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The *WTM* Genes in Budding Yeast Amplify Expression of the Stress-Inducible Gene *RNR3*

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ABSTRACT

Cellular responses to DNA damage and inhibited replication are evolutionarily conserved sets of pathways that are critical to preserving genome stability. To identify new participants in these responses, we undertook a screen for regulators that, when present on a high-copy vector, alter expression of a DNA damage-inducible *RNR3-lacZ* reporter construct in *Saccharomyces cerevisiae*. From this screen we isolated a plasmid encoding two closely related paralogs, *WTM1* and *WTM2*, that greatly increases constitutive expression of *RNR3-lacZ*. Moderate overexpression of both genes together, or high-level expression of *WTM2* alone from a constitutive promoter, upregulates *RNR3-lacZ* in the absence of DNA damage. Overexpressed, tagged Wtm2p is associated with the *RNR3* promoter, indicating that this effect is likely direct. Further investigation reveals that Wtm2p and Wtm1p, previously described as regulators of meiotic gene expression and transcriptional silencing, amplify transcriptional induction of *RNR3* in response to replication stress and modulate expression of genes encoding other *RNR* subunits.

MAINTAINING the integrity of the genome is essential to the survival and success of an organism. All prokaryotic and eukaryotic cells react to damage to their DNA or inhibited replication with coordinated actions designed to block progression through the cell cycle, repair any damage, and protect against future insults. Part of this response includes the transcriptional induction or repression of a set of genes involved in such processes as DNA metabolism, DNA repair, and cell cycle regulation (Zhou and Elledge 2000). In the budding yeast *Saccharomyces cerevisiae*, one of the genes most highly induced by DNA damage and replication inhibition is *RNR3*, encoding a large subunit of ribonucleotide reductase (RNR) (Ruby and Szostak 1985; Yagle and McEntee 1990). *RNR3* is highly similar to the cell-cycle-regulated *RNR1* gene (80% identity at the amino acid level) but *RNR3* is expressed primarily in response to genotoxic and replication stress (Huang *et al.* 1998). Recent evidence suggests that Rnr3p is less susceptible to allosteric inhibition than Rnr1p, possibly allowing the rapid production of deoxynucleotides for DNA damage repair (Domkin *et al.* 2002). *RNR2* and *RNR4*, encoding small catalytic subunits of the RNR enzyme complex, are also DNA damage inducible.

Mammalian cells exhibit DNA damage and replication stress responses that share many features with those of *S. cerevisiae*, many of whose players have been linked to cancer susceptibility (for review, see Melo and Toczyski 2002). Interestingly, one of the human genes induced by DNA damage is *p53R2*, a homolog of yeast *RNR2* and *RNR4* (Tanaka *et al.* 2000). Failure to induce *p53R2* results in sensitivity to genotoxic agents such as ultraviolet (UV) radiation and adriamycin (Tanaka *et al.* 2000). The numerous RNR regulatory mechanisms activated in both yeast and humans upon DNA damage or inhibited replication, including transcriptional induction, production of alternate subunits, and changes in subcellular localization (Yao *et al.* 2003), attest to the importance of this enzyme in these responses.

The partially characterized pathway leading to induction of *RNR3*, *RNR2*, and *RNR4* after DNA damage or replication stress relies heavily on a transcriptional repressor protein known as Crt1p or Rfx1p that binds directly to their upstream regions. A kinase cascade including the Mec1p, Rad53p, and Dun1p protein kinases culminates in the phosphorylation of Crt1p, resulting in derepression of Crt1 target genes (Huang *et al.* 1998; Zhou and Elledge 2000). Experimental evidence suggests, however, that there are regulators of the transcriptional response to DNA damage that remain unidentified (Huang *et al.* 1998; Gasch *et al.* 2001; Horak *et al.* 2002). Thus we undertook a screen to identify new regulators of the DNA damage transcriptional response, specifically the canonical DNA damage-inducible gene *RNR3*. This screen identified two potential novel *RNR3*

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regulators, *W*D40 repeat-containing *t*ranscription *m*odulator (Wtm)1p and Wtm2p. The Wtm proteins are a family of three proteins in *S. cerevisiae* that are believed to have roles in transcriptional regulation and silencing (Pemberton and Blobel 1997). The third member of this family, Ume1p (Wtm3p), has recently been found to be associated with the Sin3p/Rpd3p histone deacetylase complex (Gavin *et al.* 2002; Ho *et al.* 2002; Kurdistani *et al.* 2002; Mallory and Strich 2003), and direct binding of Ume1p to Rpd3p contributes to repression of meiotic genes (Mallory and Strich 2003).

We demonstrate here that Wtm1p and Wtm2p also influence *RNR3* expression. Simultaneous overexpression of *WTM1* and *WTM2* leads to increased transcription of *RNR3* independent of DNA damage or replication inhibition. When *WTM2* alone is overexpressed at very high levels, even in the absence of Wtm1p, even greater expression of *RNR3* occurs in conjunction with Wtm2p's association with the *RNR3* promoter. Deletion of *WTM2* attenuates *RNR3-lacZ* induction by some types of stress, implicating this gene in the DNA damage and replication stress response. *wtm*Δ mutations further influence transcription of both *RNR2* and *RNR1* reporters, implying a possible broader role in transcriptional regulation.

MATERIALS AND METHODS

Screen for regulators: Yeast strain TSR30-23, containing two to four copies of the *RNR3-lacZ* fusion at the *RNR3* locus, has been described (Ruby and Szostak 1985). This strain was transformed with a library of yeast genomic sequences on a 2μ plasmid vector (YEp13) (Nasmyth and Reed 1980), and transformants were screened for increased or decreased β-galactosidase activity. A total of 13,800 colonies resulting from the 20 independent transformations were replica plated onto X-gal indicator plates. Among these, a total of 15 colonies were either darker or lighter blue than the control strain containing empty vector.

To ascertain whether altered reporter activity resulted from the introduced plasmids, strains were analyzed for cosegregation of the altered phenotype with the plasmid *LEU2* marker. Strains were grown on nonselective media to bring about plasmid loss and *leu*− subclones were rescreened for β-galactosidase activity. The plasmid present in high-activity strain T19, pSR50, was isolated by transforming bacteria with T19 DNA as previously described (Guthrie and Fink 1991) and selecting for ampicillin resistance. The plasmid was retested for activation activity in strain TSR30-15, containing a single copy of the *RNR3-lacZ* reporter integrated at the *RNR3* locus. The region from the *Bgl*II site in *YOR228C* to the *Stu*I site between *WTM2* and *WTM1* was subcloned and sequenced and found to be identical to the reported genomic sequence.

Oligodeoxynucleotides: The following oligodeoxynucleotides were synthesized: oSG1 (5′-GTTTATTACGTAGTAAAA GTTGCATG-3′), oSG8 (5′-TAGGATCCAGAAGGAAACACTC AAGG-3′), oSG9 (5′-AGGCTCGAGACGCTGACACGAAAAAC GAA-3′), oSG12 (5′-GCGGATCCATGGCGAAAAGCAAATCC AG-3′), oSG14 (5′-CCGCTCGAGTTAGCGGCCGCAATCGTC GTAACCTCTGCCAAT-3′), oSG15 (5′-GCCGTGGCTAGTTT CTTCTTA-3′), oSG16 (5′-CGTAGGCAGATAACTTGGCTT-3′), oSR121 (5′-AAGCAGCTTTACAGATCAATGGCGG-3′), oSR122 (5′-CGCCCTCCTTACTCATTGAGAAAAAGG-3′) oSR325 (5′-ACTACGGCGCCAAGATGAAGCGACGATGGAA-3′), oSR326 (5′-CGTCGCTTAAGATTCTTTGCTCACCGAAGGAT-3′), oSR405 (5′-AATAGGATCCATGCCAAAAAAGGTTTGGAAAT CA-3′), oSR406 (5′-GTGGCTCGAG*TTA*CTATTCGCTTTCCT CGGTATA-3′), oSR463 (5′-TTAAGTCTAGAGCTGGC-3′), oSR464 (5′-CAGCTCTAGACTTAAGGCC-3′), Act1-699 (5′-GC CTTCTACGTTTCCATCCA-3′), and Act1-r851 (5′-AAGAGT AACCACGTTCACTCAAGAT-3′).

Plasmid constructions: A total of 5.4 kb of the pSR50 insert on a *Cla*I–*Xho*I fragment was subcloned into *Cla*I/*Sal*I-cleaved YEp24 to generate plasmid pSR53 and to remove the *URA3* marker. The 1.7-kb *Bam*HI *HIS3* fragment from pSZ63 (Orr-Weaver *et al.* 1981) was then inserted into the *Bam*HI site of pSR53 to create pSR54. Plasmid pSR61 was made by cutting pSR54 with *Xho*I and *Bst*EII, filling in the ends, and religating.

