Involvement of the PP2C-Like Phosphatase Ptc2p in the DNA Checkpoint Pathways of *Saccharomyces cerevisiae*

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ABSTRACT

RAD53 encodes a conserved protein kinase that acts as a central transducer in the DNA damage and the DNA replication checkpoint pathways in *Saccharomyces cerevisiae*. To identify new elements of these pathways acting with or downstream of *RAD53*, we searched for genes whose overexpression suppressed the toxicity of a dominant-lethal form of *RAD53* and identified *PTC2*, which encodes a protein phosphatase of the PP2C family. *PTC2* overexpression induces hypersensitivity to genotoxic agents in wild-type cells and is lethal to *rad53*, *mec1*, and *dun1* mutants with low ribonucleotide reductase activity. Deleting *PTC2* specifically suppresses the hydroxyurea hypersensitivity of *mec1* mutants and the lethality of *mec1*Δ. *PTC2* is thus implicated in one or several functions related to *RAD53*, *MEC1*, and the DNA checkpoint pathways.

EUKARYOTIC cells have evolved complex mechanisms for coping with DNA damage or the inhibition of DNA replication. These surveillance mechanisms, termed checkpoints, ensure that the integrity of the genome is intact before allowing cell division to proceed (for reviews, see Hartwell and Weinert 1989; Elledge 1996; Weinert 1998). In unicellular organisms, failure of the restraints imposed by the checkpoints results in genomic instability, increased mutation rates, and ultimately death if cells continue to divide unchecked. In mammals, disruptions of checkpoint pathways are believed to be important at early stages of carcinogenesis (Hartwell and Kastan 1994). Strong evidence for a link between checkpoints and cancer comes from studies of ATM, the gene mutated in the cancer-prone disease ataxia telangiectasia (reviewed in Morgan and Kastan 1997).

The understanding of checkpoint pathways is presently most advanced in the yeast *Saccharomyces cerevisiae*. Several classes of DNA checkpoints have been described. One pathway blocks chromosome segregation if DNA replication is incomplete (Weinert 1992; Allen *et al.* 1994). The other pathways induce cell cycle arrests at the G1/S and G2/M transitions, respectively, and a slowing of S phase in case of DNA damage (Weinert and Hartwell 1988; Siede *et al.* 1993; Paulovich and Hartwell 1995; Weinert 1998).

The components of the DNA checkpoint machinery fall into three categories: sensors, transducers, and targets (Weinert 1998). The sensor class includes *RAD9*, *RAD17*, *RAD24*, and *MEC3*, which are required for response to DNA damage (Lydall and Weinert 1995; Paulovich *et al.* 1997; de la Torre-Ruiz *et al.* 1998), and *POL2*, *RFC5*, and *DPB11*, which are specifically involved in the response to the inhibition of DNA replication (Araki *et al.* 1995; Navas *et al.* 1995). The corresponding proteins are thought to recognize DNA damage or stalled replication forks and to generate a signal relayed through Mec1p to various checkpoint elements, including the protein kinases Rad53p and Dun1p and the metaphase-anaphase regulator Pds1p (Sanchez *et al.* 1996; Sun *et al.* 1996; Gardner *et al.* 1999). The protein kinases Mec1p and Rad53p act as transducers in all DNA checkpoint pathways and transmit signals to downstream targets, leading to the transcription of genes involved in DNA replication and repair (Aboussekhra *et al.* 1996; Kiser and Weinert 1996; Navas *et al.* 1996) and to the activation of effectors that slow or halt the cell cycle, allowing time for the replication and repair processes.

The essential genes *MEC1* and *RAD53* are believed to encode central transducers in the DNA checkpoint pathways. Mec1p belongs to a kinase superfamily that also includes the human Atm and Atr proteins and a *Schizosaccharomyces pombe* homologue, Rad3 (Bentley *et al.* 1996). *TEL1* is another *S. cerevisiae* gene that exhibits some functional redundancy and sequence similarity with *MEC1*. *tel1*Δ mutants are not checkpoint defective, but *mec1*Δ *tel1*Δ double mutants are more sensitive to DNA damage than a *mec1* mutant, and overexpression of *TEL1* can suppress some *mec1* defects (Morrow *et al.* 1995; Sanchez *et al.* 1996). *MEC1* and *TEL1* are placed upstream of *RAD53* in the DNA checkpoint pathways as Rad53p phosphorylation in response to DNA damage or replication blocks is *MEC1* and *TEL1* dependent (Sanchez *et al.* 1996; Sun *et al.* 1996).

