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Distinct Roles of Yeast *MEC* and *RAD* Checkpoint Genes in Transcriptional Induction after DNA Damage and Implications for Function

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In eukaryotic cells, checkpoint genes cause arrest of cell division when DNA is damaged or when DNA replication is blocked. In this study of budding yeast checkpoint genes, we identify and characterize another role for these checkpoint genes after DNA damage–transcriptional induction of genes. We found that three checkpoint genes (of six genes tested) have strong and distinct roles in transcriptional induction in four distinct pathways of regulation (each defined by induction of specific genes). *MEC1* mediates the response in three transcriptional pathways, *RAD53* mediates two of these pathways, and *RAD17* mediates but a single pathway. The three other checkpoint genes (including *RAD9*) have small (twofold) but significant roles in transcriptional induction in all pathways. One of the pathways that we identify here leads to induction of *MEC1* and *RAD53* checkpoint genes themselves. This suggests a positive feedback circuit that may increase the cell's ability to respond to DNA damage. We make two primary conclusions from these studies. First, *MEC1* appears to be the key regulator because it is required for all responses (both transcriptional and cell cycle arrest), while other genes serve only a subset of these responses. Second, the two types of responses, transcriptional induction and cell cycle arrest, appear distinct because both require *MEC1* yet only cell cycle arrest requires *RAD9*. These and other results were used to formulate a working model of checkpoint gene function that accounts for roles of different checkpoint genes in different responses and after different types of damage. The conclusion that the yeast *MEC1* gene is a key regulator also has implications for the role of a putative human homologue, the ATM gene.

INTRODUCTION

Regulatory controls called checkpoints arrest cell division following damage to either chromosomes or spindle-associated structures (Hartwell and Kastan, 1994). Arrest allows the cell time for repair before further cell cycle progression and division. The DNA-responsive checkpoints, sensitive to DNA damage or blocked replication, act in multiple phases of the cell cycle (G1, S, or G2 phases; Siede *et al.*, 1993; Weinert and Hartwell, 1993; Allen *et al.*, 1995; Paulovich and Hartwell, 1995). Checkpoint genes have been genetically dissected in fission and budding yeasts (Murray, 1992; Sheldrick and Carr, 1993), and in budding yeast include seven genes to date: *RAD9*, *RAD17*, *RAD24*, *MEC1*, *MEC3*, *RAD53*, and *POL2* (Weinert and Hartwell, 1988; Weinert and Lydall, 1993; Weinert *et al.*, 1994; Siede *et al.*, 1993; Allen *et al.*, 1995; Navas *et al.*, 1995). How checkpoint gene products mediate the cell's response to DNA damage is not understood.

DNA damage causes changes in gene expression as well as in cell cycle progression. Here we define roles for yeast checkpoint genes in gene expression, namely the transcriptional induction of damage-inducible genes. Damage-inducible genes have been identified in most cell types, including bacteria, mammals, and yeast (McClanahan and McEntee, 1984; Ruby and Szostak, 1985; Witkin, 1991; Gottlieb and Jackson,

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1994) and often encode proteins known to be involved in DNA repair. In budding yeast, these genes include *RAD2* (excision repair; Robinson *et al.*, 1986), *RAD54* (recombinational repair; Cole *et al.*, 1987), *CDC9* (DNA ligase; Johnston and Nasmyth, 1978), *RNR2* (small subunit of ribonucleotide reductase; Elledge and Davis, 1989a,b; Elledge and Davis, 1990), and *RNR3/DIN1* (large subunit of ribonucleotide reductase; Elledge and Davis, 1989a; Yagle and McEntee, 1990). Other damage-inducible genes have functions whose relevance to DNA repair is unclear (e.g., *UBI4*, protein degradation; Treger *et al.*, 1988). The transcriptional induction of damage-inducible genes represents another mechanism that, like cell cycle arrest, serves to augment DNA repair.

Although the mechanisms underlying arrest and transcriptional induction after DNA damage in eukaryotic cells are not well understood, those in bacteria, called the SOS response, are well understood (Witkin, 1991). Certain themes in this bacterial paradigm seem relevant for the eukaryotic responses. In bacteria, the recA gene is required for both transcriptional and cell division responses, which are mechanistically linked; the transcriptional response (RecA-mediated, transcriptional derepression of an inhibitor) results in arrest of cell septation. In eukaryotic cells, specific genes also mediate both the transcriptional and cell cycle responses (e.g., p53 in mammalian cells (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992) and *MEC1* and *RAD53* in budding yeast (Weinert *et al.*, 1994; Allen *et al.*, 1995; this report). However, the two responses are not obligatorily linked in eukaryotic cells. At least for the G2 checkpoint in yeast, transcriptional induction is not required for arrest after DNA damage; arrest can occur in the presence of a protein synthesis inhibitor, cycloheximide (Weinert and Hartwell, 1990). Thus, how specific genes mediate multiple and apparently distinct responses is unknown.

Here, we define which yeast checkpoint genes mediate transcriptional induction. We identify the surprisingly complex roles of six checkpoint genes in transcriptional induction of five damage-inducible genes and show that the multiple pathways of induction include the checkpoint genes themselves. Although the responses are large in number and appear genetically complex, there is a surprisingly regular pattern of regulation, with respect to roles of checkpoint genes and types of DNA damage. These results, coupled with our other observations that some checkpoint gene products process DNA damage (Lydall and Weinert, 1995), lead to a working model of checkpoint gene functions.

MATERIALS AND METHODS

Yeast and Bacterial Strains

Strains used in this study are shown in Table 1. Yeast media and genetic methods were standard (Sherman *et al.*, 1986). Most experiments were performed in cells grown in YEPD, rich media. Plasmids were propagated in the bacterial strain DH5α and introduced into yeast cells by lithium acetate transformation (Schiestl and Gietz, 1989; Ausubel *et al.*, 1990).

Strain Constructions

In some experiments, we used strains that contained a deletion of the *RAD16* gene to enhance the level of transcriptional induction. *RAD16* was deleted by transplacement with a *rad16Δ::URA3* fragment (from pBLY22; Schild *et al.*, 1992), and the genomic structure of the Rad$^-$ Ura$^+$ transformants was confirmed by Southern analysis (Ausubel *et al.*, 1990). Strains containing null alleles of *RAD17*, *RAD24*, and *MEC3* will be described elsewhere (Lydall and Weinert, 1995). *rad9Δ* strains, marked with either *LEU2* or *HIS3*, were previously described (Weinert and Hartwell, 1988, 1990).

Table 1. Strains used in this study

Strain[a]	Genotype
TWY397[b]	MATa his7 leu2 trp1 ura3
GKY997-5-4	MATα cdc28-1 rad16Δ::URA3 his7 ura3
TWY177	MATa mec1-1 his3 leu2 trp1 ura3
GKY952	MATa mec1-1 rad16Δ::URA3 his3 leu2 trp1 ura3
TWY180	MATα mec3-1 his7 trp1 ura3
GKY997-21-3	MATa mec3-1 rad16Δ::URA3 his7 trp1 ura3
GKY974-1-2	MATa mec3ΔG::URA3[c] rad16Δ::URA3 leu2 his3 trp1 ura3
GKY976-12-2	MATa mec3ΔG::URA3[c] rad9Δ::LEU2 rad16Δ::URA3 leu2 his3 trp1 ura3
TWY127	MATa rad9Δ::URA3 ade2 can1 sap3 trp1 ura3
TWY398	MATa rad9Δ::LEU2 his7 leu2 trp1 ura3
DLY195[c]	MATα rad9Δ::HIS3 his3 leu2 trp1 ura3
GKY942	MATa rad9Δ::LEU2 rad16Δ::URA3 his7 leu2 trp1 ura3
GKY978-27-3	MATα rad9Δ::LEU2 rad16Δ::URA3 rad17Δ::LEU2 leu2 his3 trp1 ura3
GKY978-30-4	MATα rad9Δ::LEU2 rad16Δ::URA3 rad17Δ::LEU2 leu2 his7 trp1 ura3
GKY975-10-2	MATα rad9Δ::LEU2 rad16Δ::URA3 rad24Δ::TRP1 leu2 his3 trp1 ura3
GKY941	MATa rad16Δ::URA3 his7 leu2 trp1 ura3
GKY954	MATa rad16Δ::URA3 rad17-1 his7 leu2 trp1 ura3
GKY977-5-2	MATα rad16Δ::URA3 rad17Δ::LEU2 leu2 his3 trp1 ura3
GKY977-1-3	MATα rad16Δ::URA3 rad17Δ::LEU2 leu2 his3 trp1 ura3
GKY998-1-1	MATa rad16Δ::URA3 rad24-1 leu2 trp1 ura3
GKY973-1-4	MATα rad16Δ::URA3 rad24Δ::TRP1 leu2 his3 trp1 ura3
GKY944	MATα rad16Δ::URA3 rad53[d] trp1 ura3
GKY953	MATa rad16Δ::URA3 rad53[d] his7 trp1 ura3
TWY323	MATa rad17-1 his7 leu2 trp1 ura3
DLY196[c]	MATa rad17Δ::LEU2 his3 leu2 trp1 ura3
TWY300	MATα rad24-1 his7 leu2 trp1 ura3
TWY312	MATa rad53[d] his7 trp1 ura3
TWY178	MATα rad53[d] trp1 ura3

[a] All strains were generated in this study and are congenic with A364a, unless otherwise noted;

[b] (Weinert *et al.*, 1994);

[c] (Lydall and Weinert, 1995);

[d] The *rad53* allele used here was identified previously as *mec2-1*.

