for $TAF_{II}s$.

The results presented above indicate that the Ssn6–Tup1 complex is required for the TAF_{II} dependence of the RNR promoters; therefore, we explored the possibility that the transcription of all *SSN6/TUP1*-regulated genes relies upon *TAF90* and *TAF145* function. *DDR48* and *CTT1* are two *SSN6/TUP1*-regulated genes that are induced by heat shock and high osmolarity (Schmitt and McEntee, 1996; Marquez *et al.*, 1998). *DDR48* is also inducible by MMS, but is regulated by a mechanism different from *RNR3* (Maga *et al.*, 1986; Zhou and Elledge, 1993; Kiser and Weinert, 1996; Schmitt and McEntee, 1996). Since both genes are induced by heat shock, we used the TAF_{II} depletion strategy described in Figure 1. Depletion of $TAF_{II}90p$ or $TAF_{II}145p$ did not affect the induction of either *CTT1* or *DDR48* (Figure 5B); thus, *SSN6/TUP1* intrinsically cannot confer TAF_{II}-dependent transcription to all promoters.

$TAF_{II}s$ target the Crt1 repressor protein

Both *DDR48* and *RNR3* are repressed by the Ssn6–Tup1 complex and are regulated by the DNA damage signaling

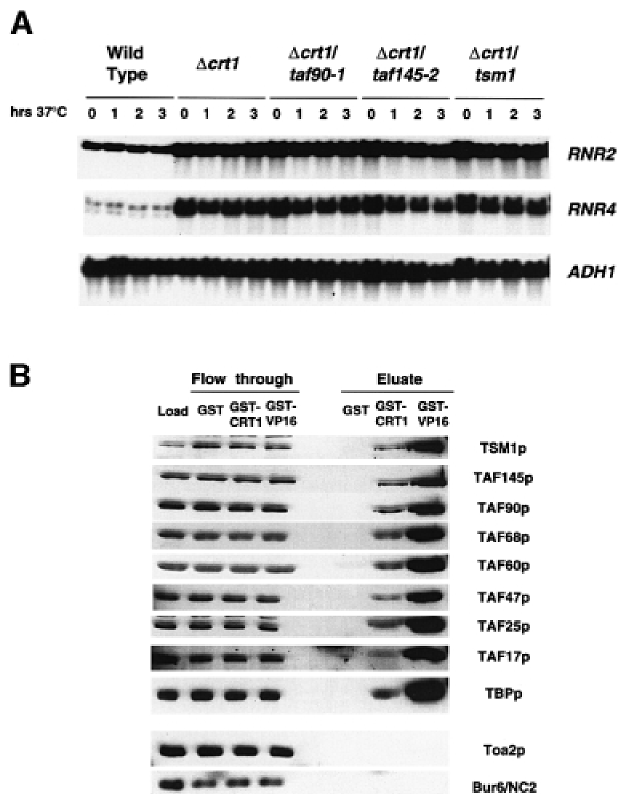


Fig. 6. Crt1 mediates the TAF_{II} dependence of the RNR genes. (**A**) Deletion of *CRT1* relieves the TAF_{II} requirement. Strains were treated as described in Figure 5A. (**B**) TFIID and Crt1 interact *in vitro*. Yeast whole-cell extracts were chromatographed on GST, GST–Crt1 and GST–VP16 affinity columns. After extensive washing, the bound proteins were eluted with high salt and were detected by western blotting. The relative amounts of material loaded onto the gel are as follows: load, 1/300 of the total; eluate, 1/35 of the total. Note that the amounts of the smaller TAF_{II}s (TAF_{II}47, TAF_{II}25 and TAF_{II}17) in the eluates appear lower than they actually are because of the spreading of the signal caused by the high salt in the eluate fractions.