Significant progress has been made in identifying

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genes involved in checkpoint control, but there is less information relating to the checkpoint effectors operating downstream of *RAD53*. In contrast to the cell cycle regulation of *S. pombe* and of mammalian cells, the inhibitory phosphorylation of the cyclin-dependent kinase Cdc28p is not involved in the checkpoint-induced arrests of cell division in *S. cerevisiae* (Amon *et al.* 1992; Sorger and Murray 1992). So far, only one potential target of the cell cycle machinery has been identified. Swi6p is modified in a *RAD53*-dependent manner in response to DNA damage, which results in the delay of entry into S phase by inhibition of *CLN* transcription (Sidorova and Breeden 1997). More data are available regarding the transcriptional activation of the *RNR* genes encoding the ribonucleotide reductase that provides desoxyribonucleotides for DNA replication and repair. Elledge and collaborators have shown that *CRT1* encodes a DNA-binding protein that recruits the general repressors Ssn6p and Tup1p to the promoters of the *RNR* genes (Huang *et al.* 1998). In response to DNA damage or the inhibition of DNA replication, Crt1p becomes hyperphosphorylated and no longer binds DNA, resulting in transcriptional induction (Huang *et al.* 1998). Crt1p hyperphosphorylation is abolished in *rad53* and *mec1* mutants, and is reduced in *dun1* mutants, demonstrating its dependence upon the checkpoint pathways. Dun1p had been characterized previously as a protein kinase whose activity was necessary for the transcriptional activation of the *RNR* genes in response to DNA damage or inhibition of replication (Zhou and Elledge 1993). It has recently been shown that Dun1p also contributes to cell cycle arrest in response to DNA damage (Gardner *et al.* 1999).

To identify new elements of the DNA checkpoint pathways acting at the level or downstream of Rad53p, we focused on the isolation of genes whose overexpression suppresses the toxicity of a dominant-lethal allele of *RAD53*. In this article, we present the isolation and analysis of the *PTC2* gene, whose product is a member of the PP2C family.

MATERIALS AND METHODS

Strains and media: Yeast strains were grown in yeast extract/peptone/dextrose with 2% glucose (YPD) or with 2% raffinose and 2% galactose (YPGal + Raf), or in synthetic defined minimal media supplemented with appropriate bases and amino acids and 2% glucose (SD) or galactose (SGal) or raffinose (SRaf). Hydroxyurea (HU; Sigma, St. Louis) was added to the media to final concentrations ranging from 10 to 150 mm. All yeast strains used in this study are listed in Table 1. All strains are congenic with W303-1A, except YPH499, which was used for the screening of the libraries. To generate the *dun1*Δ strain, Y300 was transformed with the *Xho*I-*Xba*I fragment of pZZ66 (Zhou and Elledge 1993) containing *dun1*-Δ*100::HIS3*, and His^+ transformants were checked for their sensitivities to 150 mm HU. *PTC2* was disrupted by PCR targeting using either the *Kluyveromyces lactis URA3* gene (Langle-Rouault and Jacobs 1995) or the *kanMX* cassette (Wach *et al.* 1994). The *URA3* and the *kanMX* cassettes were amplified by PCR with the primers PTC2D5 (5′-ACTATTCCATTGTTGT ATAAATATAGAGAACCAGAAAAAGAAAAACGTGATTTG CTTAAGAATT-3′) and PTC2D3 (5′-GGTTCGTATATAGGTA TGTATATATAATGAAGGATGGAAGATCCTGTAGTTTCTG GTTTTTAAAT-3′), and PTC2D5KAN (5′- ACTATTCCATTG TTG TATAAAATATAGAGAACCAGAAAAAGAAAAGCTTCG TACGCTGCAGGTCGAC -3′) and PTC2D3KAN (5′- GGTTCG TATATAGGTATGTATATATAATGAAGGATGGAAGATCCT ATCATCGATGAATTCGAGCTCG -3′), respectively, and the amplication products were used to transform the selected strains. All *PTC2* disruptions were confirmed by PCR on genomic DNA.