Analysis of G2 Delay after DNA Damage

To determine cell cycle delay following UV irradiation, mid-log (1×10^6 to 8×10^6 cells/ml) haploid cells were synchronized by a 3-h incubation at 23°C with 100 μg/ml of the microtubule poison methyl benzimadazol-2yl-carbamate (MBC) (see below). The MBC-arrested cells were then plated on agar plates, UV irradiated at 40 J/m^2, washed twice with YEPD media to remove the MBC, and resuspended in liquid media. Aliquots were removed, fixed, and stained with 4,6-diamino-2-phenylimide; recovery from UV-induced G2 delay was thus determined by scoring the percentage of cells in each cell cycle phase by their cell/nuclear morphologies (Weinert and Hartwell, 1988, 1990; see below).

Transcriptional Induction Experiment

Transcriptional induction of specific genes was determined in cells under three growth conditions: cells growing asynchronously, cells arrested in the G1 phase, or cells arrested in the G2 phase. The rationale for these conditions is discussed in the RESULTS. Asynchronous cells were those grown in rich media to $2–5 \times 10^6$ cells/ml, G2 cells were isolated by synchronizing cells by incubation with 100 μg/ml of the microtubule inhibitor MBC (Sigma, St. Louis, MO; diluted in rich YEPD media from a 10 mg/ml stock in DMSO) for 3 h at 23°C, and G1 cells were isolated by synchronizing MAT**a** cells with 5×10^{-7} M α-factor mating pheromone for 2 h at 23°C. Cells were then collected by centrifugation and resuspended in a smaller volume of YEPD (rich) media, with continued presence of cell cycle inhibitors as noted. One-half of each culture, about 10^7 cells, was plated immediately on a YEPD agar plate and UV irradiated once at 80 J/m^2 using a Stratagene Stratalinker 1800, or treated in liquid with 0.01% methyl methanesulfonate (MMS; Sigma), treated with 0.2 M hydroxyurea (HU; Sigma), or left untreated. After UV irradiation, cells were washed off the plate into liquid YEPD media, again with cell cycle inhibitors as noted. Following a subsequent 2-h incubation at 23°C, aliquots of each culture were collected for cell/nuclear morphology and cell viability assays, and RNA purification.

We found that the density of cells on plates for UV irradiation was important. For example, there was a two to threefold difference between the UV-induced transcript levels measured from wild-type cells plated at cell densities differing fivefold in cell number (2.1-fold induction when cells were plated at 10^7 cells per plate versus no increase above basal level when cells were plated at a density of 5×10^7 cells per plate). The difference in induction levels was not reflected in cell viabilities, which were similar at the two cell densities (40% and 45%, respectively). Therefore, we were careful to plate the same number of cells within any given experiment (within twofold).

Note that in the experiment shown in Figure 2A, *rad16Δ* and *rad16Δrad9Δ* mutants have similar absolute levels of *RNR3* induction. However, because the basal level of *RNR3* mRNA in the *rad16Δrad9Δ* mutant was higher than in the *rad16Δ* mutant, the relative level of induction is actually twofold lower in *rad9Δ* mutants compared with *RAD9*. The results shown here for these two strains do not come from within a single experiment and several factors that contribute to *RNR3* transcript levels were not equivalent. Subsequent experiments controlled for these factors and showed that basal levels of *RNR3* expression do not vary significantly between the different strains analyzed and that cell plating density at the time of irradiation is important.

We determined the average induction ratios for mutant cells compared with wild-type cells using data taken from single experiments (same day growth and same time irradiation, RNA preparations, and blots). Although absolute induction levels did vary from experiment to experiment, the induction ratios of mutant strains compared with wild-type strains remained constant. For example, UV-specific *RNR3* induction ratios for *rad16ΔMEC$^+$*, *rad16Δmec1–1*, and *rad16Δrad9Δ*, respectively, were measured in four separate experiments as follows: 14.6, 0.9, 7.2; 16.5, 0.8, 9.1; 10.9, 1.0, 7.0; and, 10.2, 0.7, 5.7. These data show that absolute fold induction can vary, yet relative values vary little. We normalized data within each single experiment to an average wild-type strain value (representing two to seven experiments). Using the above data example, the average *RNR3* induction ratio for *rad16Δ MEC$^+$* is 13.05; therefore, the induction ratios for the mutant strains in the first experimental set would be each multiplied by 13.05/14.6, the second set by 13.05/16.5, etc. Averages (and the standard deviation from these) of the relative induction ratios for the checkpoint mutant strains in two to seven experiments are presented in Table 2. Normalization accounts for the twofold differences in absolute induction levels between experiments, and allows us to demonstrate a statistical significance to the twofold differences in relative induction ratios between wild-type and mutant strains for certain types of DNA damage.

Importantly, the basal levels between different strains within one experiment typically do not vary by more than 10%. Therefore, comparing induction ratios between strains is essentially comparing the difference in yield of transcript induced by damage. Note that induction levels reported as ≤1.0, like those seen in *mec1* above, mean that induced levels are not significantly different from basal levels and that, because *DDR48* is not induced well by UV irradiation, we present only the *DDR48* transcriptional response to HU and MMS treatment.

RNA Preparation and Analysis

We prepared RNA by the glass bead method (Caponigro *et al.*, 1993) and separated total RNA on denaturing formaldehyde gels. Northern blot analysis used RNA transferred to Zetaprobe GT membranes (Bio-Rad, Richmond, CA) (Thomas, 1980), followed by hybridization with several DNA probes that were ^{32}P-labeled by the random hexamer-priming method (Feinberg and Vogelstein, 1983). mRNA levels were quantitated by counting β decays emitted from each band with a Betascope (Herrick *et al.*, 1990), subtracting the background counts, and dividing by the counts in a constitutive transcript band, from which background had also been subtracted. Normalization was to the levels of the constitutively expressed *PRT1* gene, which encodes protein 1 (Hereford *et al.*, 1981; Weinert and Hartwell, 1990), and/or to the levels of the constitutively expressed *URA3* gene (Zhou and Elledge, 1992).

The control transcript probe *PRT1* (and a cell cycle-regulated transcript probe *H2A*) were isolated on a single *Sac*I fragment from YpTRT1 (Meeks-Wagner and Hartwell, 1986). The 2.0-kb *UBI4* and 2.1-kb *DDR48* transcript probes were obtained by *Hin*dIII digest of pKHUbi4 and pBR48, respectively (McClanahan and McEntee, 1986; Treger *et al.*, 1988). *RNR3*/*DIN1* was probed using a 1.7-kb *Bam*HI fragment of pBR1600 (Yagle and McEntee, 1990). The checkpoint gene transcripts were identified by probes as follows: *RAD9*, a 4.0-kb *Pvu*II fragment of pTW039 (Weinert and Hartwell, 1988); *RAD17*, a 1.9-kb *Bam*HI/*Xba*I fragment of pDL179 (Lydall and Weinert, 1995); *RAD24*, a 1.5-kb *Hin*dIII/*Bgl*II fragment of pRSRAD24 (Lydall and Weinert, 1995); *MEC1*, a 7.5-kb *Sac*I/*Sal*I fragment of pRSMEC1 (Gardner and Weinert, unpublished observations); and *RAD53*, a 2.9-kb *Eco*RI fragment of pSK35 (Kim and Weinert, unpublished data). All fragments were purified from an agarose gel plug using GeneClean, by the manufacturer's specifications (Bio101, La Jolla, CA).

Induction in cdc28–1 Mutants

Asynchronous mid-log cultures of *rad16Δ* (GKY941) and *rad16Δcdc28–1* (GKY997–5-4) mutants were split in half–one-half was kept at 23°C and the other was shifted to 36°C (the restrictive temperature for the *cdc28–1* allele) for a 2-h incubation before UV irradiation. Cells were collected by centrifugation and each of these cultures was then divided in half again: half remained untreated and the other was UV irradiated as described above (cultures from 36°C were plated onto prewarmed plates for irradiation), and then maintained at the permissive or restrictive temperatures following

Table 2. Relative transcriptional induction ratios (induced level divided by basal level; a value of ≤1.0 represents no induction) following (A) UV-induced damage or (B) HU- and MMS-induced damage. For all the UV-induced damage experiments (A), the strains were in a *rad16Δ* background to enhance the UV-induced signal. All MMS- and HU-induced damage experiments used *RAD16*$^+$ strains. We have also analyzed transcriptional induction in checkpoint null alleles with similar results in response to UV-damage (*RNR3* induction ratios of 5.2, 4.9, and 3.0 for *rad16Δrad17Δ*, *rad16Δrad24Δ*, and *rad16Δmec3ΔG*, respectively).