Because pSR54 lacks both a matched vector control and a convenient multicloning site, an alternate plasmid containing the same insert was generated for further manipulation and analysis. Plasmid pSG12 was generated by inserting the *Bam*HI–*Nar*I fragment from pSR53, containing the entire genomic insert as well as part of the tetracycline resistance gene from YEp24, into *Bam*HI/*Cla*I-cleaved pRS424 (Christianson *et al.* 1992). Plasmid pSG11 was created by single-stranded mutagenesis (Kunkel *et al.* 1991) using oligonucleotide oSG1 to change the first two codons of *YOR228C* to stop codons and introduce a *Sna*BI site for screening. The 0.6-kb *Nco*I–*Nde*I fragment from pSG11, containing the mutated region, was inserted into *Nco*I/*Nde*I cleaved pSG12 to create pSG13 and sequenced in both pSG12 and pSG13. Plasmid pSG3 was generated by inserting the 5.1-kb *Eag*I–*Bam*HI segment from pSR54 into 2μ vector pRS425 (Christianson *et al.* 1992). pSG5 was then made by cutting pSG3 with *Bsa*BI and *Stu*I and religating; pSG6 was made by cutting pSG3 with *Sma*I and *Pml*I and religating.

pSE788 and pSE836, 2μ plasmids containing *RNR2(UAS)-CYC1-lacZ* and *RNR1(UAS)-CYC1-lacZ* reporters, respectively, were gifts from Steve Elledge (Elledge and Davis 1989; Zhou and Elledge 1992). To generate a matching *RNR3(UAS)-CYC1-lacZ* reporter, the upstream activating sequence (UAS) of *RNR3* (from −646 to −114 relative to the major transcription start site) was amplified by PCR with oligos oSG8 and oSG9. The amplified DNA was cut with *Bam*HI and *Xho*I and inserted into *Bgl*II/*Xho*I-cut pSE836 to replace the *RNR1* UAS and generate pSG20.

For overexpression of *WTM2* under the control of the *GPD* promoter, the coding region was amplified by PCR from genomic DNA with oligos oSG12 and oSG13, cut with *Bam*HI and *Xho*I, and inserted into the *Bam*HI/*Sal*I-cleaved vector pG-3 (Schena *et al.* 1991) to create pSGX. For overexpression of TAP-tagged *WTM2* under the control of the *GPD* promoter, the same region was amplified from plasmid pSG3 with oligos oSG12 and oSG14, which introduced a unique *Not*I site just before the translational stop codon of *WTM2*. This product was cut with *Bam*HI and *Xho*I and inserted into *Bam*HI/*Sal*I-cleaved pG-3 to create plasmid pSGY. DNA encoding the TAP tag flanked by *Not*I sites was then amplified with oSR325 and oSR326 using plasmid pFA62X (Gould *et al.* 2004) as template and cloned into the *Not*I site of pSGY to create pJW1. Plasmid pSR336 encoding TAP-tagged, truncated, mutant Wtm2p was constructed by cutting pJW1 with *Apa*I and *Sac*II and ligating in hybridized oligos oSR463/464. For overexpression of *WTM1* by the *GPD* promoter in plasmid pKL6, the same cloning strategy was used as for pSGX except that the *WTM1* ORF was amplified with oligos oSR405 and oSR406 with pSG7 as template.

All plasmids created by *in vitro* mutagenesis or PCR were sequenced.

Yeast strains and media: Most strains used in this work were derived from DSR741-3B (Ruby and Szostak 1985), an S288C derivative, and are listed in Table 1. Null mutations in the

TABLE 1

Strains used in this study

Strain name	Genotype	Source
DSR741-3B	*MAT***a** *leu2-2,112 his3-11,15 trp1 ura3*	Ruby and Szostak (1985)
	Strains derived from DSR741-3B	
TSR30-15	*RNR3-lacZ* (1 copy)	Ruby and Szostak (1985)
TSR30-23	*RNR3-lacZ* (2–4 copies)	Ruby and Szostak (1985)
T19	*RNR3-lacZ* (2–4 copies) + pSR50	Ruby and Szostak (1985)
TSG1	*RNR3-lacZ* (1 copy), *wtm1wtm2*Δ*::HIS3*	This study
TSG10	*RNR3-lacZ* (1 copy)	This study
TSG12	*RNR3-lacZ* (1 copy), *wtm1*Δ*::HIS3*	This study
TSG19	*RNR3-lacZ* (1 copy) + pSG5 (*WTM1*) + pRS423	This study
TSG20	*RNR3-lacZ* (1 copy) + pSG5 (*WTM1*) + pSR61 (*WTM2*)	This study
TSG21	*RNR3-lacZ* (1 copy) + pRS425 + pRS423	This study
TSG23	*RNR3-lacZ* (1 copy) + pRS425 + pSR61 (*WTM2*)	This study
TSG41	pRS424	This study
TSG42	pSG12 (*WTM2* + *WTM1*)	This study
TSG78	pSG20 (*RNR3-CYC1-lacZ*) + pRS424	This study
TSG80	pSG20 (*RNR3-CYC1-lacZ*) + pSG12 (*WTM2* + *WTM1*)	This study
TSG85	*RNR3-lacZ* (1 copy) + pRS425 + pG-3	This study
TSG86	*RNR3-lacZ* (1 copy) + pRS425 + pSGX (*GPD-WTM2*)	This study
TSG88	*RNR3-lacZ* (1 copy) + pSG5 (*WTM1*) + pG-3	This study
TSG89	*RNR3-lacZ* (1 copy) + pSG5 (*WTM1*) + pSGX (*GPD-WTM2*)	This study
TSG94	*RNR3-lacZ* (1 copy) + pG-3	This study
TSG95	*RNR3-lacZ* (1 copy) + pSGX (*GPD-WTM2*)	This study
TSG97	*RNR3-lacZ* (1 copy), *wtm1wtm2*Δ*::HIS3* + pG-3	This study
TSG98	*RNR3-lacZ* (1 copy), *wtm1wtm2*Δ*::HIS3* + pSGX (*GPD-WTM2*)	This study
TSG100	*RNR3-lacZ* (1 copy), pSGX (*GPD-WTM2*)	This study
TSG107	pSGX (*GPD-WTM2*)	This study
TSG125	*DIN7-lacZ* (1 copy) + pRS423	This study
TSG126	*DIN7-lacZ* (1 copy) + pSR54 (*WTM2* + *WTM1*)	This study
TSG133	pSGX (*GPD-WTM2*)	This study
TSG134	pG-3	This study
TSG1257	*RNR3-lacZ* (1 copy), *wtm2*Δ*::LEU2*	This study
TSR2048	*RNR3-lacZ* (1 copy) + pSR54 (*WTM2* + *WTM1*)	This study
TSR2051	*RNR3-lacZ* (1 copy) + pRS423	This study
TSR2147	pSE788 (*RNR2-CYC1-lacZ*) + pRS424	This study
TSR2148	pSE836 (*RNR1-CYC1-lacZ*) + pRS424	This study
TSR2150	pSE788 (*RNR2-CYC1-lacZ*) + pSG12 (*WTM2* + *WTM1*)	This study
TSR2151	pSE836 (*RNR1-CYC1-lacZ*) + pSG12 (*WTM2* + *WTM1*)	This study
TSR2167	pSE788 (*RNR2-CYC1-lacZ*)	This study
TSR2169	pSE836 (*RNR1-CYC1-lacZ*)	This study
TSR2170	*wtm1wtm2D::HIS3* + pSE788 (*RNR2-CYC1-lacZ*)	This study
TSR2172	*wtm1wtm2D::HIS3* + pSE836 (*RNR1-CYC1-lacZ*)	This study
TSR2386	*wtm2*Δ*::LEU2* + pG-3	This study
TSR2387	*wtm2*Δ*::LEU2* + pJW1 (*GPD-WTM2-TAP*)	This study
TSR2425	pLGSD5 (*GAL10-CYC1-lacZ*) + pRS424	This study
TSR2427	pLGSD5 (*GAL10-CYC1-lacZ*) + pSG12 (WTM2 + WTM1)	This study
TSR2441	RNR3-lacZ + pKL6 (GPD-WTM1)	This study
TSR2451	*wtm2*Δ*::LEU2* + pSR336 (*GPD-wtm2-TAP*)	This study
Y80	*MAT***a**, *can1-100, ade2-1, his3-11, leu2-3,112, trp1-1, ura3-1*	Huang *et al.* (1998)
	Strains derived from Y80	
Y301	*rad53-21*	Huang *et al.* (1998)
TSG46	*RNR3-lacZ* (1 copy) + pRS424	This study
TSG47	*RNR3-lacZ* (1 copy) + pSG13 (*WTM2* + *WTM1*)	This study
TSG48	*rad53-21, RNR3-lacZ* (1 copy) + pRS424	This study
TSG49	*rad53-21, RNR3-lacZ* (1 copy) + pSG13 (*WTM2* + *WTM1*)	This study

WTM genes were generated by gene replacement (Rothstein 1983). *wtm1Δ::HIS3* mutants contain the *HIS3* gene between bases 56 and 1186 of the 1312-bp ORF. *wtm2Δ::LEU2* mutants contain the *LEU2* gene between bases 107 and 613 of the 1405-bp ORF. In *wtm1wtm2Δ::HIS3* strains (*wtmΔ12*), the region from base 107 of *WTM2* to 994 of *WTM1* is replaced by the *HIS3* gene. All strains with integrated mutations were confirmed by Southern analysis (data not shown). Media for yeast strain manipulations were made as described (Guthrie and Fink 1991). Hydroxyurea (HU) (Sigma, St. Louis) was made up as a 1-M stock solution in water and stored at −20°; this solution was added directly to the culture to the desired concentration.