Construction of the tetO-RAD53-GFP fusion: The RAD53 and the green fluorescent protein (GFP) moieties were first amplified separately by PCR, using as templates the pJA98 (Allen *et al.* 1994) and the pYGFP3 plasmids, with the primers RAD53-10 (5′-CCCAGCTTTGTTTAAACATGGAAAATATTA CACAACCCACACAG-3′) and RAD53-11 (5′-GAATAATTCTT CACCTTTAGACATCGAAAATTGCAAATTCTCG-3′), and GFPA (5′-ATGTCTAAAGGTGAAGAATTATTCACTGG-3′) and GFPB (5′-AACGACGGCCAGTGAATTCGAG-3′), respectively. The PCR products were ethanol precipitated and resuspended in TE. Aliquots of both were then used as templates for the sewing PCR reaction with the primers RAD53-10 and GFPB. After digestion with *Pme*I and *Pst*I, this ultimate PCR product was cloned into the *Pme*I-*Pst*I sites of pCM183 (Gari *et al.* 1997) behind the tetracycline operator tetO.

Library screening: RAD53-GFP is lethal to both the Y300 and the YPH499 strains at 37° on glucose and at 30° on galactose. However, RAD53-GFP is harbored by a *TRP1* plasmid, and the Y300 *TRP1* allele *trp1-1* has a high reversion rate ($\sim 10^{-4}$) that was incompatible with a screening for suppressors. We therefore performed the initial screenings with YPH499, and later checked the suppressors' activity in both strains. We screened a genomic library built in the multicopy vector pFL44 (Stettler *et al.* 1993) and a library consisting of cDNAs under the control of the *GAL1* promoter (Liu *et al.* 1992) under two conditions: at 33° on galactose and at 37° on glucose. YPH499 cells containing RAD53-GFP were transformed using the lithium acetate procedure (Ito *et al.* 1983) and were incubated directly at the indicated temperature. A total of 40,000 and 60,000 clones transformed with the genomic and the cDNA libraries, respectively, were screened for growth at 33° on galactose, and after verification, only the cDNA library yielded two suppressor constructs, M1-15 and M3-11, encoding the same protein, Ptc2p. A total of 40,000 clones transformed with the genomic library were screened on glucose at 37°, and *CRT1* was then isolated as a suppressor of RAD53-GFP.

β-Galactosidase assays: pZZ13, a plasmid harboring the *RNR3-lacZ* reporter gene (Zhou and Elledge 1992), was introduced into wild-type cells (Y300) in combination with either an empty vector (pRS314; Sikorski and Hieter 1989) or a vector bearing RAD53-GFP. Liquid β-galactosidase assays were carried out on yeast cells that were grown overnight in selective medium, diluted into fresh medium, and grown to mid-log phase. β-Galactosidase assays with the colorimetric substrate *o*-nitrophenyl-β-galactopyranoside were performed as described in Zhou and Elledge (1992). Assays were carried out in triplicate and averaged.

Testing the sensitivity to HU, UV, and *PTC2* overexpression: To test the cells' sensitivity to *PTC2* overexpression, transformants containing the *GAL1-PTC2* construct M1-15 or the empty vector pRS316 (Sikorski and Hieter 1989) were first selected on SD-URA plates, streaked onto SRaf-URA medium, and ultimately tested on SGal-URA plates with or without HU. The measurement of UV and HU sensitivity was routinely performed as follows. Overnight precultures were diluted to

TABLE 1

Strains used in this study

Strains	Genotype	Reference/Source
YPH499	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 trp1-Δ63 ura3-52 lys2-801*	Sikorski and Hieter (1989)
Y300	*MAT***a** *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5*	Allen *et al.* (1994)
Y301	As Y300 *rad53-21*	Allen *et al.* (1994) Sanchez *et al.* (1996)
Y306	As Y300 *mec1-21*	Allen *et al.* (1994) Sanchez *et al.* (1996)
MCM134	As Y306 *ptc2Δ::URA3*	This study
MCM135	As Y306 *ptc2Δ::URA3*	This study
Y601	As Y300 *rad53Δ::HIS3* + pJA92 (*CEN4, URA3, RAD53*)	Desany *et al.* (1998)
Y602	As Y300 *mec1Δ:: HIS3* + pBAD45 (*CEN4, URA3, MEC1*)	Desany *et al.* (1998)
MCM190	As Y602 *ptc2Δ::kanMX*	This study
MCM191	As Y602 *ptc2Δ::kanMX*	This study
MCM123	As Y300 *dun1Δ::HIS3*	This study
W1588-4C	*MAT***a** *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*	Zhao *et al.* (1998)
U952-3B	As W1588-4C *sml1Δ::HIS3*	Zhao *et al.* (1998)
U953-61A	As W1588-4C *mec1Δ::TRP1 sml1Δ::HIS3*	Zhao *et al.* (1998)
U960-5C	As W1588-4C *rad53Δ::HIS3 sml1-1*	Zhao *et al.* (1998)