Table 2A. Transcript induction ratio from UV damage

	Class B					Class A	
	RNR3			*UBI4*		*MEC1*	*RAD53*
Genotype	cycling	G1	G2	cycling	G1		
MEC$^+$ *RAD16*$^+$	4.0 ± 0.3	3.0 ± 0.3	ND	ND	ND	6.2 ± 1.2	ND
MEC$^+$ *rad16Δ*	13.1[a]	8.3[a]	11.4[a]	14.8[a]	7.0[a]	9.1[a]	5.2[a]
mec1-1 *rad16Δ*	0.9 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	12.0 ± 1.9	7.3 ± 0.6	0.8 ± 0.3	1.4 ± 0.3
rad53 *rad16Δ*	0.8 ± 0.3	0.8 ± 0.2	0.9 ± 0.3	12.4 ± 1.5	7.8 ± 0.8	3.7 ± 0.5	3.6 ± 0.5
rad17-1 *rad16Δ*	6.1 ± 0.9	4.2 ± 0.2	5.0 ± 0.5	5.0 ± 0.8	ND	3.5 ± 1.2	3.6 ± 1.0
rad9Δ *rad16Δ*	7.4 ± 0.8	3.4 ± 0.2	5.6 ± 0.7	8.6 ± 0.8	4.5 ± 0.4	4.1 ± 1.2	3.6 ± 0.5
rad24-1 *rad16Δ*	5.1 ± 0.6	3.4 ± 0.2	4.3 ± 0.1	8.5 ± 0.6	ND	3.9 ± 1.8	ND
mec3-1 *rad16Δ*	4.8 ± 1.1	2.4 ± 0.2	4.2 ± 0.2	5.4 ± 0.7	ND	ND	3.6 ± 0.8

Table 2B. Transcript induction ratio from HU and MMS damage

	Class B				Class C	
	RNR3		*UBI4*		*DDR48*	
Genotype	HU	MMS	HU	MMS	HU	MMS
MEC$^+$ *RAD16*$^+$	5.0[a]	5.1[a]	2.3[a]	4.6[a]	3.1[a]	2.6[a]
mec1-1 *RAD16*$^+$	0.7 ± 0.3	1.4 ± 0.5	2.2 ± 0.1	4.5 ± 1.0	1.0 ± 0.1	0.8 ± 0.2
rad53 *RAD16*$^+$	ND	0.9 ± 0.2	ND	5.6 ± 1.2	ND	0.8 ± 0.3
rad17-1 *RAD16*$^+$	5.6 ± 1.1	ND	2.0 ± 0.4	3.6 ± 0.4	0.6 ± 0.5	0.9 ± 0.1
rad9Δ *RAD16*$^+$	5.0 ± 0.1	2.4 ± 0.5	2.4 ± 0.1	4.0 ± 0.1	2.2 ± 0.1	2.0 ± 0.1
rad24-1 *RAD16*$^+$	4.2 ± 0.5	2.7 ± 0.4	2.2 ± 0.1	3.1 ± 0.6	2.4 ± 0.6	ND
mec3-1 *RAD16*$^+$	ND	2.9 ± 0.4	ND	4.7 ± 1.0	ND	2.3 ± 0.4

Levels of RNA were determined by counting β decays in the bands on a Northern as described in MATERIALS AND METHODS. Samples were taken either from cycling cells unless otherwise stated, from cells arrested and maintained in G1 by α-factor treatment, or from cells arrested and maintained in G2 by MBC treatment. In each column of each table, averages of normalized data from two to seven experiments are presented. Data were normalized as discussed in MATERIALS AND METHODS. ND, not determined. Strains used here include: TWY397 (*MEC*$^+$*RAD16*$^+$), GKY941 (*MEC*$^+$*rad16Δ*), TWY177 (*mec1-1*), GKY952 (*rad16Δmec1-1*), TWY312 (*rad53*), GKY953 (*rad16Δrad53*), TWY398 (*rad9Δ*), GKY942 (*rad16Δrad9Δ*), TWY323 (*rad17-1*), GKY954 (*rad16Δrad17-1*), TWY300 (*rad24-1*), GKY998-1-1 (*rad16Δrad24-1*), TWY180 (*mec3-1*), and GKY997-21-3 (*rad16Δmec3-1*).

[a] Denotes values that represent the average transcript induction ratio of the non-checkpoint mutant strain (either *MEC*$^+$ or *rad16Δ*) and are the standard to which all values within that column were normalized (see MATERIALS AND METHODS).

UV irradiation. Samples of each culture were taken after 2 h for RNA preparation, and cell viability and cell/nuclear morphology assays.

Cell Viability and Cellular/Nuclear Morphology Assays

Cells exposed to various mutagens were streaked for single cells on YEPD agar plates and the percentage of cells (out of at least 100) forming microcolonies of about 50 cell bodies or more after about 24 h at 23°C was determined.

We determined nuclear and cell morphologies by fluorescence and light microscopy of cells fixed in 70% ethanol for at least 1 h at 4°C, washed three times with water, sonicated, and stained with 4,6-diamino-2-phenylimide (Pringle *et al.*, 1989). At least 100 cells were analyzed per sample and cell cycle phase-associated morphologies were classified as unbudded (G1 phase), small budded (S phase), large budded with an undivided nucleus (G2 phase), or large budded with a divided nucleus (post-anaphase) (Weinert and Hartwell, 1990).

RESULTS

Pathways of Cell Cycle Arrest

Previous studies described the roles of specific genes in S phase arrest following a block to DNA replication or in G2 phase arrest following DNA damage (Weinert *et al.*, 1994). A view of these pathways is shown in Figure 1 (adapted from Weinert *et al.*, 1994). For the purposes of this study, we refer collectively to the *RAD9*, *RAD24*, *RAD17*, and *MEC3* genes as the G2-specific checkpoint genes, as they form a class of genes distinct from *MEC1* and *RAD53* in both cell cycle arrest and transcriptional regulation phenotypes. However, the G2-specific checkpoint genes probably all act in the G1 checkpoint as well (Siede *et al.*, 1993, 1994).

Characterization of Transcriptional Regulation Pathways

We chose to analyze roles of checkpoint genes in transcriptional regulation of the *RNR3* gene. *RNR3* encodes a subunit of ribonucleotide reductase, an enzyme involved in DNA metabolism (Elledge and Davis, 1990; Yagle and McEntee, 1990), and has the following properties: 1) its transcript has low basal mRNA levels with relatively high inducible levels (Ruby and Szostak, 1985; Elledge and Davis, 1990); 2) it is not induced by general cell stress like heat shock or high cell density, which do induce other transcripts (Ruby and Szostak, 1985; Yagle and McEntee, 1990); and 3) increased levels of *RNR3* transcript following damage are probably due to elements in the promoter and not to decreased RNA degradation, because the promoter region confers similar induction properties to heterologous genes (Yagle and McEntee, 1990; Zhou and Elledge, 1992).

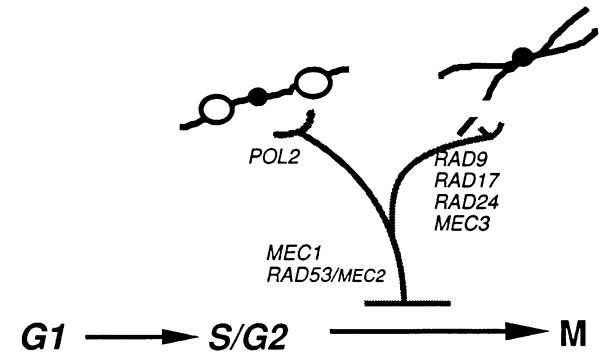

Figure 1. Roles of checkpoint genes in cell cycle arrest. The roles of specific genes in arrest at the S phase checkpoint in cells blocked in DNA replication and at the G2 checkpoint in cells with DNA damage. *MEC1* and *RAD53* are required for arrest at both checkpoints. *RAD53* was previously identified independently as *MEC2* by us, and *SPK1* and *SAD1* by other researchers (see text).

In our initial studies, we found that our wild-type strains supported only a fourfold induction of *RNR3* transcription after UV irradiation. We reasoned that we might enhance the transcriptional induction signal by slowing the cell's ability to repair UV lesions, and repair mutants defective in either dimer incision or excision might delay repair sufficiently to enhance transcriptional induction. Therefore, we screened a panel of incision/excision repair mutants (*rad1*, *rad2*, *rad4*, *rad10*, *rad7*, *rad12*, *rad14*, and *rad16*; Friedberg, 1985) and found that indeed *rad16* mutants, defective in excision repair (Friedberg, 1985), showed a three- to fivefold increase in transcript level, compared with that seen in wild-type cells. We obtained similar qualitative results after UV in *RAD16*$^+$ strains but the levels of induction did not allow a reliable assessment of subtle effects (our unpublished observations). A *rad16* mutation was therefore introduced into checkpoint mutant cells for studies using UV damage (see MATERIALS AND METHODS).

Upon evaluation of the time course of *RNR3* induction, we found that 2 h of post-irradiation time yielded the highest level of transcript in all three strains tested, including the *rad16* mutant, the *rad9rad16* double mutant, and the *RAD16*$^+$ wild-type strains (Figure 2A). For subsequent studies comparing levels between wild-type and mutants cells, we therefore measured transcript levels at 2 h post-UV and compared the level of transcript to that seen in unirradiated cells.