β-Galactosidase assays: Unless otherwise indicated, cells were grown overnight in YPD or appropriate selective medium containing 2% glucose to an OD_{600} of 1–4, diluted to an OD_{600} of 0.3, and allowed to continue growing for 3 hr at 30° prior to any experimental treatment. After the time period indicated, cells were collected by centrifugation and cell pellets frozen on dry ice and stored at −80°. β-Galactosidase activity in the cell pellets was determined by the glass bead method and expressed in Miller units as previously described (Ruby *et al.* 1983). The general linear model (GLM) ANOVA procedure and Dunnet's comparisons to control (Figure 3A) or Tukey's multiple-comparison method (all others) contained in Minitab (Version 13) were used to analyze activity data. To equalize the variances, data were log transformed prior to analysis.

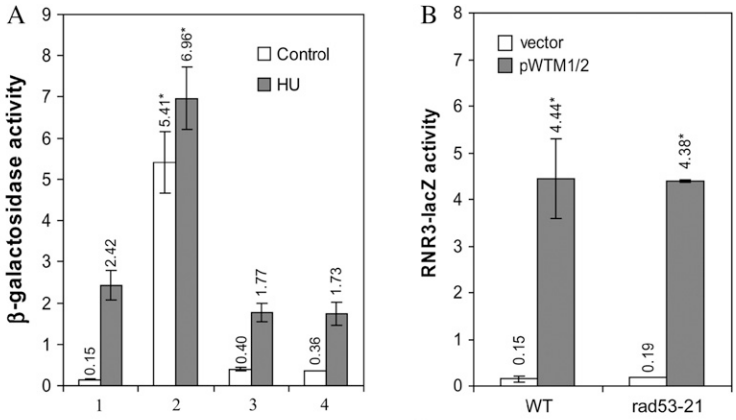
Northern blots: RNA was extracted as previously described (Vijayraghavan *et al.* 1989) and sample integrity was assayed on a Bioanalyzer according to the manufacturer (Agilent). RNA samples were fractionated by electrophoresis in a 1.25% agarose gel with formaldehyde, transferred to Genescreen (New England Nuclear, Boston), and hybridized as previously described (Maniatis *et al.* 1982; Ruby 1999). Probes were labeled with ^{32}P-dATP (ICN) by random priming (Feinberg and Vogelstein 1983). Quantitative analysis was performed with a STORM imager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software. GLM ANOVA in Minitab was used to compare RNA levels.

Chromatin immunoprecipitation assays: Three independent yeast isolates containing p*GPD-WTM-TAP* and one or two negative control strains (with either untagged or no Wtm2p) were used for each of two independent chromatin preparations and chromatin immunoprecipitation (ChIP) repetitions. Crosslinking with dimethyl adipimidate (Pierce, Rockford, IL) for 1 hr followed by formaldehyde for 16–18 hr was performed as described for Ume1/Wtm3p (Kurdistani *et al.* 2002; Kurdistani and Grunstein 2003). All subsequent lysate preparatory steps were performed at 4°. Whole-cell lysates were prepared by glass bead breakage in ice-cold lysis buffer (200 mM KCl, 1% Triton X-100, 0.1% sodium deoxycholate, 20 mM Tris pH 7.4, and 5 mM $MgCl_2$) supplemented with a 100-fold dilution of protease inhibitor HALT (Pierce). The lysate was drained from the glass beads by centrifugation in a clinical centrifuge for 1 min at 3000 rpm after which the crosslinked chromatin was sedimented at 40,000 × *g* for 3 min (Kurdistani and Grunstein 2003). The crosslinked chromatin was resuspended in lysis buffer, sonicated to an average size of 500 bp, and separated from debris by centrifugation for 1 hr at 40,000 × *g*. Supernatant aliquots were diluted 100-fold in water and measured spectrophotometrically at 260 nm. Immunoprecipitations were performed with 450 absorbance units of sample incubated overnight at 4° with 30 μl of IgG-Sepharose-6 beads (Amersham, Arlington Heights, IL) equilibrated in lysis buffer. After four washes in wash buffer (300 mM KCl, 1% Triton X-100, 0.1% sodium deoxycholate, 20 mM Tris–HCl, pH 7.4, and 5 mM $MgCl_2$), the beads were incubated in elution buffer (0.1 M $NaHCO_3$, 1% SDS, and 0.2 M NaCl) for 1 hr at 65° and then 1 hr at 75° to elute the bound material while simultaneously reversing the formaldehyde crosslinks (Solomon and Varshavsky 1985). Nucleic acids in the supernatant were precipitated, then treated sequentially with RNAseA and proteinase K as described (Kuo and Allis 1999), after which they were phenol extracted and ethanol precipitated. Inputs (110 absorbance units) were diluted in elution buffer and similarly heated and processed. Detection was by PCR with HotStarTaq polymerase (QIAGEN, Valencia, CA) and oligo pairs oSG15/16, oSR121/122, and ActI-699/851r for 1 cycle of 95° for 10 min, 50° for 45 sec, and 72° for 1 min followed by 24 cycles of 95° for 1 min, 50° for 45 sec, and 72° for 1 min. Template dilutions showed that all amplifications were in the linear range. PCR products were fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining. Dilutions of PCR products were measured by Southern blot hybridization with radiolabeled probes, scanning with a STORM imager, and quantitating with ImageQuant software. Fold enrichment was calculated as the ratio of the levels for Wtm2-TAP *vs.* those for the negative control. The one-sample *t*-test and ANOVA in Minitab were used to compare fold enrichments to the null predicted ratio of one and for multiple comparisons, respectively.

For one ChIP repetition, two and three dilutions of the selected and total input samples, respectively, were also analyzed by real-time PCR with Sybr green master mix containing ROX in a 7900 HT cycler (Applied Biosystems, Foster City, CA) and quantitated via the standard curve method according to the supplier. Standard and dissociation curves showed that the primer pairs had equal efficiency and that each pair produced a single PCR product, respectively. Comparable results were obtained with real-time PCR as with Southern hybridization of PCR products.

RESULTS

Screen for *RNR3* regulators: To identify potential *trans*-acting regulators of the *RNR3* (*DIN1*) gene, we initiated a screen for high-copy activators or repressors of *RNR3* transcription. A library of yeast sequences on a 2μ plasmid vector (YEp13) (Nasmyth and Reed 1980) was used as this vector is maintained at 20–50 copies per cell (Old and Primrose 1994). The library was introduced into a yeast strain containing multiple copies of an *RNR3-lacZ* reporter integrated at the *RNR3* chromosomal locus (Ruby and Szostak 1985), and transformants were screened for increased or decreased β-galactosidase activity. Among 15 strains with altered reporter activity, 4 strains were identified in which this activity segregated with the plasmid marker. One transformant in particular, T19, exhibited much higher β-galactosidase activity than the parent strain even in the absence of a DNA-damaging agent and was chosen for further analysis. The plasmid pSR50 was isolated from this strain and observed to cause a clear increase in *RNR3-lacZ* activity on X-gal plates upon retransformation into a yeast strain with a single copy of the integrated *RNR3-lacZ* reporter (data not shown). Furthermore, levels of endogenous *RNR3* transcript were elevated in the presence of this plasmid in a strain lacking the reporter (data not shown, but see Figure 2).