an OD of 0.1 and grown for an additional 4–5 hr. Tenfold dilutions were then spotted on YPD plates with or without HU. Sets of YPD plates were irradiated by UV light at 20–120 J/m^2 using a Stratalinker 1800. Next, plates were incubated at 30° for 3 days. For each genotype tested, at least two independent strains were assayed. To quantify more precisely the UV sensitivity of wild-type cells overexpressing *PTC2*, the following procedure was adopted. Wild-type cells containing either the empty vector pRS316 or the *GAL1-PTC2* construct M1-15 were grown to mid-log phase in SRaf-URA medium, plated onto SGal-URA plates at an appropriate dilution (~2000 cfu/plate), and irradiated by UV light. Percent survival was then determined relative to that of unirradiated controls.

Analysis of Rad53p phosphorylation: Wild-type and *dun1Δ* cells containing either the empty vector pRS316 (Sikorski and Hieter 1989) or the *GAL1-PTC2* construct were grown overnight in SRaf-URA medium and diluted to an OD of 0.4 in the same medium, to which galactose was added to a final concentration of 2%. The cultures were grown for an additional 2.5 hr and split into two, whereupon one-half was treated with HU (0.2 m final) and the other half was left untreated. All cells were then allowed to grow for a further 2 hr before extraction. Yeast extracts were prepared as described (Vialard *et al.* 1998), by glass bead beating in 20% trichloroacetic acid (TCA), washing the glass beads in 5% TCA, and combining the wash with the lysate. The protein suspension was then pelleted, resuspended in 1× Laemmli loading buffer (pH 8.8), boiled for 5 min, pelleted, and the supernatant was retained as a whole-cell extract. For Western blotting, proteins were separated on 10% SDS-PAGE with an acrylamide:bis-acrylamide ratio of 30:0.4 and transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL) by electroblotting (Bio-Rad, Richmond, CA). Goat polyclonal antibody raised against a polypeptide corresponding to an amino acid sequence mapping at the C terminus sequence of Rad53p [RAD53 (yC-19); Santa Cruz Biotechnology] was incubated with the nitrocellulose membranes at a 1:1000 dilution in Tris-buffered saline containing 0.1% Tween-20 and 5% (w/v) milk overnight. Secondary horseradish peroxidase-conjugated anti-goat antibody (Santa Cruz Biotechnology) was incubated for 1 hr at a 1:5000 dilution and the blot was revealed by chemiluminescence (Amersham).

RESULTS

A dominant lethal allele of *RAD53*: We reasoned that we might be able to isolate mutant alleles of *RAD53* that constitutively arrest the cell cycle even in the absence of DNA lesions or replication blocks. Such alleles would be lethal, and screening for suppressors should reveal elements of the checkpoint pathways acting downstream or at the level of Rad53p. Fortuitously, one allele of this sort was generated through the construction of a RAD53-GFP fusion. A translational fusion containing the entire coding sequence of *RAD53* and the sequence of the GFP was produced (see materials and methods) and placed under the control of the regulatable tetracycline operator tetO [tetO activity is repressed by tetracycline or derivatives such as doxycycline (Gari *et al.* 1997)].

The functionality of the tetO-RAD53-GFP construct was tested by its ability to complement a *rad53Δ* deletion. Y601, a *rad53Δ* mutant containing a wild-type copy of *RAD53* on a *URA3*-marked plasmid (Desany *et al.* 1998), was transformed either with an empty vector or with a plasmid bearing RAD53-GFP. Only transformants containing the RAD53-GFP construct were able to lose the *URA3* plasmid with the wild-type copy of *RAD53* and to form colonies resistant to 5-fluoro-orotic acid [5-FOA; a compound that is toxic to cells containing the *URA3* gene (Boeke *et al.* 1984)], demonstrating that RAD53-GFP is functional (data not shown). We were also able to detect the RAD53-GFP protein by fluorescence in the nucleus, confirming previous reports of nuclear localization of Rad53p (see Zheng *et al.* 1993; data not shown). Overexpression of *RAD53* affects the cell cycle (Zheng *et al.* 1993; Allen *et al.* 1994) and we confirmed this result (Figure 1A).