We imagine that the defect in DNA repair introduced by the *rad16* mutation increases the level of transcript by allowing DNA breaks to accumulate. If DNA breaks do accumulate, arrest at the G2 checkpoint should be extended in *rad16* mutants compared with wild-type cells. We tested this prediction by analyzing the extent of delay at the G2 checkpoint after UV irradiation (see MATERIALS AND METHODS). *rad16* mutants indeed had a longer delay at the G2 checkpoint than did wild-type cells, and the delay is completely checkpoint dependent (i.e., the delay is abolished in *rad9* mutants; Figure

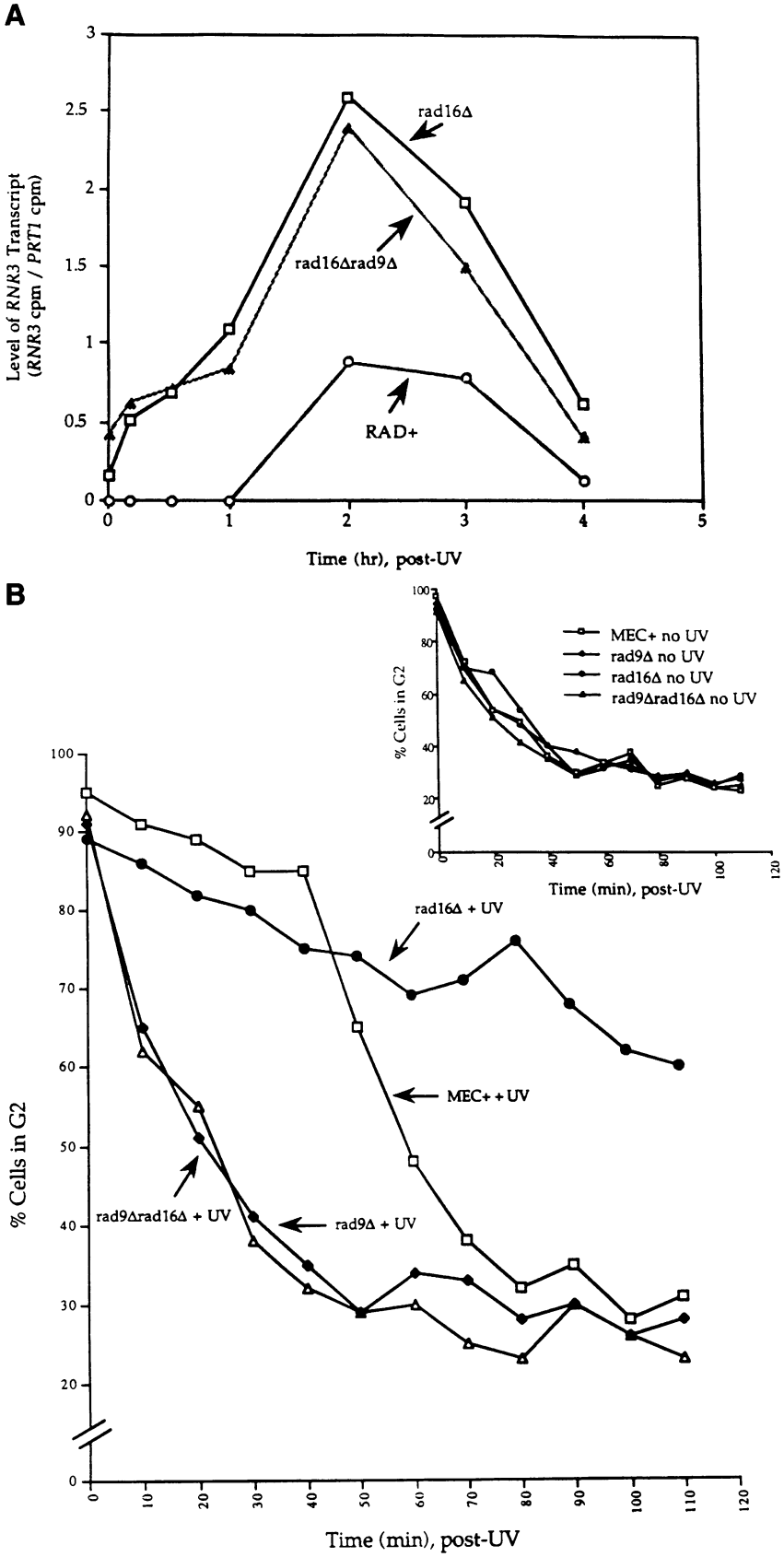

Figure 2. The transcription and cell cycle arrest responses to DNA damage. (A) Graph of *RNR3* transcript levels following UV irradiation in several strains, determined from counted β decays in Northern blot bands as described in legend for Figure 3. Results shown are from individual experiments and are consistent with other results, with respect to the kinetics of transcriptional induction. Following UV irradiation at 80 J/m^2, samples were taken at several time points. The data used for Figure 2A were not collected in a single experiment nor were they controlled for cell number; therefore, the relative levels of induction between the strains are not typical for these strains (see MATERIALS AND METHODS). (B) Graph of UV-induced cell cycle delay. *MEC*$^+$*rad16Δ* and *rad16Δrad9Δ* cells were arrested in G2 by MBC treatment, plated, and UV irradiated at 40 J/m^2 in the light. The progression of cells out of G2, following removal of MBC, was assessed by analysis of nuclear morphology of cells fixed and stained for DNA morphology. The percentage of cells with an undivided nucleus in the G2 phase is shown. The results of a single experiment are shown and were similar to at least two other experiments. Unirradiated controls for each strain exhibited no delay (see inset). Strains are: *MEC*$^+$*RAD16*$^+$ (TWY397), *MEC*$^+$*rad16Δ* (GKY941), *rad9Δ* (TWY127), and *rad16Δrad9Δ* (GKY942).

2B; Weinert and Hartwell, 1993). The extended delay also indicates that the *RAD16* gene is not essential for the checkpoint and validates the use of a *rad16* mutation to increase the sensitivity of the transcriptional assays. The relative kinetics of arrest and transcription seen in Figure 2, A and B, are addressed in the DISCUSSION.

Roles for MEC1 and RAD53 in Damage-inducible Transcription of RNR3

Using the enhanced sensitivity of *rad16* mutants, we measured the *RNR3* transcript levels in checkpoint mutant and wild-type cells following UV irradiation. Checkpoint-proficient, Rad16p-deficient (i.e., wild type, with respect to checkpoint genes) cells induced *RNR3* about 10-fold above basal levels following UV irradiation (Figures 2A and 3; Table 2A). In contrast, *mec1–1* and *rad53* mutants were defective for the induction of *RNR3* transcription by UV damage and by other DNA damaging agents as well (discussed below). The defect in transcription of *RNR3* was not due to an altered time course of induction because we did not see *RNR3* transcript in mutant cells within 10 min or 2 h after induction, nor did we see *RNR3* transcripts induced for up to 6 h post-UV in an extensive analysis of *rad16Δrad53* mutants (our unpublished observations). The failure to induce *RNR3* transcripts was also not simply a consequence of low viability of mutant cells because the *UBI4* transcript was successfully induced by the same dose of UV in both *rad16Δmec1–1* and *rad16Δrad53* mutant cells (Table 2A). (The *rad53* mutant allele used in these studies was identified previously as *mec2–1*; Weinert *et al.*, 1994. Because *RAD53* has now been isolated independently and given four different names, we use the initial gene designation *RAD53* to minimize further confusion).

In contrast to the important roles played by *MEC1* and *RAD53*, the G2-specific genes (*RAD9*, *RAD24*, *MEC3*, and *RAD17*) had only a minor role in *RNR3* induction. The UV-induced level of *RNR3* transcript was reproducibly reduced about twofold in *rad16Δrad9Δ*, *rad16Δrad17–1*, *rad16Δrad24–1*, and *rad16Δmec3–1* mutants compared with checkpoint-proficient cells (Table 2A; Figure 3). We asked whether this twofold reduction in *RNR3* transcription was due either to residual activity of the non-null alleles or to functional redundancy among the G2-specific genes. We tested null mutants of the four G2-specific checkpoint genes and also found a twofold reduction in *RNR3* transcript levels, similar to that seen in the presumed point mutants, compared with wild-type cells (see legend to Figure 3). A similar twofold reduction in transcript abundance after UV was seen in all double checkpoint-mutant combinations tested. The twofold effect on transcriptional regulation in G2-specific checkpoint mutants thus appears to be due to a pathway common to all four genes. (The specific double checkpoint mutants tested included *rad16Δrad9Δrad17Δ* *rad16Δrad9Δrad24Δ*, and *rad16Δrad9Δmec3Δ* because, by other criteria, *RAD9* and the other three G2-specific genes are in distinct DNA repair epistasis groups; Lydall and Weinert, 1995).

To test whether the *MEC1* and *RAD53* transcriptional roles are specific to UV damage, we examined mutants for transcriptional induction of *RNR3* in response to other DNA damaging agents–MMS, an alkylating agent, and HU, an inhibitor of ribonucleotide reductase that blocks DNA replication (Harder and Follmann, 1990). We examined transcriptional induction by MMS or HU treatment in *RAD16*$^+$ cells, because *RAD16* is not involved in repair of MMS- or HU-induced damage. As in response to UV damage, *mec1* and *rad53* mutants failed to induce *RNR3* in response to MMS or HU treatment. In MMS, the *rad9*, *rad17*, *rad24*, and *mec3* mutants induced *RNR3* to only slightly reduced levels; however, induction by HU occurred to levels similar to those seen in wild-type cells (Table 2B). *MEC1* and *RAD53* therefore appear to play general roles in transcriptional induction of *RNR3*, not restricted to a particular type of DNA lesion.