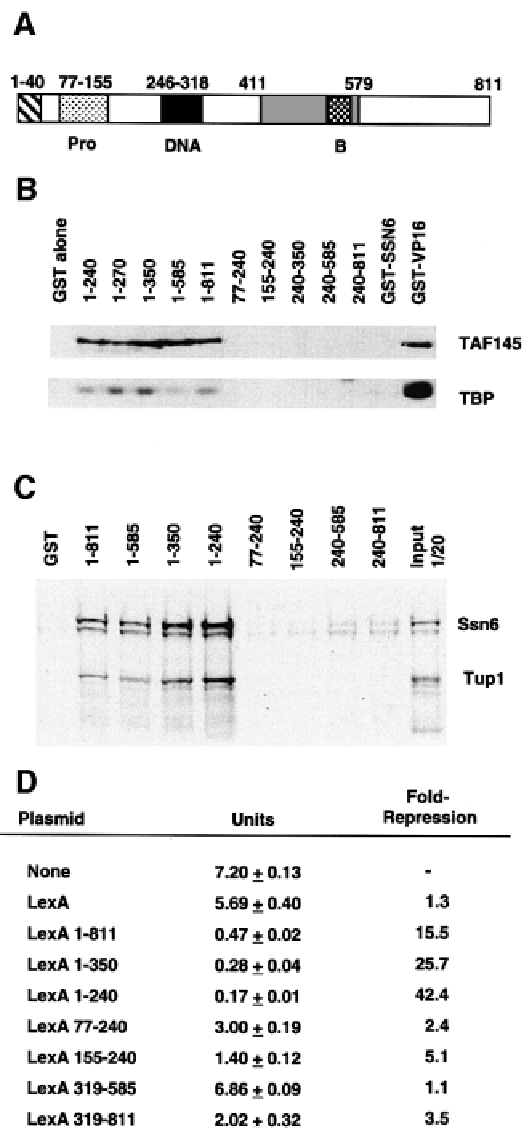
pathway, but their distinct expression patterns are mediated by different sequence-specific DNA-binding proteins. The expression of *DDR48* is dependent upon Msn2/4, and that of *RNR3* upon Crt1 (Schmitt and McEntee, 1996; Huang *et al*., 1998), suggesting that the TAF_{II} dependence of the RNR genes is specified by Crt1. We tested this hypothesis by analyzing the expression of *RNR2* and *RNR4* in double TAF_{II}^{ts-}/*Δcrt1* strains using the strategy described in Figure 5A. Yeast strains containing a deletion of *CRT1* have elevated levels of RNR mRNA compared with wild-type cells (Figure 6A, compare lane 1 with lane 5), which is in agreement with the findings of a published report (Huang *et al*., 1998). Similarly to what was observed in double TAF_{II}^{ts-}/*Δssn6* and TAF_{II}^{ts-}/*Δtup1* strains, shifting the TAF_{II}^{ts-}/*Δcrt1* mutants to 37°C failed to result in significant reductions in the expression of *RNR2* and *RNR4* (Figure 6A). Identical results were obtained in the double mutants treated with MMS (data not shown).

TFIID is believed to function by interacting with sequence-specific DNA-binding proteins and general transcription factors (Burley and Roeder, 1996; Lee and Young, 1998), and it is likely that it regulates the RNR genes by interacting with Crt1. We explored the likelihood of this mechanism by examining the ability of a glutathione *S*-transferase (GST)–Crt1 affinity column to retain subunits of TFIID from whole-cell extracts. GST and GST–VP16 columns were used as negative and positive controls, respectively. As expected, both the GST–Crt1 and GST–VP16 columns retained subunits of TFIID, namely TAF_{II}170/Tsm1p, TAF_{II}145p, TAF_{II}90p, TAF_{II}68p, TAF_{II}60p, TAF_{II}47p, TAF_{II}25p, TBPp and TAF_{II}17p (Figure 6B). However, neither column retained a subunit of TFIIA (Toa2) or yeast NC2 (Bur6p), two TBP-binding transcription factors that are distinct from TFIID. The retention of TAF_{II}s on the VP16 column was significantly higher, but since the quantity of fusion protein contained on each column was equalized based on protein content, rather than molar amounts, it is difficult to judge the relative affinity of TFIID for Crt1 versus VP16. The stoichiometry of the TAF_{II}s eluting from the GST–Crt1 column seemed to be equivalent because the fraction of each TAF_{II} bound to the column (eluate) was similar to that contained in the starting material (load). Since both Crt1 and TFIID bind to DNA, we verified that this interaction is not dependent on nucleic acids by repeating the chromatography in the presence of the DNA-intercalating agent ethidium bromide (data not shown). These results support the idea that Crt1 mediates the TAF_{II} dependence of the RNR gene promoters.