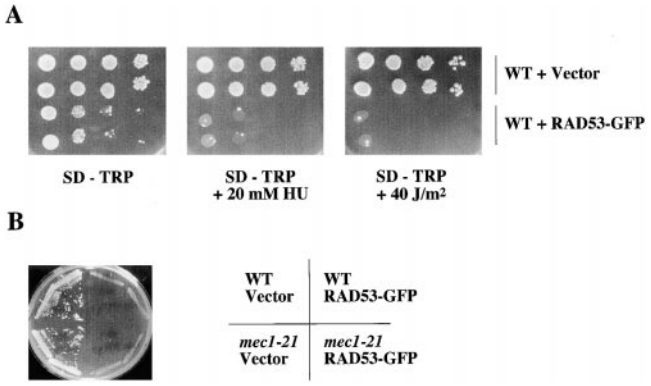

Figure 1.—(A) The expression of the RAD53-GFP construct is deleterious to cell growth regardless of the medium and the temperature, and is lethal in the presence of hydroxyurea or after UV irradiation. Wild-type cells (WT, Y300) containing either an empty vector (pCM183) or the RAD53-GFP construct were grown overnight in SD-TRP medium containing doxycycline (1 μg/ml) and diluted to an OD of 0.1 in the same medium without doxycycline. The cultures were grown for an additional 4 hr, and 10-fold serial dilutions were then spotted on SD-TRP plates with or without HU or UV treatment. Plates were then incubated at 30° and examined after 3 days. (B) The expression of the RAD53-GFP construct is lethal to both wild-type and *mec1* cells on a medium containing galactose. Wild-type (WT, Y300) and *mec1* (*mec1-21*, Y306) cells harboring either an empty vector (pCM183) or the RAD53-GFP construct were selected on SD-TRP medium containing 1 μg/ml doxycycline and then streaked onto SGal-TRP plates without doxycycline. Plates were incubated at 30° and examined after 4 days.

We also found that RAD53-GFP expression in wild-type cells was lethal under conditions of genotoxic stress, including UV irradiation or the presence of HU, a drug that stalls replication forks by limiting deoxyribonucleotide availability through inhibition of ribonucleotide reductase (RNR) activity (Figure 1A). This feature prevented us from testing directly the proficiency of RAD53-GFP in executing *RAD53* checkpoint functions. We therefore examined whether RAD53-GFP could activate by itself the transcription of a *RNR3-lacZ* reporter gene (Zhou and Elledge 1992). The activities of wild-type cells with or without RAD53-GFP reached 74 ± 26 and 6.9 ± 0.5 β-gal units, respectively. Rad53-GFP thus behaved as a hyperactive Rad53p and seemed to trigger, in the absence of genotoxic stress, physiological events normally induced by DNA damage or replication blocks. The events elicited by Rad53-GFP appear to be deleterious to cell growth under normal conditions, and, interestingly, not only did they not protect the cells against genotoxic stress, but they were even lethal in that case, probably because of their lack of regulation. We also found that the expression of RAD53-GFP was lethal in the absence of genotoxic stress at 37° on glucose and at 30° on galactose. Arrest of cell division was not uniform (as determined by microscopic observation, data not shown), was independent of *MEC1* (Figure 1B), and was partially suppressed by a deletion of *DUN1* (data not shown), further demonstrating that the toxicity of Rad53-GFP was due to the triggering of events located downstream of Rad53p. We used this characteristic of RAD53-GFP to attempt to isolate new elements of the pathways controlled by *RAD53*. We sought checkpoint-negative regulators by searching for genes whose overexpression suppresses RAD53-GFP toxicity.