We considered the possibility that transcriptional defects in *mec1* and *rad53* mutants could be secondary consequences of the differences in cell cycle positions of wild-type and checkpoint mutant cells with DNA damage. Recall that wild-type cells with DNA damage arrest, while *mec1* and *rad53* cells do not. One possible consequence of a failure by mutant cells to arrest is that a single-stranded DNA break may be converted into a double-stranded DNA break by DNA replication. In this case, differences between cycling and arrested cells could be because the transcriptional response to different lesions differs. To rule out such indirect effects, we evaluated transcriptional induction in cells that were not cycling (synchronized in either the G1 or G2 phases) and found qualitatively similar results to those in cycling cells (Table 2A; our unpublished observations). The overall levels of transcript in damaged G1 cells were reduced (Table 2A; Elledge and Davis, 1990). We conclude that the transcriptional defects in *mec1* and *rad53* mutants are most likely due to mutant proteins and not to an indirect effect on the cell cycle.

Finally, the demonstration that *MEC1* and *RAD53* induced transcription in G1 cells (Table 2A) shows that each gene must function in the G1 phase (as well as in the S and G2 phases, as established previously; Weinert *et al.*, 1994). A role for *RAD53* in cell cycle arrest in G1 was also shown previously (Allen *et al.*, 1995).

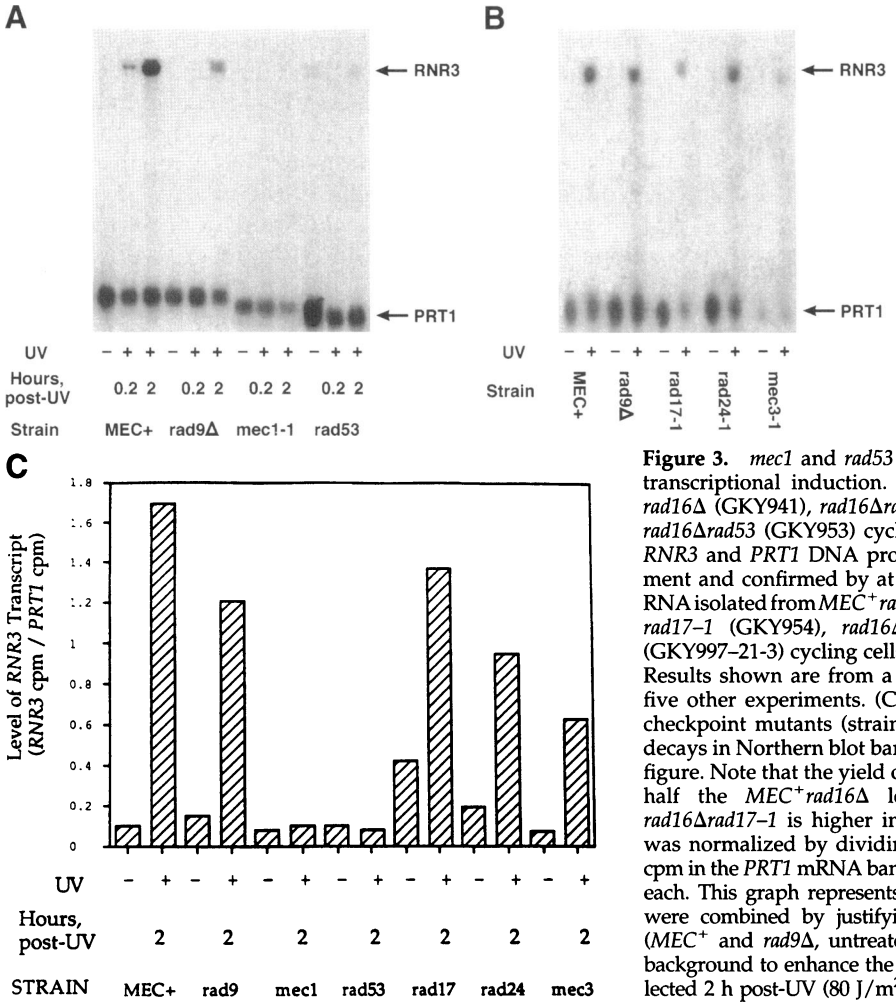

Figure 3. *mec1* and *rad53* mutants are defective for UV-induced *RNR3* transcriptional induction. (A) Northern of RNA isolated from *MEC*$^+$ *rad16*Δ (GKY941), *rad16*Δ*rad9*Δ (GKY942), *rad16*Δ*mec1–1* (GKY952), and *rad16*Δ*rad53* (GKY953) cycling cells UV irradiated and hybridized with *RNR3* and *PRT1* DNA probes. Results shown are from a single experiment and confirmed by at least five other experiments. (B) Northern of RNA isolated from *MEC*$^+$*rad16*Δ (GKY941), *rad16*Δ*rad9*Δ (GKY942), *rad16*Δ *rad17–1* (GKY954), *rad16*Δ*rad24–1* (GKY998–1-1), and *rad16*Δ*mec3–1* (GKY997–21-3) cycling cells UV irradiated and hybridized as in panel A. Results shown are from a single experiment and confirmed by at least five other experiments. (C) Graph of *RNR3* transcript levels in several checkpoint mutants (strains noted above), determined from counted β decays in Northern blot bands, like those shown in panels A and B of this figure. Note that the yield of induction in *rad16*Δ*rad17–1* is actually about half the *MEC*$^+$*rad16*Δ level because the level of uninduced in *rad16*Δ*rad17–1* is higher in this experiment. The *RNR3* transcript level was normalized by dividing the cpm in the *RNR3* mRNA band by the cpm in the *PRT1* mRNA band, after background cpms were subtracted from each. This graph represents the data from two separate experiments that were combined by justifying data points common to each experiment (*MEC*$^+$ and *rad9*Δ, untreated and irradiated). All strains were in *rad16*Δ background to enhance the UV-induced signal. Induced samples were collected 2 h post-UV (80 J/m^2), unless otherwise indicated.

Checkpoint-mediated Inducible RNR3 Transcription Does Not Require CDC28 Function

The *RNR3* transcriptional role of checkpoint genes provides a phenotype with which to evaluate the roles of other genes thought to mediate checkpoints. The p34$^{cdc2+/CDC28}$ has been implicated in checkpoint-mediated cell cycle arrest in many other organisms, including fission yeast, frogs, and mammals, although not in budding yeast (Enoch and Nurse, 1990; Nurse, 1990; Li and Murray, 1991; Murray, 1991, 1992; Amon *et al.*, 1992; Sorger and Murray, 1992; Heald *et al.*, 1993). An assay of a transcriptional role of *CDC28*, the fission yeast *cdc2*$^+$ gene homologue, is also reasonable because *CDC28* is known to regulate HO gene expression through phosphorylation of the Swi5p transcriptional activator (Moll *et al.*, 1991). We therefore tested whether induction of *RNR3* required *CDC28* and found that it did not; induction of *RNR3* transcripts after UV damage was similar in temperature-sensitive *cdc28–1* mutant cells grown at the permissive or restrictive temperature (23°C or 36°C, respectively; Figure 4).

Some Checkpoint Genes Are Transcriptionally Induced by DNA Damage

We tested whether the checkpoint genes themselves might be induced by DNA damage because, if so, they could be used to describe additional transcriptional pathways. We found that transcripts encoded by *MEC1*, *RAD53*, *RAD17*, and *RAD24* genes were each increased by UV irradiation, while the transcript from the *RAD9* gene was not (Figure 5, A and B). A previous study reported that the *RAD9* transcript was also not induced by x-irradiation (Weinert and Hartwell, 1990). The *MEC3* transcript remains to be analyzed. We have scanned the DNA sequences of these inducible genes for *cis*-acting sequences that might be essential for their transcriptional induction. Only *RAD53* contains an identifiable DRE (damage responsive element; Zheng *et al.*, 1993), a degenerate promoter ele-

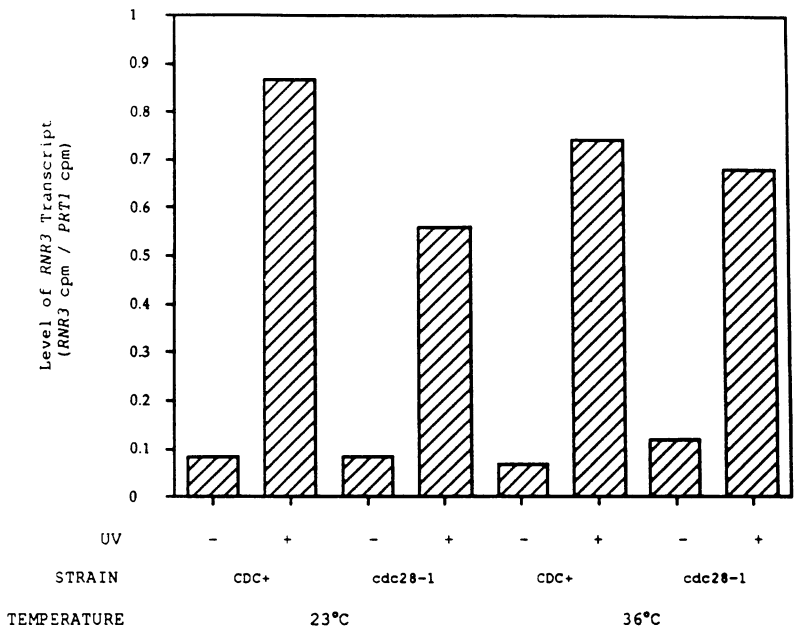

Figure 4. *RNR3* transcriptional induction following UV irradiation is not dependent on *CDC28*. Graph of *RNR3* transcript levels in *rad16Δcdc28–1* mutant (GKY997–5-4) and *rad16ΔCDC28*$^+$ (GKY941) cells, determined from counted β decays in Northern blot bands as described in legend for Figure 3. *CDC28* was inactivated before UV irradiation by a 2-h pre-incubation at 36°C, the restrictive temperature for the *cdc28–1* allele (78% of *rad16Δcdc28–1* cells were arrested in G1 after these two h) and cultures were maintained at 36°C for the duration of the experiment. Results shown are from a single experiment and were confirmed by two other experiments. Induced samples were collected 2 h post-UV (80 J/m^2). All strains were in *rad16Δ* background to enhance the UV-induced signal.