Column	1	2	3	4
Plasmid	vector	pSR54	vector	pSR54
Reporter	RNR3-lacZ	RNR3-lacZ	DIN7-lacZ	DIN7-lacZ

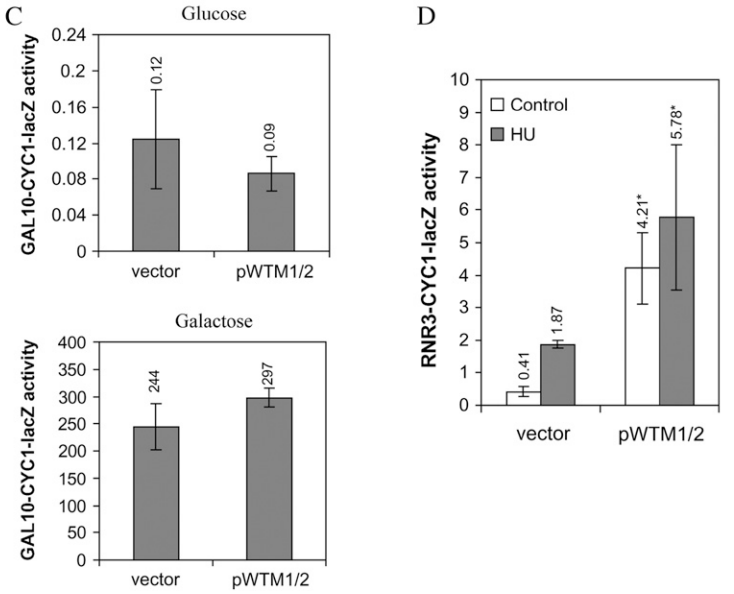


FIGURE 1.—High-copy *WTM1* and *WTM2* increase expression of both integrated and extrachromosomal *RNR3-lacZ* reporters, independent of Rad53p activity. (A) Strains containing either an *RNR3-lacZ* or a *DIN7-lacZ* integrated reporter and transformed with pSR54 (a high-copy plasmid containing a genomic DNA insert encoding *WTM2* and *WTM1*) or empty vector were grown to midlog phase in synthetic medium and then incubated for 3 hr with or without 100 mM HU. β-Galactosidase activities are the means of four independent isolates with error bars and asterisks indicating standard deviations and statistically significant differences, respectively. Effect of pSR54 on activity: $P < 0.001$ for *RNR3-lacZ*, not significant for *DIN7-lacZ*. (B) Isogenic *rad53-21* and wild-type (wt) strains containing an integrated *RNR3-lacZ* reporter and either a plasmid encoding both *WTM1* and *WTM2* (pWTM1/2) or empty vector were assayed for β-galactosidase activity in midlog phase. Values represent means of two independent isolates; $P < 0.001$ for effect of pWTM1/2 on both strains. (C) Cells containing a plasmid-based *GAL10-CYC1-lacZ* reporter and either a plasmid expressing *WTM1* and *WTM2* or empty vector were grown in media containing glucose (top) or galactose (bottom) and assayed for β-galactosidase activity in midlog phase. Values represent means of three and six independent isolates for strains with empty vector and a plasmid expressing *WTM1* and *WTM2*, respectively. Effects of pWTM1/2 and empty vector on reporter activity were not significant. (D) Cells containing a plasmid-based *RNR3-CYC1-lacZ* reporter and either a plasmid expressing *WTM1* and *WTM2* or empty vector were assayed for β-galactosidase activity in midlog phase. Values represent means of four independent isolates; $P < 0.001$ for pWTM1/2 *vs.* vector. Strains used: (A) TSR2051, TSR2048, TSG125, and TSG126; (B) TSG46, TSG47, TSG48, and TSG49; (C) TSR2425 and TSR4247; (D) TSG78 and TSG80.

The plasmid pSR50 contains an insert of ~5.6 kb of yeast genomic DNA. Sequencing revealed that this plasmid contains a segment of DNA from *S. cerevisiae* chromosome XV that includes the complete coding sequences of the paralogous *WTM2* and *WTM1* genes, which are 61% identical to each other at the amino acid level and reside on the chromosome as a tandem repeat separated by 990 bp of noncoding DNA. Approximately 5.4 kb of the pSR50 insert was subcloned into another 2μ vector and transformation of this construct (pSR54) into yeast containing an *RNR3-lacZ* reporter reconfirmed that the presence of this sequence on a high-copy vector leads to activation of the reporter. Quantitative assays demonstrated the increase to be 36-fold in untreated cells, greater than the 16-fold increase resulting from treatment with 100 mM HU, a replication inhibitor known to activate the DNA damage response (HUANG *et al.* 1998) ($P < 0.001$) (Figure 1A). Treatment of pSR54-containing cells with HU resulted in minimal added increases in reporter activity (Figure 1A), although much higher levels of activity can be elicited from this reporter with DNA-damaging agents such as methyl methanesulfonate (MMS) (data not shown). This suggests that there may be significant overlap in the mechanisms by which HU and pSR54 activate *RNR3* expression.

One possible explanation for these observations is that the overexpression of *WTM2* and/or *WTM1* somehow produces DNA damage or interferes with DNA replication and thereby indirectly induces *RNR3*. To test this possibility, we examined the effects of pSR54 on the expression of another transcript, *DIN7* (*DIN3*), which is inducible by a variety of DNA-damaging agents as well as replication inhibition (RUBY and SZOSTAK 1985; MIECZKOWSKI *et al.* 1997). Strains containing a *DIN7-lacZ* reporter exhibit no increase in β-galactosidase

activity when transformed with plasmid pSR54, suggesting that the presence of this plasmid does not cause DNA damage or otherwise initiate a general DNA damage response (Figure 1A). Induction of the *DIN7-lacZ* reporter in the presence of HU is also unaffected by *WTM1* and *WTM2* overexpression (Figure 1A). Finally, induction of *RNR3-lacZ* by *WTM1* and *WTM2* overexpression is unaffected in cells containing an inactivating mutation in *RAD53* (rad53-21, which eliminates kinase activity), a major upstream player in the DNA damage and replication inhibition responses (Figure 1B) (Allen *et al.* 1994; Huang *et al.* 1998). These data, along with the observation that *WTM1* and *WTM2* overexpression has no discernible effect on cell growth, viability, or sensitivity to agents including HU, MMS, and UV radiation (data not shown), indicate that this overexpression does otherwise induce the DNA damage response.

Another possible explanation for these observations is that overexpression of Wtm1p and Wtm2p, both WD40 repeat proteins, nonspecifically interferes with the function of Tup1p, another WD40 repeat protein. To test this hypothesis, we examined the effect of *WTM1* and *WTM2* overexpression on a *GAL1/GAL10* reporter that is also repressed by the Ssn/Tup1 complex (reviewed in Smith and Johnson 2000). We found that expression of the *GAL10-CYC1-lacZ* reporter, on the 2μ plasmid pLGSD5 (Guarente *et al.* 1982), was unaffected by *WTM* gene overexpression (Figure 1C).

To further characterize the mechanism of action for *WTM1* and *WTM2*, we constructed a 2μ plasmid with the UASs of *RNR3* driving expression of the *CYC1-lacZ* reporter, such that no *RNR3* coding sequence is present in the expressed message. This UAS region from −646 to −114 relative to the major transcription start site spans from the 3′ end of the neighboring gene *FIS1* to the TATA box of *RNR3*, including all three Crt1p recognition sites (X-boxes). As expected, introduction of this plasmid [*RNR3(UAS)-CYC1-lacZ*] into yeast results in β-galactosidase activity that is inducible by HU (Figure 1D; $P < 0.03$) as well as the alkylating agent MMS (data not shown). Cotransformation of cells containing the *RNR3(UAS)-CYC1-lacZ* reporter with a plasmid encoding *WTM1* and *WTM2* (pWTM1/2) results in 10-fold higher β-galactosidase activity than cotransformation with empty vector (Figure 1D; $P < 0.001$). A matched control reporter plasmid lacking the *RNR3* upstream sequences yields very low activity regardless of DNA damage, replication stress, or *WTM1* and *WTM2* overexpression (data not shown). This demonstrates that the *RNR3* upstream sequences are both necessary and sufficient for activation by pWTM1/2.

A number of factors other than transcription can potentially influence the activity of a *lacZ* reporter construct (Ruby *et al.* 1983), so we wished to determine whether a high-copy vector containing *WTM1* and *WTM2* has an effect on the endogenous *RNR3* gene. We measured expression of *RNR3* in yeast transformed with either a plasmid harboring *WTM1* and *WTM2* or a matched empty vector. RNAs were extracted from cells grown to midlog phase in appropriate selective synthetic medium and analyzed by Northern blotting. Little *RNR3* transcript is present in untreated cells containing empty vector, while a clear band is present in cells overexpressing *WTM1* and *WTM2* (Figure 2). The increase is estimated to be 2.3-fold ($P = 0.003$) but, given the low message level in cells containing empty vector, the effect is difficult to quantify accurately. This increase is smaller than the 30-fold activation of the *lacZ* reporter, but expression effects are often amplified in β-galactosidase reporter assays (Ruby and Szostak 1985; S. W. Ruby, unpublished observations), possibly due to the tetrameric structure of the active enzyme (Jacobson *et al.* 1994). These results demonstrate that