TFIID binds to a region of Crt1 required for Ssn6–Tup1 binding and repression

We next sought to map the region of Crt1 that is required for TFIID binding. Crt1 is a member of a family of highly conserved DNA-binding proteins characterized by the similarities within their DNA-binding and C-terminal dimerization/oligomerization domains (Emery *et al*., 1996). A schematic diagram of Crt1 is presented in Figure 7A. GST 'pull-down' experiments were performed in whole-cell extracts using full-length Crt1 and its derivatives, and the binding of TFIID was monitored by immunoblotting for TAF_{II}145p and TBP. The results of Figure 7B show that all of the derivatives containing the first 240 amino acids of Crt1 retained TAF_{II}145p and TBP as well as full-length protein; however, the DNA-binding domain and the entire C-terminus were dispensable for binding. The N-terminus of Crt1 contains a proline-rich domain, and since proline-rich domains in some gene-regulatory proteins interact with human TFIID (Chiang and Roeder, 1995), we tested whether or not this region binds to yTFIID. However, the proline-rich domain of Crt1 is not sufficient for interacting with TFIID because a derivative containing this region and amino acids up to the DNA-binding domain [GST–Crt1(77–240)] failed to retain TFIID subunits in this assay (Figure 7B). From this analysis, we conclude that TFIID interacts with the N-terminus of Crt1.

We next determined whether the region of Crt1 that binds to TFIID is important for its function as a transcriptional repressor. Crt1 binds to Ssn6 and Tup1; however, the region required for this function is not known (Huang *et al*., 1998). We therefore used the GST–Crt1 derivatives described above to identify the region that binds to Ssn6 and Tup1. The results shown in Figure 7C demonstrate that both Ssn6 and Tup1 bound to the



Plasmid	Units	Fold-Repression
None	7.20 ± 0.13	-
LexA	5.69 ± 0.40	1.3
LexA 1-811	0.47 ± 0.02	15.5
LexA 1-350	0.28 ± 0.04	25.7
LexA 1-240	0.17 ± 0.01	42.4
LexA 77-240	3.00 ± 0.19	2.4
LexA 155-240	1.40 ± 0.12	5.1
LexA 319-585	6.86 ± 0.09	1.1
LexA 319-811	2.02 ± 0.32	3.5

Fig. 7. Mapping of Crt1. (**A**) Domains of Crt1. Amino acids 1–40 are dispensable for Crt1 function (Huang *et al*., 1998). The locations of the proline-rich domain, 77–155; DNA-binding domain, 246–318; a conserved 'B' box and a region displaying homology to numerous yeast and metazoan genes, 411–579 are indicated in the diagram. (**B**) Mapping of the TFIID interaction region. A 50 μg aliquot of GST derivatives bound to glutathione–agarose beads was incubated with yeast whole-cell extracts, washed and eluted as described in Materials and methods. TFIID binding was detected by western blotting for TAF$_{II}$145p and TBP. (**C**) Mapping of the Ssn6–Tup1 interaction region. A 20 μg aliquot of GST derivatives bound to glutathione–agarose beads was incubated with *in vitro* translated Ssn6 and Tup1, washed and eluted as described in Materials and methods. The binding of Ssn6 and Tup1 was detected by fluorography of SDS–polyacrylamide gels. (**D**) Identification of the repression domain of Crt1. Plasmids expressing Crt1–LexA DNA-binding domain fusion proteins were transformed into BY4705 cells containing the reporter plasmid JK101 (Brent and Ptashne, 1985) and grown in liquid SC-raffinose medium. β-galactosidase activities were measured in protein extracts prepared from at least three independent isolates. The results shown are from the same experiment; however, the average fold repression and SEMs from three independent experiments were: LexA–Crt1(1–811), 16.5 ± 1.8; LexA–Crt1(1–240) 37.5 ± 3.2; LexA–Crt1(1–350) 21.4 ± 1.6.

N-terminus of Crt1(1–240). Moreover, there is a correlation between the region required for Ssn6 and Tup1 and TFIID binding because neither GST–Crt1(77–240) nor GST–Crt1(155–240) was capable of interacting with Ssn6 or Tup1. In these experiments, Ssn6 and Tup1 were co-translated; however, identical results were obtained when Ssn6 or Tup1 binding was analyzed individually (not shown).