ment that has been implicated in DNA damage-inducible transcription of some genes, including *RAD2*, *RNR1*, *RNR2*, and *RNR3* (Elledge and Davis, 1989b; Yagle and McEntee, 1990; Siede and Friedberg, 1992). We have not as yet demonstrated that the increase in transcript levels of the checkpoint genes is due to increased transcription as opposed to a decrease in mRNA turnover. However, as shown using DNA damage-inducible promoter fusions, transcriptional induction of other damage-inducible genes is typically the result of increased transcription (including *RNR3* [Elledge and Davis, 1989a] and *UBI4* and *DDR48* [McEntee, personal communication]). The *cis*-acting elements involved in transcriptional induction of *MEC1*, *RAD17*, and *RAD24* remain to be identified.

The role of transcriptional induction of checkpoint genes seems in one sense puzzling because increased expression of proteins after DNA damage is not required for arrest at the G2 checkpoint (see INTRODUCTION). However, transcriptional induction is often associated with genes involved in DNA repair, suggesting checkpoint genes may play a role in DNA repair directly (a hypothesis that is addressed more explicitly elsewhere; Lydall and Weinert, 1995). Here, we note additional observations suggestive of roles some checkpoint genes may have in DNA repair. We found that *rad53* mutations had strong genetic interactions with *rad16Δ*, a DNA repair mutation. *rad16Δ rad53* double mutants showed a reduced cell viability without irradiation and greater UV sensitivity after irradiation, as compared with either single mutant (Table 3). We also identified more subtle genetic interactions between *rad16Δ* and two G2-specific gene mutants, *rad9Δ* and *rad17Δ*. Surprisingly, we saw no *rad16Δ* interaction with *mec1–1*. The nature of these genetic interactions is unknown; however, one explanation that we favor is that genetic interaction with *rad16Δ* reveals roles for some checkpoint genes in DNA repair. These putative repair pathways have not been defined, however.

Multiple Pathways of Checkpoint Gene-mediated Transcriptional Regulation

The *RNR3* transcriptional induction by *MEC1* and *RAD53* further distinguishes their roles from those of *RAD9*, *RAD17*, *RAD24*, and *MEC3*. To continue the dissection of checkpoint gene functions, we tested four additional damage-inducible genes, including *UBI4*, *DDR48*, *MEC1*, and *RAD53*. Previously, *UBI4* and *DDR48* were used to identify the transcriptional role of *DUN1* and *RAD53* (Zhou and Elledge, 1993; Allen *et al.*, 1995; Figure 6).

Transcriptional regulation of the four additional genes in six checkpoint mutants describes a remarkably complex yet regular pattern of regulation (Table 2, A and B, and results summarized in a working model presented in Figure 6). We found a total of four transcriptional pathways. One pathway was independent of all of our checkpoint genes and resulted in *UBI4* induction. Checkpoint genes regulated three pathways, resulting in induction of three distinct classes of transcripts, termed here Class A, B, and C transcripts. The Class A pathway required only the *MEC1* gene of the six checkpoint genes analyzed and resulted in *MEC1* and *RAD53* induction. The Class B pathway required both *MEC1* and *RAD53* and resulted in *RNR3* induction, whereas the Class C path-

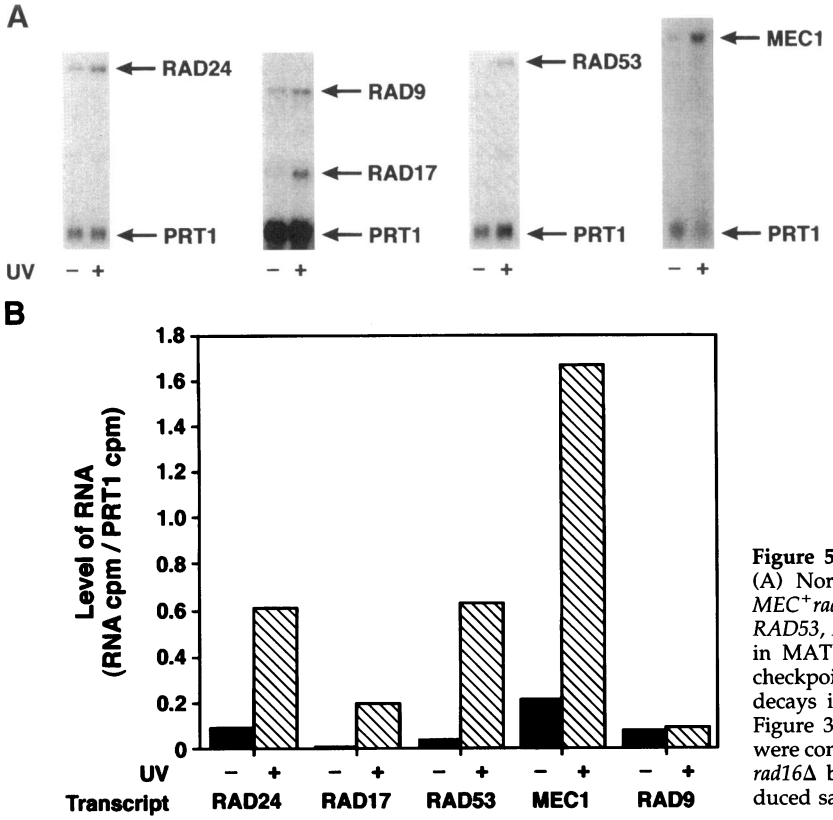

Figure 5. Several checkpoint genes are damage-inducible. (A) Northern blots of RNA isolated from UV-irradiated *MEC*$^+$*rad16*Δ (GKY941) cells and hybridized with *MEC1*, *RAD53*, *RAD9*, or *RAD17*, and *PRT1* DNA probes (described in MATERIALS AND METHODS). (B) Graph of induced checkpoint gene transcript levels, determined from counted β decays in Northern blot bands as described in legend for Figure 3. Results shown are from a single experiment and were confirmed by two other experiments. All strains were in *rad16*Δ background to enhance the UV-induced signal. Induced samples were collected 2 h post-UV (80 J/m^2).

way required *MEC1*, *RAD53*, and *RAD17* to induce *DDR48* transcription. The other three checkpoint mutants, *rad9*, *rad24*, and *mec3*, had about a twofold effect on induction of all classes of transcript, a phenotype addressed further below.

These results delineate the functions of *MEC1* and *RAD53*, which were previously classed together based on their similar phenotypes (Weinert *et al.*, 1994). *RAD53* had some limited role in the Class A transcriptional pathway because induction of Class A transcripts was reduced twofold in the *rad53* mutant, whereas in the *mec1* mutant, Class A transcripts were not detectably induced (Table 2A). This implies that *MEC1* is required for *RNR3* induction while *RAD53* is not. However, this conclusion is based on the analysis of non-null alleles of both *MEC1* and *RAD53*. We could not use null alleles because *MEC1* and *RAD53* are both essential genes (Zheng *et al.*, 1993; Kato and Ogawa, 1994; Nasr *et al.*, 1994; Allen *et al.*, 1995; Kim and Weinert, unpublished observations). The *mec1* and *rad53* mutant alleles used are equally defective for other checkpoint gene-mediated responses (e.g., *RNR3* transcription [Table 2A] and arrest at the S and G2 checkpoints [Weinert *et al.*, 1994]). Therefore, our results indicate either that *MEC1* and *RAD53* have different roles, or that both genes are required for Class A transcriptional induction but the *rad53* allele tested here retains that function while the *mec1–1* allele does not.

Significant Twofold Effects in rad9, rad24, rad17, and mec3 Checkpoint Mutants

We have taken much effort to evaluate the twofold lower induction levels of transcripts after UV treatment in the G2-specific mutants. The twofold effects were statistically significant and were pervasive (seen in all mutants, with many different transcripts; Table 2, A and B; see Transcriptional Induction Experiments section of MATERIAL AND METHODS). *RNR3* induction, but not *UBI4* or *DDR48* induction, was also reduced twofold in G2-specific checkpoint mutants after MMS-treatment. We think induction of *UBI4* and *DDR48* may be complicated for reasons unrelated to the G2-specific checkpoint gene functions. Induction of *UBI4* may occur in response to cell stress in addition to DNA damage, and alkylation may induce such stress. In addition, the levels of *DDR48* induction may not have been sufficient to reliably detect twofold differences.