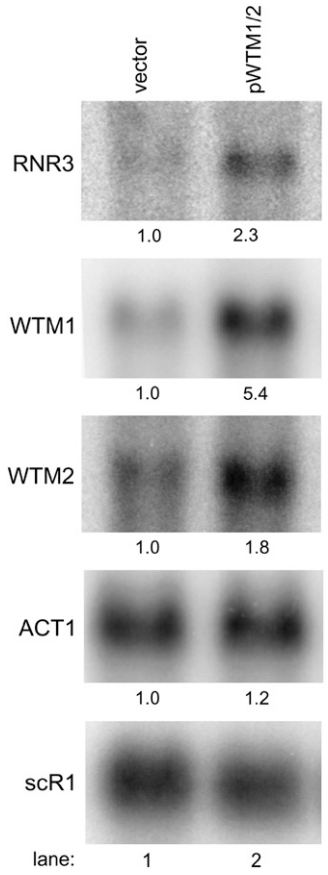
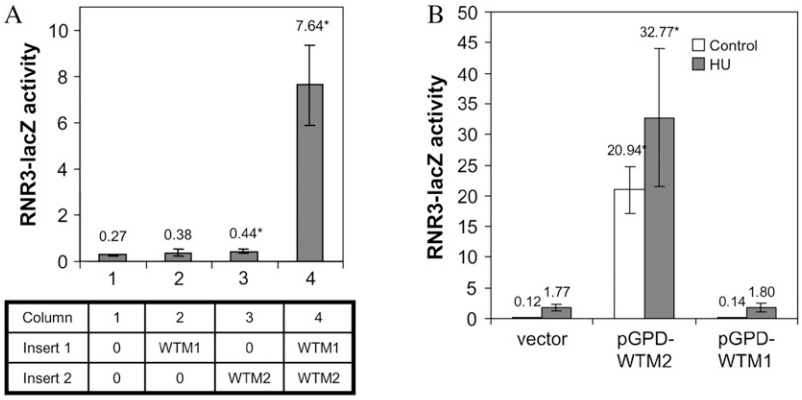
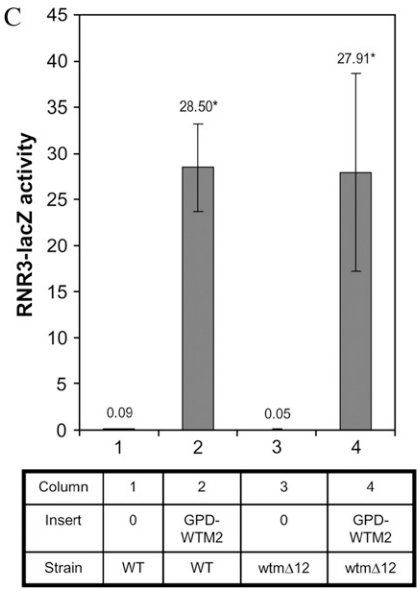


Figure 2.—High-copy *WTM1* and *WTM2* increase expression of the native *RNR3* gene. Cells containing empty vector (lane 1) or a plasmid encoding both *WTM1* and *WTM2* (lane 2) and grown in selective medium were harvested in midlog phase for RNA extraction and Northern blot analysis. The same blot was probed sequentially for *RNR3*, *WTM1*, *WTM2*, *ACT1*, and *scR1* (a loading control). Numbers below each band indicate the mean of three independent isolates run on the same gel relative to untreated vector control and normalized to *scR1*; all coefficients of variation were <30%. *P*-values for pWTM1/2 *vs.* vector: *RNR3*, $P = 0.003$; *WTM1*, $P < 0.001$; *WTM2*, $P = 0.001$; *ACT1*, not statistically significant. Strains used: TSG41 and TSG42.





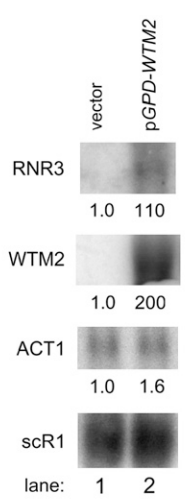


FIGURE 3.—Expression levels of both *WTM1* and *WTM2* influence *RNR3-lacZ* expression. (A) Overexpression of *WTM1* and *WTM2* genes individually on high-copy (2μ) vectors indicates that both genes together maximally stimulate *RNR3-lacZ* expression. Each strain contains a single, integrated copy of *RNR3-lacZ* and two high-copy plasmids, either empty vector or a plasmid encoding a single *WTM* gene (as indicated in the table). These strains were grown to midlog phase in selective medium and assayed for β-galactosidase activity. The means and standard deviations of five independent isolates are plotted. Effect relative to empty vector: *WTM1*, not statistically significant; *WTM2*, $P = 0.04$; *WTM1* + *WTM2*, $P < 0.001$. (B) High-level expression of *WTM2* alone increases *RNR3-lacZ* expression. Strains containing a single, integrated copy of *RNR3-lacZ* and p*GPD-WTM1* (a high-copy plasmid with *WTM1* under control of the constitutive *GPD* promoter), p*GPD-WTM2* (a high-copy plasmid with *WTM2* under control of the constitutive *GPD* promoter), or a control vector were assayed for β-galactosidase activity. The means and standard deviations of three independent isolates are plotted: $P < 0.001$ for effect of *GPD-WTM2*; *GPD-WTM1* effect is not statistically significant. (C) Stimulation of *RNR3-lacZ* by high-level *WTM2* expression does not require the endogenous *WTM1* gene. Wild-type or *wtmΔ12* cells containing an integrated *RNR3-lacZ* reporter were transformed with plasmid p*GPD-WTM2* and assayed for β-galactosidase activity. The means and standard deviations of four independent isolates are plotted: $P < 0.001$ for p*GPD-WTM2*; effect of *wtmΔ12* mutation on p*GPD-WTM2* stimulation is not statistically significant. (D) High-level expression of *WTM2* alone increases *RNR3* gene expression. Strains containing high-copy plasmid p*GPD-WTM2* or empty vector were grown to midlog phase and their RNAs were extracted and subjected to Northern blot analysis. The same blot was probed sequentially for *RNR3*, *WTM1*, *WTM2*, *ACT1*, and *scR1* (a loading control). Numbers below each band indicate the mean of three independent isolates relative to untreated vector control and normalized to *scR1*. Coefficients of variation were ≤23%. *P*-values for p*GPD-WTM2 vs.* vector: *RNR3*, $P = 0.01$; *WTM2*, $P = 0.01$; *ACT1* is not significantly different. Strains used: (A) TSG21, TSG19, TSG23, and TSG20; (B) TSG94, TSG95, and TSR2441; (C) TSG94, TSG95, TSG97, and TSG98; (D) TSG133 and TSG134.

the increased expression of *RNR3-lacZ* by pWTM1/2 reflects a genuine, although smaller, increase in *RNR3* mRNA levels, but do not entirely rule out the possibility that there are also post-transcriptional effects.

The same blot probed with *WTM1* and *WTM2* gene sequences demonstrates that their transcripts are elevated fivefold ($P < 0.001$) and twofold ($P = 0.001$), respectively, in strains harboring pWTM1/2, whereas RNA levels of control genes *ACT1* and *scR1* are unchanged (Figure 2). *ACT1* encodes actin protein and *scR1* (small cytoplasmic RNA 1) (FELICI *et al.* 1989) is a polIII transcript gene used as a loading and normalization control (SHARMA *et al.* 2003) (Figure 2).

Dosage levels of both *WTM1* and *WTM2* influence *RNR3-lacZ* expression: A series of experiments was undertaken to establish whether both *WTM1* and *WTM2* contribute to the observed *RNR3-lacZ* activation. Assays of several derived constructs suggested that disruption of either the *WTM1* or the *WTM2* regions of pSR54 interfered with increased expression of the *RNR3-lacZ* reporter (data not shown). When *WTM1* and *WTM2* were each introduced on separate plasmids with different selectable markers, *RNR3* activation was comparable to the original construct (30-fold increase, $P < 0.001$) (Figure 3A). Cells overexpressing *WTM1* or *WTM2* alone exhibit relatively little change, although the *WTM2* plasmid does cause a small but reproducible and statistically significant increase in *RNR3-lacZ* expression ($P = 0.03$) (Figure 3A). This implies that both *WTM1* and *WTM2* must be present at high copy to increase the *RNR3-lacZ* reporter transcription.

In addition to the complete coding sequences of *WTM2* and *WTM1*, the pSR54 insert also contains much (696 of 909 bp) of the coding sequence of a divergently transcribed uncharacterized ORF, *YOR228C*, whose removal from the plasmid results in less robust increases

in *RNR3-lacZ* expression (data not shown). However, when the start codon of *YOR228C* was mutated to a stop codon in a plasmid otherwise containing the full insert, the effect on *RNR3-lacZ* activation was minimal (data not shown). Thus it appears that the protein product of *YOR228C* is not necessary to bring about *RNR3-lacZ* activation, but that its coding region contains sequences important for *WTM2* expression. This conclusion is bolstered by Northern analysis revealing that deletion of this region from the plasmid results in a 50% decrease in *WTM2* message (data not shown).