The repression domain of Crt1 was identified by analyzing the ability of LexA DNA-binding domain–Crt1 fusion proteins to repress a promoter containing LexA operator sites *in vivo* (Brent and Ptashne, 1985). We found that the entire coding region of Crt1 repressed the reporter gene >15-fold when fused to the LexA DNA-binding domain [LexA–Crt1(1–811); Figure 7D]. Derivatives containing only the N-terminus, LexA–Crt1(1–240), or the N-terminus and DNA-binding domain, LexA–Crt1(1–350), were more effective than LexA–Crt1(1–811), repressing the reporter gene up to 40- and 25-fold, respectively. We speculate that the weaker repression by LexA–Crt1(1–811) compared with the shorter derivatives may result from the presence of Crt1's DNA-binding and/or oligomerization domain, which may interfere with its ability to interact with the promoter. An intact N-terminus of Crt1 is required for its transcriptional repression function because neither LexA–Crt1(77–240) nor LexA–Crt1(155–240) repressed the reporter gene significantly (Figure 7D). None of the derivatives tested enhanced transcription from the reporter gene. Western blotting of cell extracts using antibodies to the LexA DNA-binding domain revealed that all derivatives accumulated to levels within 2-fold of each other (not shown). Our mapping studies of Crt1 indicate that the N-terminus of Crt1 is required for transcriptional repression, Ssn6–Tup1 binding and TFIID binding.

Discussion

We report that *TAF68*, *TAF90* and *TAF145* are required for the induction of the RNR genes by DNA damage. Unlike the *TAF145*-dependent ribosomal protein and cyclin genes, expression of the RNR genes requires multiple TAF$_{II}$s. However, despite showing an overall sensitivity to TAF$_{II}$ mutations and depletion, the individual RNR genes showed different sensitivities to these conditions. *RNR4* seemed to be the least sensitive and, interestingly, it has the weakest consensus and the fewest DREs in its promoter region compared with the other two genes (Huang *et al*., 1998); thus, it is less tightly regulated by Crt1 and DNA damage. This is consistent with our mapping of the TAF$_{II}$ dependence to the DREs and Crt1 function. The regulation of the RNR genes is also different from that of the previously characterized *TAF145*-dependent genes (Shen and Green, 1997; Walker and Green, 1997; Tsukihashi *et al*., 2000) in that the TAF$_{II}$-dependent region maps to the upstream repression sequences, the DREs. Our results indicate that TFIID has other functions in addition to promoter recognition and selectivity in yeast, which have not been firmly established by previous work. Three recent studies identified *TAF17* and *TAF68* as putative co-activators of Gcn4-mediated transcription (Apone *et al*., 1998; Krishnamurthy *et al*., 1998; Moqtaderi *et al*., 1998); however, it is likely that this activity is attributed to their function in the SAGA HAT complex. Mutation of SAGA-specific components affects the Gcn4-mediated expression of *HIS3* (Tr +13) and *HIS4*

(for review see Grant *et al*., 1998b), but depletion or mutation of TAF_{II}s not contained in SAGA does not (Moqtaderi *et al*., 1996, 1998; our unpublished data). Moreover, the SAGA complex, but not TFIID, can interact with Gcn4 (Drysdale *et al*., 1998; Grant *et al*., 1998a). In light of these observations, it is unlikely that TFIID functions as a co-activator of Gcn4-mediated transcription. We argue that the effects we observe on the RNR promoters are mediated by TFIID based upon the following criteria: (i) expression of these genes is affected by mutation or depletion of the TFIID subunit *TAF145*; (ii) their expression is not affected by the deletion of SAGA-specific components (our unpublished data); and (iii) TFIID binds to Crt1 *in vitro*. Our conclusion is dependent upon the widely accepted view that TAF_{II}145p is specific for TFIID. It is a formal possibility that TAF_{II}145p functions outside of the context of TFIID but, without evidence to support this, our interpretations are valid.

A seemingly obvious explanation for the TAF_{II} dependence of the RNR promoter is that TFIID has limited access to the repressed promoter, and it requires multiple TAF_{II}–DNA contacts to stabilize its interaction with the core promoter (for reviews see Burley and Roeder, 1996; Verrijzer and Tjian, 1996; Lee and Young, 1998). Mutant TFIID may be unable to access the promoter under repressive conditions but, when repression is relieved, it can do so. Our data suggest that the mechanism is not this simple. If true, we expect that all strongly repressed promoters, especially Ssn6–Tup1-regulated promoters, would have a similar requirement for TAF_{II}s, and that the core promoter would mediate the TAF_{II} dependence. Our results indicate that neither of these is true.