There are several important trends in these twofold effects. First, the G2-specific genes specifically enhanced the transcriptional response to UV and MMS, but not to HU. Gene induction in HU-treated cells in

Table 3. Synthetic phenotypes are seen in some *rad16Δ checkpoint* double mutants

	Cell viability (% cells forming microcolonies at 23°C)		
		+ UV	
Genotype	no UV	10 J/m^2	20 J/m^2
rad16Δ	94.2 ± 3.6	61.5 ± 2.5	24.3 ± 2.0
mec1-1	89.3 ± 3.5	52.0 ± 3.6	16.0 ± 1.7
rad53	87.7 ± 2.3	61.7 ± 6.2	26.4 ± 7.6
rad16Δ mec1-1	88.5 ± 4.9	55.5 ± 2.5	14.0 ± 2.8
rad16D rad53	69.3 ± 2.7	<1.0	<1.0
rad9Δ	95.5 ± 0.5	72.3 ± 3.8	62.7 ± 2.1
rad17Δ	91.0 ± 1.0	73.5 ± 2.5	51.3 ± 0.9
rad16Δ rad9Δ	93.5 ± 2.5	45.7 ± 4.5	3.5 ± 2.5
rad16Δ rad17Δ	95.5 ± 1.5	46.7 ± 5.0	3.0 ± 3.0

Cell viabilities on rich media were determined as described in MATERIALS AND METHODS for two separate strains of each genotype, except for *mec1-1, rad16Δ, rad17Δ, rad16Δmec1-1,* and *rad16Δrad9Δ* (which were determined with single strains). All results reflect at least two experiments. The average percentage of viable cells and standard deviations are shown. Strains analyzed include: GKY941 (*rad16Δ*), TWY177 (*mec1-1*), TWY312 and TWY178 (*rad53*), GKY952 (*rad16Δmec1-1*), GKY944 and GKY953 (*rad16Δ rad53*), TWY127 and DLY195 (*rad9Δ*), DLY196 (*rad17Δ*), GKY942 (*rad16Δ rad9Δ*), and GKY977-5-2 and GKY977-1-3 (*rad16Δ rad17Δ*).

all G2-specific mutants was similar to that seen in wild-type cells (Table 2B). The basis for this difference is unknown, although we suggest that the type of lesion may be important (see DISCUSSION).

Second, *UBI4* induction is *MEC1* and *RAD53* independent (in cycling as well as noncycling cells), yet *UBI4* induction was reduced about twofold in all G2-specific mutants (Table 2A). This identifies a function for G2-specific checkpoint genes that is independent of *MEC1* and *RAD53*. The roles of the G2-specific genes in *UBI4* induction was specific for UV treatment and not seen in cells treated with HU. This emphasizes the damage specificity of G2-specific checkpoint gene function noted above.

DISCUSSION

A Working Model of Checkpoint Gene Function

We describe here roles for checkpoint genes in multiple transcriptional regulatory pathways of damage-inducible genes. Taken together with results of earlier studies (Weinert and Hartwell, 1988, 1993; Weinert *et al.*, 1994; Zhou and Elledge, 1993; Allen *et al.*, 1995; Navas *et al.*, 1995; Lydall and Weinert, 1995), these results suggest a model of checkpoint gene functions. The model in Figure 6 accounts for each of the following observations: 1) five damage responses (three of transcriptional induction and two of cell cycle arrest); 2) roles of seven different checkpoint genes; 3) different types of DNA damage; and finally 4) the G2-specific checkpoint genes have roles in processing DNA damage (Lydall and Weinert, 1995). The model summarizes observations and contains features that are speculative.

After DNA damage (Figure 6A), transcriptional induction of some genes, like *UBI4*, occurs independently of checkpoint proteins. The exact nature of the initial DNA damage is unknown, although after UV it could be a small gap generated by excision. Checkpoint proteins then respond to this damage to mediate either transcriptional and/or arrest responses. We suggest Mec1p mediates each response in conjunction with specific checkpoint proteins, most prominently Rad53p. The additional checkpoint proteins increase or extend Mec1p function, perhaps by altering DNA damage. Similarly, after DNA replication is blocked (Figure 6B), we suggest a delayed replication fork also contains a DNA damage structure sufficient for most

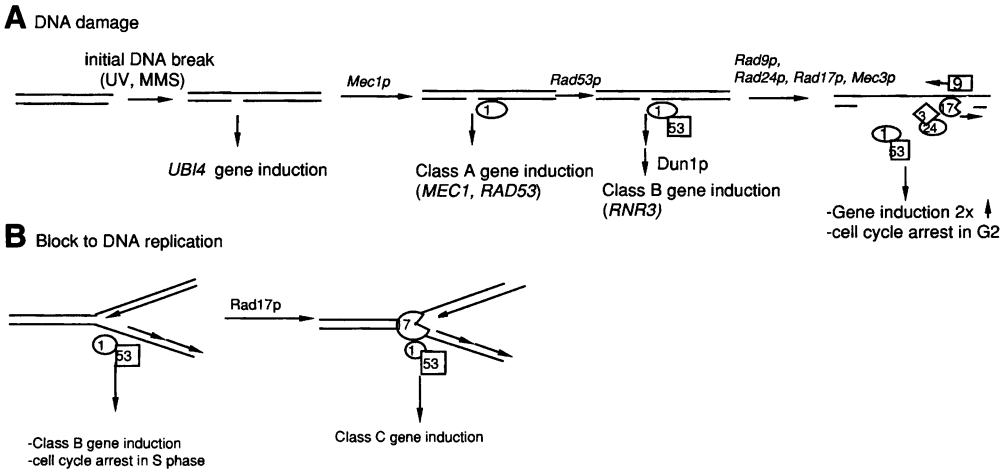

Figure 6. A hierarchy of checkpoint gene function. (A) DNA damage caused by UV or MMS leads to activation of response pathways requiring specific subsets of checkpoint genes. (B) A stalled replication fork causes activation of response pathways requiring a different subset of checkpoint genes. The hypothesis that *MEC1* and *RAD53* have different roles in transcriptional induction of Class A transcripts has the caveat that studies were performed on non-null alleles (see DISCUSSION).

transcriptional and arrest responses mediated by Mec1p and Rad53p.

DNA Damage Activates at Least Four Transcriptional Pathways in Yeast

The multiple transcriptional pathways may reflect different roles of corresponding genes in DNA repair. The gene products resulting from the Class A response pathway, Mec1p and Rad53p, may augment the cell's general response to DNA damage by increasing the cell's sensitivity to DNA breaks and mediating multiple responses. Gene products from the Class B pathway, defined by *RNR3*, may serve to enhance the cell's ability to repair damage by increasing dNTP pools via the ribonucleotide reductase activity. The molecular function of the gene products from the Class C pathway, defined by *DDR48*, is not well understood, although *ddr48* mutants do have defects in DNA metabolism (Treger and McEntee, 1990).

The analysis of induction of *DDR48* gave an unexpected result. In HU-treated cells, *RAD17* was required for *DDR48* induction but not for cell cycle arrest (Table 2B; Weinert and Hartwell, 1993). Because *RAD17* encodes a putative 3′-5′ exonuclease (Lydall and Weinert, 1995), we suggest that S phase arrest and transcriptional induction of *DDR48* are activated by different lesions. The lesion activating *DDR48* transcription requires degradation (processing) by Rad17p, while the lesion activating arrest does not (Figure 6B). Of note, arrest and transcriptional induction of Class B genes (like *RNR3*) after HU treatment also require *POL2* (Navas *et al.*, 1995); however, the role of *POL2* in *DDR48* induction has not been tested.

The Primary Role of Mec1p and an Analogous Role of DNA-PK

After DNA damage (by UV or MMS) or a block to DNA replication (by HU), Mec1p mediates transcriptional induction that can be independent of other checkpoint proteins. Because Mec1p can respond alone, this suggests a simple model in which Mec1p can associate with DNA breaks (independently of other checkpoint proteins tested). Although speculative, the hypothesis that Mec1p associates with DNA breaks is supported by analogy with the mammalian protein DNA-PK. The Mec1p yeast protein shares both protein sequence and functional similarity to DNA-PK (Kato and Ogawa, 1994; Gardner and Weinert, unpublished data), a DNA damage-activated protein kinase from mammalian cells (see reviews in Anderson, 1993; Gottlieb and Jackson, 1994). DNA-PK is a large protein of 350 kDa, as is Mec1p, and both proteins contain a so-called phosphatidylinositol-3 (PI-3) kinase/protein kinase domain (Dhand *et al.*, 1994; Stack and Emr, 1994; Hartley *et al.*, 1995; Gardner and Weinert, unpublished data). Notably, DNA-PK has a protein kinase activity that requires activation by DNA breaks (Gottlieb and Jackson, 1993). The nature of the activating DNA substrate has been well studied and appears to involve a double-stranded/single-stranded DNA junction (Gottlieb and Jackson, 1993; Morozov *et al.*, 1994). In vitro, DNA-PK binds to two other proteins called Ku (p70 and p80). The Ku subunits bind directly to DNA breaks, thereby activating the associated DNA-PK protein kinase activity (Gottlieb and Jackson, 1993). Among the in vitro substrates of DNA-PK are transcription factors (see reviews in Anderson, 1993; Gottlieb and Jackson, 1994). The in vivo significance of these in vitro findings is unknown, although changes in phosphoproteins were recently reported (Boubnov and Weaver, 1995). The in vitro studies implicating roles for DNA-PK and Ku in DNA metabolism are supported by in vivo studies; mutants in DNA-PK (*scid* mice and XRCC7 hamster cell lines) and Ku (XRCC5 hamster cell lines) are radiation sensitive and have defects in V(D)J recombination (Rathmell and Chu, 1994; Taccioli *et al.*, 1994; Blunt *et al.*, 1995). Whether these mutant cells have cell cycle defects has not been reported.