Screening for genes whose overexpression suppresses RAD53-GFP toxicity: Among the suppressors we isolated was *CRT1*, which encodes a negative regulator of *RNR* gene transcription (Huang *et al.* 1998). This result validated our screen, since negative regulators of the checkpoint pathways were exactly the kind of suppressor we were expecting. Screening a library consisting of cDNAs under the control of the *GAL1* promoter (Liu *et al.* 1992) at 33° on galactose yielded two independent constructs encoding Ptc2p, a protein phosphatase of the PP2C family. Both constructs contained the full-length cDNA corresponding to *PTC2*. The sequences started 42 bp (M1-15) and 293 bp (M3-11) before the translation initiation codon, respectively. The M1-15 clone proved to be a more efficient suppressor of RAD53-GFP and was therefore selected for further analyses.

***PTC2* overexpression induces hypersensitivity to genotoxic agents in wild-type cells:** Because we imagined that overexpression of *PTC2* suppressed the lethality of Rad53-GFP by blocking one of its downstream effects, we expected that Ptc2p would also partially block the action of Rad53p in the presence of genotoxic agents. Indeed, overexpression of *PTC2* increased sensitivity to DNA damage or replication blocks (Figures 2 and 3). Overexpression of *PTC2* slowed cell growth in the absence of HU, as had already been reported by Welihinda *et al.* (1998), and was lethal when 20 mm HU was added to the medium. Increasing the concentration of HU to 50 mm decreased further the residual cell growth (Figure 2). *PTC2* overexpression also caused a marked decrease in cell viability after UV irradiation (Figure 3).

In the presence of HU or of DNA-damaging agents, Rad53p triggers several responses for sustaining cell viability. To understand which Rad53p-dependent pathways are affected by *PTC2* overexpression, we monitored cell cycle progression of wild-type cells overexpressing *PTC2* after irradiation by UV light. Cells were blocked in G1 phase by α-factor treatment and were UV irradiated. Wild-type cells overexpressing *PTC2* arrested normally in G1 after the UV treatment and remained blocked for the next 4 hr, exhibiting no defect in the mechanism of checkpoint arrest (Figure 4). This was in contrast to *rad53-21* cells, which resumed their cell cycle ~1 hr after UV irradiation. Similar results were obtained when cells were treated with γ irradiation or HU treatment (data not shown). We conclude that the hypersensitivity of *PTC2*-overexpressing cells to genotoxic agents was

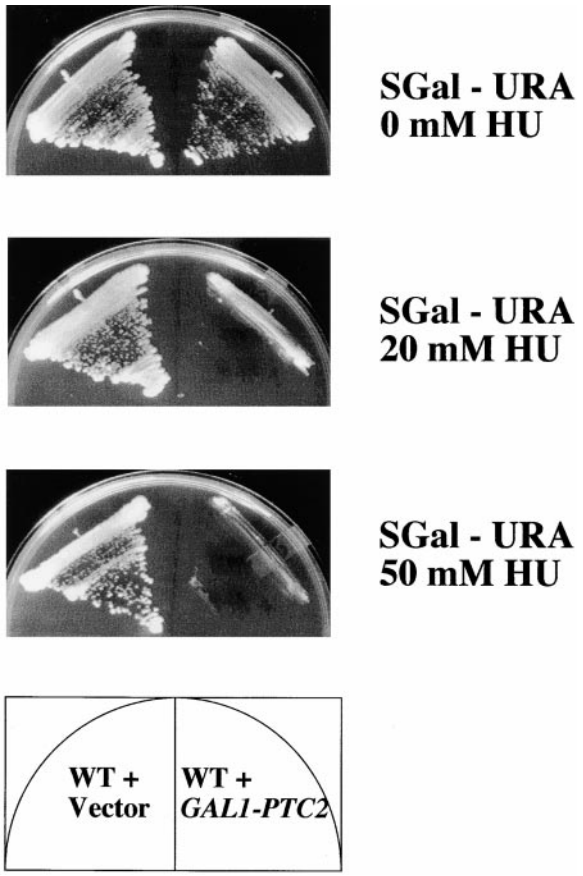

Figure 2.—*PTC2* overexpression is lethal to wild-type cells growing in the presence of hydroxyurea. Y300 (WT, wild-type) cells containing either the empty vector pRS316 (Sikorski and Hieter 1989) or the *GAL1-PTC2* construct M1-15 were grown onto SRaf-URA plates and then streaked on SGal-URA plates with various concentrations of HU.