The small increase in *RNR3-lacZ* when *WTM2* is overexpressed alone (Figure 2A) suggests that *WTM2* might be capable of acting independently. While Wtm2p is constitutively expressed at much lower levels than Wtm1p, they are thought to interact with a 1:1 stoichiometry in a large nuclear complex (Pemberton and Blobel 1997), so Wtm2p dosage could be limiting for the phenotypic effect seen when *WTM1* and *WTM2* are simultaneously overexpressed from their native promoters. Therefore, we constructed high-copy plasmids p*GPD-WTM1* and p*GPD-WTM2* in which expression of *WTM1* and *WTM2*, respectively, is individually driven by the constitutive *GPD* promoter (Schena *et al.* 1991) and tested the ability of these plasmids to increase expression of either the integrated, single-copy *RNR3-lacZ* reporter or the intact *RNR3* gene. While high-level expression of *WTM2* dramatically increases *RNR3* reporter activity, comparable high-level expression of *WTM1* has no significant effect on the reporter (Figure 3B). Simultaneous overexpression of *WTM1* does not further increase the *RNR3-lacZ* reporter expression stimulated by p*GPD-WTM2* (data not shown) nor does the deletion of the chromosomal *WTM1* and *WTM2* genes impair it (Figure 3C). Plasmid p*GPD-WTM2* also increases RNA levels from the normal chromosomal copy of *RNR3* at least 100-fold (Figure 3D). Thus, when expressed at these very high levels, Wtm2p can act alone to stimulate *RNR3* expression.

Normally, Rnr3p is present at less than one-tenth the levels of Rnr1p and contributes little to the synthesis and maintenance of deoxynucleotide (dNTP) pools as it has very low specific activity (Domkin *et al.* 2002), but the increased *RNR3* expression that we observe with highly expressed *WTM2* might alter dNTP levels, which would manifest as increased sensitivity or resistance to replication inhibitors or DNA-damaging treatments. Others have shown that increased dNTP pools raise resistance to some DNA-damaging treatments such as UV radiation and MMS (Chabes *et al.* 2003). However, cells overexpressing *WTM2* on p*GPD-WTM2* grow as well as wild-type cells and are comparably sensitive to HU, MMS, and UV radiation as the empty vector control (data not shown). Thus, some other factor must be limiting resistance to these treatments.

Wtm2p acts directly at the *RNR3* promoter: We hypothesized that increased *RNR3* expression by highly expressed *WTM2* could be due to a direct association of Wtm2p with the *RNR3* promoter. To test this possibility, we used ChIP to investigate whether highly expressed Wtm2p localizes to the promoter of the intact, normal chromosomal copy of *RNR3*. We added a sequence for an epitope (TAP) tag onto the 3′ end of *WTM2* expressed under the *GPD* promoter in the high-copy plasmid p*GPD-WTM2-TAP* and confirmed that the tagged protein could activate expression of the integrated *RNR3-lacZ* reporter to the same extent as the untagged form (data not shown). Cells containing p*GPD-WTM2-TAP* and a deletion of the chromosomal copy of *WTM2* were then subjected to ChIP, along with two negative controls: cells with p*GPD-WTM2* and therefore untagged Wtm2p or vector without *WTM2*. We found that the upstream region of *RNR3* is enriched 6.2-fold in Wtm2-TAP-selected samples relative to the negative controls ($P < 0.01$) and 2-fold relative to the control genes *ACT1* (encoding actin protein) and *SNR7* (encoding U5 RNA) ($P = 0.002$) (Figure 4, A and B). These two control genes were used because they are actively transcribed in mitotically growing cells. As another control, we expressed a mutant form of the Wtm2-TAP protein in which only the first one-third of Wtm2p is present. This mutant protein, when expressed via the *GPD* promoter, neither increases *RNR3-lacZ* expression (data not shown) nor immunoprecipitates the *RNR3* promoter above the levels of the negative controls (Figure 4). Thus, association of Wtm2p with the *RNR3* promoter correlates with its ability to increase constitutive *RNR3* expression.

Finally, we looked at the effects of HU treatment on chromatin association of Wtm2-TAP. In cells treated with HU, the association of overexpressed Wtm2-TAP with the *RNR3* promoter did not change significantly (data not shown), consistent with the observation that HU exposure only moderately increases *RNR3* expression when *WTM2* is highly expressed (data not shown). When Wtm2-TAP is expressed at normal levels from the *WTM2* chromosomal locus, we found no statistically significant association with the *RNR3* promoter in either untreated or HU-treated cells (data not shown).

We conclude that when Wtm2p is highly expressed, it associates with the *RNR3* upstream regulatory region and increases its transcription, rather than indirectly influencing *RNR3* expression. However, other factors in addition to high levels of Wtm2p may also be involved in increasing *RNR3* expression as Wtm2p associates with at least two other genes, *ACT1* and *SNR7*, although to a lesser extent than with *RNR3*. *WTM2* overexpression does not, however, significantly affect *ACT1* or U5 RNA levels in untreated cells (Figures 2 and 3D and data not shown).

Role of the Wtm proteins in the response to replication stress: Since *RNR3* is expressed at appreciable levels in mitotically growing cells only subsequent to DNA damage or replication inhibition (Ruby and Szostak 1985; Jia *et al.* 2002), we hypothesized that the Wtm proteins are involved in the DNA damage or

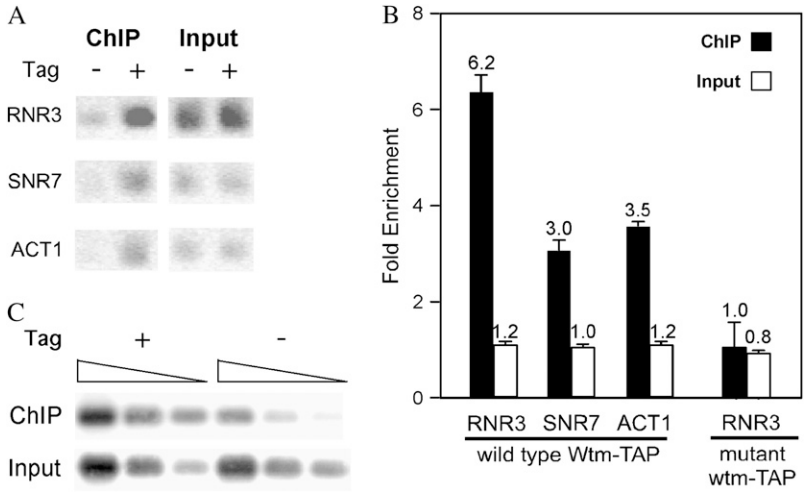


FIGURE 4.—Highly expressed Wtm2p associates with the upstream region of *RNR3*. Chromatin immunoprecipitations (ChIP's) with IgG sepharose were performed on extracts of cells with a chromosomal *wtm2* deletion and plasmid p*GPD-WTM2-TAP* expressing epitope-tagged Wtm2p. Control extracts were from cells with untagged Wtm2p, no Wtm2p, and a truncated, mutant, epitope-tagged wtm2. (A) Quantitative PCR products from one representative ChIP experiment. Input and ChIP samples were assayed using primers for the upstream region of *RNR3* and for the coding regions of *SNR7* and *ACT1*. Products fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining are shown as the inverse image. (B) Bar graph of quantitative PCR results. Dilutions of PCR products were fractionated by agarose gel electrophoresis and measured by Southern analyses. The data are represented as signals from tagged full-length, wild-type Wtm2p or mutant wtm2p samples normalized to those of untagged or no Wtm2p samples. Solid and open bars represent ChIP and input samples, respectively. The mean and standard deviations of three independent isolates, each measured in two independent chromatin preparations and experiments, are plotted for wild-type Wtm2p-TAP: $P < 0.01$ for *RNR3*, *SNR7*, and *ACT1* enrichments, each being different from the background ratio of 1.0; $P = 0.002$ for *RNR3* enrichment being different from those of *SNR7* and *ACT1*; there were no statistically significant differences for input samples. Coefficients of variation were 16% for *RNR3* ChIP and ≤10% for all other samples. Strains used: TSR2386, TSR2387, TSG107, and TSR2451. (C) Southern blot analysis of PCR products from amplification reactions using three different amounts of starting template. Template dilutions (from left to right: 1×, 0.25×, and 0.1×) from ChIP and total input samples from strains with full-length, wild-type Wtm2p-TAP or the untagged control were amplified by PCR and then analyzed by Southern blotting with the upstream region of *RNR3* as probe. Strains used: TSR2386 and TSR2387.

replication stress responses. If the Wtm proteins are genuine regulators of *RNR3* induction by stress, mutants lacking the *WTM* genes should exhibit defects in this response. We therefore generated *wtm1*Δ, *wtm2*Δ, and *wtm1*Δ*wtm2*Δ (hereafter referred to as *wtm*Δ*12*) mutants by gene replacement (see MATERIALS AND METHODS) and tested their ability to survive and to increase *RNR3-lacZ* reporter expression in response to replication stress and DNA-damaging treatments.