Given the sequence and phenotypic similarities of DNA-PK to the yeast Mec1p, we suggest Mec1p may also recognize DNA breaks, perhaps in association with other yeast proteins yet to be identified, and then phosphorylate proteins that lead to changes in gene expression and cell cycle progression. The identity of Mec1p-associated proteins is unknown. However, a yeast protein similar to mammalian Ku80 has been identified; called Hdf1p, this yeast protein binds to DNA breaks in vitro (Feldmann and Winnacker, 1993). The relationship, if any, between Mec1p and Hdf1p is not obvious because *hdf1* mutants are not radiation sensitive (Weinert, unpublished observations). Whether Mec1p associated with DNA damage by a mechanism analogous to that of DNA-PK remains a major question.

The Relationship between MEC1 and RAD53

From their respective mutant phenotypes, *MEC1* and *RAD53* genes appear to play nearly equivalent and important roles in all checkpoint gene-mediated responses (Weinert *et al.*, 1994; this report). These two genes are good candidates for signalers because both encode kinases–Mec1p, a phosphatidylinositol-3/protein kinase, and Rad53p, a protein kinase (Stern *et al.*, 1991).

We place *RAD53* second to *MEC1* in a functional hierarchy because *MEC1* is required for Class A gene transcriptional induction while *RAD53* is not. The conclusion that *MEC1* and *RAD53* have different roles is

based on the assumption that the alleles tested have essentially null phenotypes for transcriptional induction of Class A transcripts. We cannot test true null alleles as both genes are essential, thus this conclusion must be tested further. Nevertheless, there is other evidence indicating at least different roles for Rad53p and MEC1p, although none indicate their order of function. For example, *rad53*, as well as *rad9* and *rad17*, have genetic interaction with *rad16*, a mutation affecting DNA repair. *mec1* does not show this genetic interaction (see Table 3). One interpretation of this genetic interaction is that Rad53p (as well as Rad9p and Rad17p) act in a DNA repair pathway distinct from that of Rad16p, and Mec1p does not act in this repair pathway. The corresponding repair pathways are not well understood (Friedberg, 1985), thus the possible roles of the checkpoint proteins are unknown.

A recent study from fission yeast lends additional circumstantial support to the notion that *RAD53* performs a subset of the *MEC1*-dependent responses. The fission yeast *cds1*$^+$ gene product is similar in sequence to the budding yeast *RAD53* gene product (Murakami and Okayama, 1995), and *cds1*$^+$ is required for only a subset of the responses that require *rad3*$^+$, the fission yeast *MEC1* homologue (Seaton *et al.*, 1992). However, it remains possible that *cds1*$^+$ is not the *bona fide* *RAD53* homologue.

In sum, in budding yeast *MEC1* seems to play a preeminent role in checkpoint gene-mediated responses. We suggest that *RAD53* plays one of three possible roles–either it is involved in processing damage (similar to *RAD9*), or it is involved more intimately in signaling (similar to *MEC1*), or both. Defining the roles of *MEC1* and *RAD53* genes will require additional genetic and biochemical tests.

Lesion Specificity of G2-specific Checkpoint Gene Functions Is Consistent with Roles in Damage Processing

We found previously that G2-specific checkpoint proteins process DNA damage (Lydall and Weinert, 1995). Here, we found that these G2-specific genes also increased the transcriptional induction of all genes tested by twofold after UV-induced damage (Table 2A). After MMS-induced DNA damage, a similar twofold effect was seen on *RNR3* (but not *UBI4* nor *DDR48*) induction (Table 2B). The absence of the G2-specific genes did not appear to effect transcriptional induction after HU-induced stalled replication. To account for roles in response to UV and MMS damage only, we suggest that different lesions are processed by different gene products; Rad9p and other checkpoint proteins process UV- or MMS-induced damage and do not process DNA damage present in HU-arrested cells (except for a minor role of *RAD17*; Figure 6B). We propose that the twofold increase after UV damage results from damage processing that either increases the number of lesions that Mec1p/Rad53p can detect or generates a more robust substrate for Mec1p/Rad53p. Finally, we found that the twofold transcriptional enhancement by G2-specific checkpoint genes occurred even with induction of *UBI4*, whose induction does not require Mec1p or Rad53p (Table 2, A and B). This uncovers a function of the G2-specific checkpoint proteins that we suggest is in processing DNA damage and that is distinct from that of Mec1p and Rad53p (Lydall and Weinert, 1995; this study). In this case, processing leads to increased *UBI4* induction by some unknown pathway.

The Relationship between Transcriptional Induction and Cell Cycle Arrest

We suggest in this model that Mec1p responds to some DNA breaks to induce only the transcriptional response and to other DNA breaks to induce the arrest responses (and perhaps transcriptional responses as well). This hypothesis is based on the following observations and arguments. First, we have tested G2 cells for both transcriptional induction and cell cycle arrest after UV irradiation and both responses require *MEC1* but only arrest requires *RAD9*. We infer, therefore, that *RAD9* and the other G2-checkpoint genes play some role in determining how Mec1p and Rad53p mediate the two responses. Second, we have shown elsewhere that Rad9p (and the other three G2-specific checkpoint proteins) has a role in processing DNA damage (Lydall and Weinert, 1995). In fact, Rad17p encodes a putative 3′-5′ exonuclease. Therefore, the G2-specific checkpoint proteins appear to act upstream of Mec1p. The differences in the downstream responses of transcriptional induction and arrest, then, appear to be at the level of DNA damage itself. We conclude that Mec1p may either recognize different DNA damage structures (e.g., a nick versus a gap) and respond differently or recognize different protein complexes on DNA and respond differently. There is precedent for a protein binding and responding uniquely to different types of damage structures; DNA-PK binds to both ssDNA and dsDNA but its protein kinase is activated only by dsDNA (Gottlieb and Jackson, 1993).

The two responses do appear distinct in other respects as well. The dose sensitivity and kinetics of the two responses differ; transcriptional induction appears slow (Figure 2A) and is dose dependent (Ruby and Szostak, 1985), while cell cycle arrest is relatively fast (Figure 2B) and dose independent (Weinert and Hartwell, 1988, 1993). For example, arrest can occur after one double-stranded DNA break, but transcriptional induction seems to require more damage (Sandell and Zakian, 1993; Kim and Weinert, unpublished data; Nejad and Weinert, unpublished observations).

Why might different types of damage induce different signals? We suggest that different types of damage may be repaired by unique pathways. An initial DNA break may be one type of repair intermediate, while processing by checkpoint proteins may allow repair by another pathway. Perhaps the initial DNA break poses little threat to cell viability and may induce changes in gene expression to facilitate repair yet fail to induce the more drastic cell physiological alteration of cell cycle arrest. However, when DNA damage is either too abundant or inherently difficult to repair, processing by checkpoint proteins may serve to signal the additional response of cell cycle arrest.

In sum, our observations suggest that the type of DNA damage and/or the different damage-associated protein complexes lead to different responses. How Mec1p and Rad53p recognize the different lesions or associated complexes to mediate the responses is unknown.

Alternative Models of Checkpoint Gene Function

Our results on roles of checkpoint proteins in transcriptional responses may have other interpretations. In one alternative interpretation, each checkpoint protein acts sequentially to mediate responses. Class B transcriptional induction, for example, occurs by sequential activity of Mec1p activating Rad53p, which induces most directly. Similarly, induction of *DDR48* transcription would require sequential activation of Mec1p, then Rad53p, and then Rad17p. In a related, collective model, checkpoint proteins would participate jointly in each response. Class B transcriptional induction, for example, would require the simultaneous function of both Mec1p and Rad53p. Some elements of both the sequential or collective functions may be incorporated in the model shown in Figure 6.

Implications for Understanding ATM Gene Function

Recently, the gene defective in individuals with ataxia-telangiectasia (AT) was identified (Savitsky *et al.*, 1995). This gene, called *ATM*, has significant sequence similarity to the budding yeast *MEC1* (Gardner and Weinert, unpublished observations; *MEC1* was identified independently as *ESR1*, [Kato and Ogawa, 1994] and as *YRB1012* [Nasr *et al.*, 1994]) and *TEL1* genes (Morrow *et al.*, 1995), and to the fission yeast *rad3*$^+$ gene (Seaton *et al.*, 1992; Carr, personal communication). AT-affected and *mec1*-mutant cells have some common cell cycle checkpoint defects, and *mec1* and *tel1* mutations show genetic interaction, suggesting some common functions (Painter and Young, 1980; Nagasawa *et al.*, 1985; Rudolph and Latt, 1989; Beamish and Lavin, 1994; Weinert *et al.*, 1994; Morrow *et al.*, 1995; Paulovich and Hartwell, 1995). Transcriptional induction may also be affected in AT cells (Papathanasiou *et al.*, 1991). ATM may play similarly complex roles in human cells as those performed by *MEC1* and *TEL1* in yeast. Whether the diverse disease symptoms in AT individuals correlate to different responses mediated by ATM will be of interest. Delineating the different roles may be accomplished by studying Mec1p, as well as Tel1p, in yeast.

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