We present evidence that the TAF_{II} dependence of the RNR genes is specified by Crt1. Studies on Crt1 clearly indicate that it is a transcriptional repressor and has no detectable gene activation activities (Huang *et al*., 1998; Figure 7D) and, thus, TAF_{II}s are acting as antirepressors, rather than as co-activators. To our knowledge, this is the first description of such activities of TAF_{II}s *in vivo*. TFIID interacts within the same region of Crt1 that is required for repression and Ssn6–Tup1 binding, and deletion of any of these three genes alleviates the TAF_{II} dependence of the RNR promoters. Therefore, the mechanism is likely to involve competition (functional or physical) between TFIID and the Ssn6–Tup1–Crt1 complex at the promoter. How the interaction of TFIID with Crt1 inhibits its activity is not clear at this time; however, it may do so by introducing a conformational change in the repression domain of Crt1 and affecting its function. Studies have shown that the binding of hTAF31 to the activation domains of VP16 and p53 causes them to fold into a conformation conducive to gene activation (Uesugi *et al*., 1997; Uesugi and Verdine, 1999). Given the architectural and, in some cases, strategical similarities between transcriptional activators and repressors, a TAF_{II}-induced conformational change in the repression domain of Crt1 is feasible.

How TAF_{II}s mediate the derepression of the RNR genes is an open question; however, the mechanism is certainly highly interdependent upon the functions of the co-repressor complex. Ssn6–Tup1 is reported to repress transcription factors directly (Herschbach and Johnson, 1993; Kuchin and Carlson, 1998; Papamichos-Chronakis *et al*., 2000), and it is feasible that TFIID is antagonizing interactions between Ssn6–Tup1–Crt1 and components of the pre-initiation complex, such as subunits of the RNA polymerase holoenzyme complex (Kuchin and Carlson, 1998; Papamichos-Chronakis *et al*., 2000). Ssn6–Tup1 can repress genes by a chromatin-mediated mechanism (Roth, 1995), and genetic analysis implicates histone modification in the derepression of the *RNR2* gene (Edmondson *et al*., 1996). A role for TFIID in the activation of the *ADH2* gene has been proposed (Komarnitsky *et al*., 1998) and, interestingly, the expression of *ADH2* requires extensive chromatin remodeling at its promoter (Verdone *et al*., 1996). Given this genetic evidence, TAF_{II}s may be involved in regulating chromatin structure. There are two models that support a chromatin-mediated mechanism. First, TFIID itself modifies histones around the promoter through TAF145p's HAT activity (Mizzen *et al*., 1996). Alternatively, TAF_{II}s may play a role in the recruitment of HAT complexes, or other chromatin remodeling activities, to the promoter. TBP binds to some SAGA acetyltransferase complex components, and recruitment of SAGA to promoters by TBP/TFIID has been presented as a targeting mechanism (for review see Grant *et al*., 1998b). Future studies aimed at analyzing the recruitment of chromatin-modifying complexes by TFIID, isolation and characterization of HAT-defective mutants of *TAF145* and the delineation of the chromatin structure of the *RNR3* promoter will further our understanding of the antirepression activities of TFIID.

Materials and methods

Yeast strains and genetic manipulations

The strains used in this study are as follows: AW41 (Ray *et al*., 1991); tbp1-1 (Cormack and Struhl, 1992); DDY547 and DDY555 (Welch *et al*., 1993); LYC-1 and LY20 (Apone *et al*., 1996); YSW87, YSW94, YJR11 and YJR18 (Walker *et al*., 1996); YJR195, as YSW93, *ssn6Δ::URA3*; YJR196, as YSW93, *tup1Δ::URA3*; YJR199, as AW41, *ssn6Δ::URA3*; YJR200, as AW41, *tup1Δ::URA3*; YJR201, as LY20, *ssn6Δ::URA3*; YJR202, as LY20, *tup1Δ::URA3*; YJR220, as YSW87, *tup1Δ::URA3*; YJR221, as YSW87, *ssn6Δ::URA3*; YJR352, as YSW87, *crt1Δ::URA3*; YJR353, as LY20, *crt1Δ::URA3*; YJR354, as YSW93, *crt1Δ::URA3*; YJR 355, as AW41, *crt1Δ::URA3*; WCS131, *Mat*α, *ade2-101*, *his3-200*, *leu2-Δ1*, *lys2-801*, *trp1Δ63*, *ura3-52*, *tsm1::TRP1*, [pRS313-*TSM1*]; WCS132, as WCS131 except [pRS313-*tsm1-1*]; BY4705, *Mat* α, *ade2Δ::hisg*, *his3Δ200*, *leu2Δ0*, *lys2Δ0*, *met15Δ0*, *trp1Δ63*, *ura3Δ0*. The *SSN6* and *TUP1* deletion strains were constructed by transforming with *Avr*II-digested pRS406-*Δssn6* or pRS406-*Δtup1* (Cooper *et al*., 1994). Deletion of *SSN6* or *TUP1* in the TAF_{II} mutants did not result in any obvious enhanced synthetic phenotypes compared with the single mutants alone (not shown). *CRT1* knockouts were constructed by transforming cells with *Eag*I–*Xho*I-digested pBScrt1Δ::*URA3*, and verified by Southern blotting and examination of RNR gene expression.