None of the *wtm*Δ strains exhibited growth defects (PEMBERTON and BLOBEL 1997) or altered sensitivity to HU, UV radiation, MMS, or ionizing radiation (data not shown). While the induction of *RNR3-lacZ* by HU is relatively unaffected in a *wtm1*Δ strain, activity is reproducibly attenuated by ~45% in a *wtm2*Δ single mutant ($P < 0.001$) and by ~25% in the double mutant ($P < 0.002$; Figure 5). This is somewhat surprising in light of data suggesting that the two proteins may act synergistically (Figure3A), but confirms our observations that Wtm2p appears to be more important in *RNR3* expression than Wtm1p. Similar observations were made in cells treated with a 5-krad dose of ionizing radiation (IR), which induces double-strand breaks as well as other lesions (BIRRELL *et al.* 2002), but no difference in induction of *RNR3-lacZ* by the alkylating agent MMS was observed (data not shown). This is consistent with previous studies that have found that different agents can activate different pathways (HUANG *et al.* 1998). Our data suggest that endogenous Wtm2p accounts for up to half the *RNR3* induction in response to some types of stress, such as HU and IR. No change in either *WTM2* or *WTM1* expression was observed by Northern blot in cells treated with HU (data not shown).

Effect on other *RNR* genes: All four genes encoding ribonucleotide reductase subunits in yeast are DNA damage-inducible, and *RNR2*, *RNR3*, and *RNR4* share a

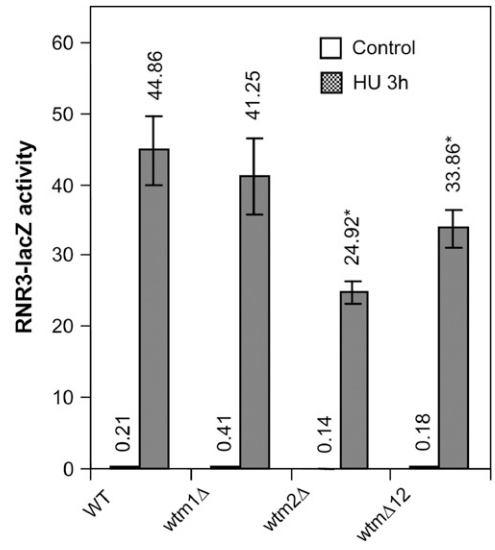
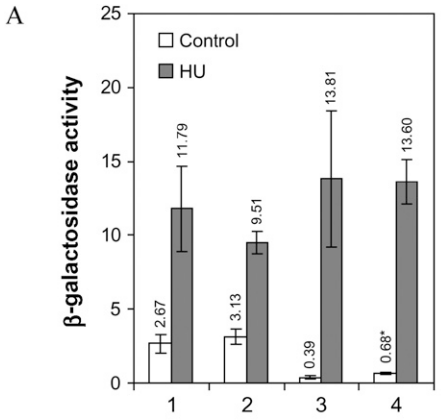


FIGURE 5.—*RNR3-lacZ* induction is attenuated in *wtm2* deletion mutants. Cells lacking *WTM1*, *WTM2*, or both genes were grown to midlog phase in YPD medium, incubated with or without 100 mM HU for 3 hr, and then assayed for β-galactosidase activity. Values indicate mean and standard deviation of four independent isolates: $P = 0.001$ and $P = 0.002$ for effects of *wtm2*Δ and *wtm*Δ*12 vs.* control on HU induction, respectively. Strains used: TSR30-15, TSG10, TSG12, TSR1257, and TSG1.

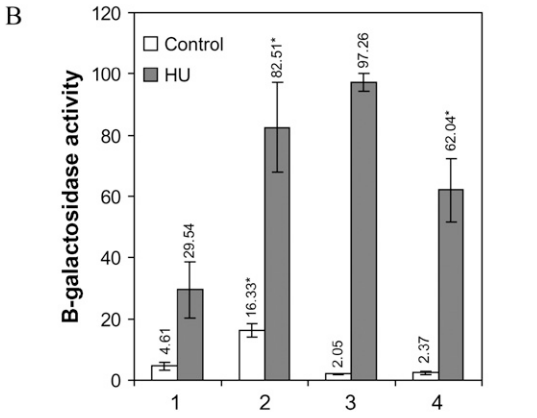
common regulatory mechanism by which this induction occurs. These three genes are repressed by the Crt1p (Rfx1p) protein, which binds to X-boxes in their promoters (Huang *et al.* 1998). Another regulatory element shared by *RNR2* and *RNR3* is a binding site for the repressive Rpa protein complex (Singh and Samson 1995). *RNR1*, on the other hand, shows significant coding sequence similarity to *RNR3* but the two genes are not generally observed to be transcriptionally coregulated (Huang *et al.* 1998; Gasch *et al.* 2001).

To see if overexpression of *WTM1* and *WTM2* has a general effect on ribonucleotide reductase production, we utilized reporter constructs to examine *RNR* gene expression. The plasmids pSE836 and pSE788 contain the upstream activating sequences of *RNR1* or *RNR2*, respectively, driving expression of the *CYC1-lacZ* fusion gene on a 2μ plasmid (Zhou and Elledge 1992). Yeast cells containing each of these reporters in addition to plasmid p*WTM1/2* overexpressing both *WTM1* and *WTM2* were grown to midlog phase and were either left untreated or exposed to HU for 4 hr. Overexpression of *WTM1* and *WTM2* results in a small (75%) but significant increase in *RNR2* reporter activity in untreated cells ($P = 0.03$) and no change in *RNR1* reporter expression (Figure 6A). For comparison, a matched reporter construct containing *RNR3* upstream sequence exhibits a 10-fold increase in β-galactosidase under identical conditions (Figure 1D).

When *WTM1* and *WTM2* are deleted rather than overexpressed, the response of the *RNR2* reporter is qualitatively similar to that of the *RNR3* reporter: its induction by HU is attenuated 30–40% in the double mutant ($P = 0.02$; Figure 6B). In contrast, the double mutant exhibits a marked (2.5- to 3.5-fold) increase in *RNR1* reporter expression in both untreated and HU-treated cells ($P < 0.001$; Figure 6B). The similarities in *RNR2* and *RNR3* reporter response when the *WTM* genes are either overexpressed or deleted suggest a common mechanism. But the fact that their deletion also affects *RNR1* (Figure 6B) and *IME2* (Pemberton and Blobel 1997) expression suggests they may have a more global role in gene regulation.



Column	1	2	3	4
Insert	none	WTM1&2	none	WTM1&2
Reporter	RNR1	RNR1	RNR2	RNR2



Column	1	2	3	4
Strain	WT	wtmΔ12	WT	wtmΔ12
Reporter	RNR1	RNR1	RNR2	RNR2

Figure 6.—Effects of *WTM1* and *WTM2* dosage on *RNR2* and *RNR1* reporter expression. Cells containing high-copy *RNR2* or *RNR1* reporter plasmids were grown in selective medium to midlog phase, incubated with or without 100 mM HU, and then assayed for β-galactosidase activity. (A) Effect of high-copy *WTM1/2* plasmid compared to empty vector at 4 hr incubation. Values indicate mean and standard deviation of four independent isolates; effect of pWTM1/2 is statistically significant only for a twofold increase in the *RNR2* reporter in untreated cells, $P = 0.03$. (B) Wild-type or *wtmΔ12* deletion strains were assayed for β-galactosidase activity after 3 hr incubation. Values indicate mean and standard deviation of four independent isolates: for the *RNR1* reporter, $P < 0.001$ for effect of *wtmΔ12* in both treated and untreated cells; for the *RNR2* reporter, $P = 0.02$ for effect of *wtmΔ12* on HU response. Strains used: (A) TSR2147, TSR2148, TSR2150, and TSR2151; (B) TSR2167, TSR2170, TSR2169, and TSR2172.

DISCUSSION

We demonstrate here that two of the *WTM* genes, previously known to influence transcriptional silencing and meiotic gene regulation (Pemberton and Blobel 1997), also modulate expression of the DNA damage and replication stress-inducible gene *RNR3*. We find that simultaneous overexpression of *WTM1* and *WTM2* produces a >30-fold increase in β-galactosidase activity in strains bearing an *RNR3-lacZ* reporter gene and a 2.3-fold increase in endogenous *RNR3* mRNA levels, as well as a 2-fold increase in *RNR2* reporter activity (Figures 1 and 6). Increased constitutive expression of the *RNR3-lacZ* reporter can also be achieved by high-level expression of *WTM2*, but not *WTM1*, alone. This stimulation by *WTM2* occurs even in the absence of *WTM1* and is associated with the presence of Wtm2p at the *RNR3* promoter (Figures 3C and 4). Reciprocally, deletion of

WTM2 attenuates the stress inducibility of an integrated *RNR3-lacZ* reporter by ~45% (Figure 5).

The most striking effect of the two *WTM* genes is that they increase *RNR3* expression when overexpressed together, or when *WTM2* is highly expressed alone, even in the absence of DNA-damaging treatments or replication stress. For this stimulation, they may partially overcome *RNR3*'s transcriptional repression, established and maintained mainly by a protein complex comprising Crt1p, Tup1p, and Ssn6p (Keleher *et al.* 1992). This complex positions nucleosomes in the upstream region of *RNR3* (Li and Reese 2001), possibly via interactions with general transcription factors (Zhang and Reese 2004) and deacetylated N-terminal regions of histones H3 and H4 (Davie *et al.* 2002). When overexpressed, the Wtm proteins could alter any one or more of these interactions to increase *RNR3* transcription. It is worth noting that Tup1p is also a WD40 repeat protein and could potentially compete with Wtm1p and Wtm2p for binding partners such as Ssn6p or Crt1p. However, Wtm1p and Wtm2p overexpression does not lead to a generalized derepression of Ssn6p/Tup1p-regulated genes, as expression of a *GAL10* reporter is not affected (Figure 1C). Furthermore, the effect of highly expressed Wtm2p is unique in that individual overexpression of *WTM1*, or the closely related paralog *UME1* (*WTM3*), under control of the *GPD* promoter does not increase *RNR3* reporter expression despite the considerable similarity between their WD40 repeat regions (Figure 3B and data not shown). The promoter specificity of the effect when *WTM1/2* are overexpressed together and the unique effect of highly expressed *WTM2*, as well as the decrease in *RNR3-lacZ* expression in the *wtm2* deletion mutant (Figure 5), suggest that Wtm2p normally plays a role in *RNR3* regulation rather than nonspecifically or artifactually interfering with Tup1p function when overexpressed. Additional experiments are required to determine the mechanism (be it derepression or activation) by which Wtm2p functions alone and in combination with Wtm1p.

There are some hints that the two *WTM* genes may increase *RNR3* transcription by participating in chromatin remodeling. As we have shown here, Wtm2p associates with the upstream region of *RNR3* when highly expressed. Others have shown that disruption of the interaction between Tup1/Ssn6 and the N termini of histones H3 and H4 partially derepresses *RNR3* and *RNR2* expression in the absence of stress (Edmondson *et al.* 1996). Although Wtm1p and Wtm2p have no clear orthologs in mammals, they are members of a subfamily of WD-repeat proteins that includes some chromatin-remodeling factors (Pemberton and Blobel 1997). For example, two members of this family, human proteins RpAp48 and RpAp46, associate with several different remodeling complexes (Loyola and Almouzni 2004). Wtm2p itself associates with the chromatin-remodeling factor Rvb1p (Jonsson *et al.* 2001; Ho *et al.* 2002), and both Wtm1p and Wtm2p are involved in regulating transcriptional silencing at some loci (Pemberton and Blobel 1997). Interestingly, the third, more distantly related yeast family member, Ume1p/Wtm3p, associates with the Rpd3p/Sin3p histone deacetylase complex (Gavin *et al.* 2002; Ho *et al.* 2002; Kurdistani *et al.* 2002) but does not copurify with Wtm1p and Wtm2p (Pemberton and Blobel 1997) or increase *RNR3-lacZ* activity when overexpressed (data not shown).

Another, not mutually exclusive, possibility is that the two overexpressed *WTM* genes alter an as yet unknown pathway or mechanism modulating *RNR* gene expression. The Wtm proteins could interact with Rpa1, another repressor of *RNR3* (Singh and Samson 1995), or other transcription factors implicated in *RNR3* regulation such as Msn4p (Martinez-Pastor *et al.* 1996; Moskvina *et al.* 1998; Tadi *et al.* 1999) and Yox1p (Horak *et al.* 2002). Interestingly, overexpression of Msn4p, a factor involved in the general stress response, results in a sixfold increase in constitutive *RNR3* RNA levels (Gasch *et al.* 2000). The roles of these factors in the DNA damage and replication stress responses, as well as their relationships to the Crt1/Tup1/Ssn6 complex, are not known, but it is apparent that multiple factors including Wtm1p and Wtm2p are involved in regulating *RNR3* expression. While it is possible that *WTM1* and *WTM2* function in different cellular processes when overexpressed *vs.* normally expressed, the simplest explanation is that they function in the same process in either condition.

One other possibility that we considered is that perturbation of *WTM* levels could, by interfering with chromatin structure or some aspect of DNA metabolism, induce a DNA damage response and indirectly lead to increased *RNR3* transcription. However, we think that this possibility is unlikely for several reasons. Most importantly, highly expressed Wtm2p localizes to the *RNR3* gene (Figure 4). Furthermore, a *DIN7-lacZ* reporter that is normally induced by DNA damage (Ruby and Szostak 1985) is unaffected by *WTM1* and *WTM2* overexpression (Figures 1). Finally, the transcriptional upregulation of *RNR3-lacZ* by *WTM1* and *WTM2* overexpression is unaffected by the *rad53-21* mutation that effectively disables the known pathway of the DNA damage and replication stress response, including *RNR3* induction, by blocking kinase activity (Allen *et al.* 1994; Craven and Petes 2000) (Figure 1B).

The combined results from experiments in which the two *WTM* genes are either overexpressed or deleted suggest that these genes are normally involved in modulating expression of the *RNR* genes. *WTM* overexpression triggers increases in *RNR3* and *RNR2* expression in unstressed cells (Figures 1, 3, and 6), while deletion reduces *RNR2* and *RNR3* induction by hydroxyurea (Figure 5) and *RNR3* induction by ionizing radiation (data not shown). As *RNR2* and *RNR3* share some regulatory factors such as Crt1p, a common regulatory role

by the Wtm proteins is plausible. *RNR1*, previously thought to be regulated differently than the other *RNR* genes (Huang *et al.* 1998), does not respond to *WTM1* and *WTM2* overexpression, but it is responsive to the *WTM* genes as their deletion increases *RNR1* expression (Figure 6). Deletion and overexpression of other regulatory factors in yeast often, but not always, result in reciprocal effects on target gene expression (Herrgard *et al.* 2003). Therefore, the simplest explanation is that *WTM1* and *WTM2* normally modulate expression of the *RNR* genes. Furthermore, their regulatory role is more global than that of the *RNR* genes, as they also affect *IME2* expression and transcriptional silencing of some genes (Pemberton and Blobel 1997). Finally, at least in the case of the *RNR2* and *RNR3* genes, *WTM1* and *WTM2* also normally amplify expression in response to inhibited replication as their deletion reduces induced levels. Additional work is necessary to determine the extent of the roles of the two *WTM* genes in the transcriptional responses to replication stress and DNA damage.

Our evidence indicates that Wtm2p localizes to the upstream region of *RNR3* and increases *RNR3* transcription when highly overexpressed, even in the absence of Wtm1p. Lower-level overexpression of *WTM2* has only a small effect on *RNR3* reporter activity; however, this activity is dramatically enhanced by overexpression of *WTM1*. These data suggest that the two proteins normally cooperate, yet each has a distinct function. Indeed, a previous study has shown that these two proteins copurify in a large, nuclear complex (Pemberton and Blobel 1997; Ho *et al.* 2002). Furthermore, two independent recent reports showed that Wtm1p associates with and retains a complex of Rnr2/Rrn4 subunits in the nucleus in normal yeast cells and shuttles this complex between the nucleus and cytoplasm in response to DNA damage or replication inhibition (Lee and Elledge 2006; Zhang *et al.* 2006). Wtm2p also associates with this complex, but a *wtm2* deletion has no effect on localization or shuttling of the complex. Furthermore, Lee and Elledge (2006) observed that when Wtm2p is overexpressed under control of a *GAL* promoter, it increases the levels of Rrn2 and Rnr4 protein, consistent with our observations here that overexpression of *WTM2* increases *RNR2* and *RNR3* expression. The combined results suggest that Wtm2p has a function different from that of Wtm1p. Intriguingly the only remarkable difference in sequence between the two proteins is a stretch of 14 charged amino acids, 13 of which are either aspartate or glutamate. This stretch is located between the first and second putative WD repeats of Wtm2p and may have a functional role as it has been conserved among some other ascomycetes retaining the two paralogs (Gish and States 1993; Balakrishnan *et al.* 2005).

Although our results indicate that Wtm2p can increase transcription, previous work with fusion proteins suggested that Wtm1p and Wtm2p repress transcription (Pemberton and Blobel 1997). It could be that nonspecific steric hindrance is responsible for the repressive activity of Wtm-LexA fusion proteins when targeted to a reporter gene by LexA binding. However, chromatin remodeling complexes such as Swi/Snf and Rvb1/Rvb2 have also been observed to act as both positive and negative regulators, depending on context (Sudarsanam *et al.* 2000; Jonsson *et al.* 2001). The Wtm proteins may also have this type of dual activity: they behave like positive regulators of silenced genes at *HM* loci and telomeres, but like negative regulators of the meiotic gene *IME2* (Pemberton and Blobel 1997) and *RNR1*. While Wtm2p acts as a positive regulator at the *RNR3* locus, Wtm1p and Wtm2p could potentially interact with chromatin in a manner that represses transcription at other loci.

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