Temperature shift experiments were typically conducted as follows: cells are grown to midlog, and the culture transferred to a shaking water bath at 37°C. Cultures were typically pre-incubated at 37°C for 45–120 min and then treated with DNA-damaging or replicative stress agents. Aliquots were taken for RNA preparation at various times thereafter. TAF_{II} depletion experiments were conducted as described previously (Walker *et al*., 1996). Aliquots were removed for RNA isolation and protein extract preparation.

Plasmid constructions

All promoter fragments were amplified by PCR from genomic DNA and inserted into the polycloning site of pRS316. For the *RNR3* and *ADH1* genes, +1 indicates the start site of transcription determined by primer extension analysis (Tornow and Santangelo, 1990; Yagle and McEntee, 1990). DRE-*ADH1-lacZ* was constructed by inserting a synthetic double-

stranded oligonucleotide (5′-tcgagTCGCCATGGCAACactTCGCCAT-GGCAACctgTCGCCATGGCAACtgaTCGCCATGGCAACt-3′). PCR primer sequences and a detailed description of the construction are available upon request. pGST–Crt1 and its derivatives were generated by inserting the coding sequence of *CRT1* into pRET3aGSTN2, which contains the GST gene driven by the T7 promoter (a gift from Dr Song Tan). Regions of *CRT1* were amplified by PCR and cloned into pEG202 (Brent and Ptashne, 1985) to produce LexA DNA-binding domain fusions. The coding regions of *SSN6* and *TUP1* were amplified by PCR and cloned into BS-KS+ for use in *in vitro* transcription/translation.

RNA analysis, western blotting and affinity chromatography

Methods for protein extraction used for western blotting, and RNA isolation and analysis were described in Walker *et al*. (1996) and Apone *et al*. (1996). The methods for affinity chromatography and yeast whole-cell extract preparation were described previously (Reese *et al*., 1994). Columns of 0.5 ml (0.8 mg/ml GST protein) were equilibrated with 0.15 M potassium acetate buffer T (Reese *et al*., 1994) supplemented with 0.003% NP-40 and protease inhibitors, and 1 ml of yeast whole-cell extract (~5 mg/ml protein) was passed 5–10 times over the column. After extensive washing with 0.15 M buffer T, proteins were eluted with 1 M NaCl in buffer T supplemented with 0.003% NP-40 and protease inhibitors. In some experiments, chromatography was performed in the presence of 300 µg/ml ethidium bromide. GST pull-downs were performed similarly except that all steps were carried out in batch. Briefly, ~50 µg of GST, GST–Ssn6 and GST–Crt1 protein were incubated with whole-cell extracts for 1.5 h at 4°C, washed four times for 10 min and eluted. The Ssn6–Tup1 interaction assays were performed as follows: 20 µg of GST–Crt1 (or mutant derivatives) were incubated with 10 µl of co-translated Ssn6 and Tup1 in 90 µl of binding buffer [20 mM HEPES–KOH pH 7.5, 150 mM potassium acetate, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol and 0.01% NP-40]. After a 60 min incubation at 4°C, the beads were collected by low speed centrifugation and washed four times for 10 min each with 300 µl of binding buffer. The bound proteins were eluted with SDS–PAGE loading buffer, separated by SDS–PAGE, stained, treated with En3Hance (Dupont-NEN), dried and exposed to film.

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