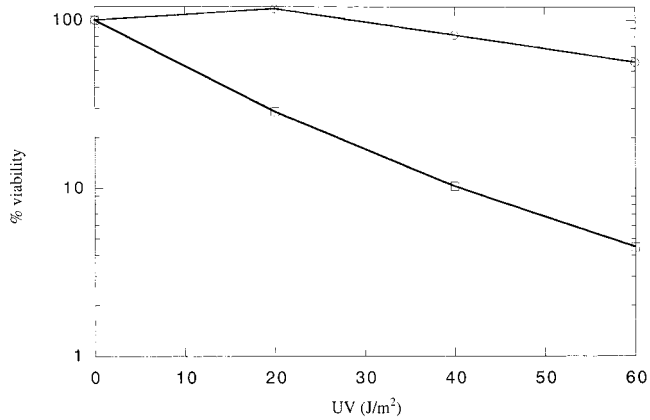

Figure 3.—UV sensitivity of wild-type cells overexpressing *PTC2*. Y300 (wild-type) cells containing either the empty vector pRS316 (open circle) or the *GAL1-PTC2* construct M1-15 (open square) were grown to mid-log phase in SRaf-URA medium, plated onto SGal-URA plates at an appropriate dilution (~2000 cfu/plate), irradiated at the indicated doses of UV light, and percentage survival was determined relative to unirradiated controls.

not caused by a deficiency in checkpoint-induced arrest of cell division.

***PTC2* overexpression is lethal to mutants with low ribonucleotide reductase activity:** As a further test of *PTC2* interaction with the DNA checkpoints, we examined checkpoint mutants for their sensitivity to *PTC2* overexpression. We found that the overexpression of *PTC2* was lethal to *rad53-21* and *mec1-21* mutants, even in the absence of genotoxic agents (Figure 5, B and C). *RAD53* and *MEC1* are both essential genes whose functions are required during a normal cell cycle, probably for ribonucleotide reductase activity, since *rad53*Δ and *mec1*Δ lethality can be suppressed by increased RNR activity (Desany *et al.* 1998; Huang *et al.* 1998; Zhao *et al.* 1998). Since *PTC2* overexpression was toxic in the *rad53-21* and *mec1-21* mutants, we hypothesized that *PTC2* function was related to ribonucleotide reductase activity. This hypothesis was supported by the observation that *PTC2* overexpression was also lethal to *dun1*Δ mutants (Figure 5D). Dun1p is a protein kinase that is required for the transcriptional activation of the *RNR* genes in response to DNA damage or DNA replication blocks and that contributes to cell cycle arrest in response to DNA damage (Zhou and Elledge 1993; Gardner *et al.* 1999). Finally, we found that *rad53*Δ *sml1-1* and *mec1*Δ *sml1*Δ double mutants were not as sensitive to *PTC2* overexpression as *rad53-21* and *mec1-21* single mutants (Figure 5, B and C). Since Sml1p has been characterized only as a negative regulator of the ribonucleotide reductase activity (Zhao *et al.* 1998), this result suggests that the sensitivity of the *rad53-21* and *mec1-21* mutants to the overexpression of *PTC2* is only due to a defect related to the ribonucleotide reductase function.

***PTC2* deletion suppresses the hypersensitivity to HU of *mec1* mutants and the lethality of *mec1*Δ:** Disrupting *PTC2* had no effects on the growth nor on the HU and UV sensitivity of wild-type cells (data not shown). We also deleted *PTC2* in the *rad53-21*, *rad53*Δ *sml1-1*, *mec1-21*, *mec1*Δ *sml1*Δ, and *dun1*Δ mutants. Interestingly, the *mec1* mutants were the only strains whose sensitivities to hydroxyurea were modified by the disruption of *PTC2*: *mec1-21 ptc2*Δ double mutants show a dramatic increase in HU resistance compared to *mec1-21* single mutants (Figure 6A). No such suppression of *mec1-21* sensitivity to UV irradiation by *PTC2* disruption was observed (data not shown). Similar results were obtained with the *mec1*Δ *sml1*Δ mutant: deleting *PTC2* increased its resistance to HU, but had no effect on its sensitivity to UV (data not shown).

We found that *ptc2*Δ was able to rescue *mec1*Δ lethality, but not *rad53*Δ lethality (Figure 6B; data not shown). *PTC2* was disrupted in Y601, a *rad53*Δ mutant containing a wild-type copy of *RAD53* on a *URA3* plasmid, and in Y602, a *mec1*Δ mutant containing a wild-type copy of

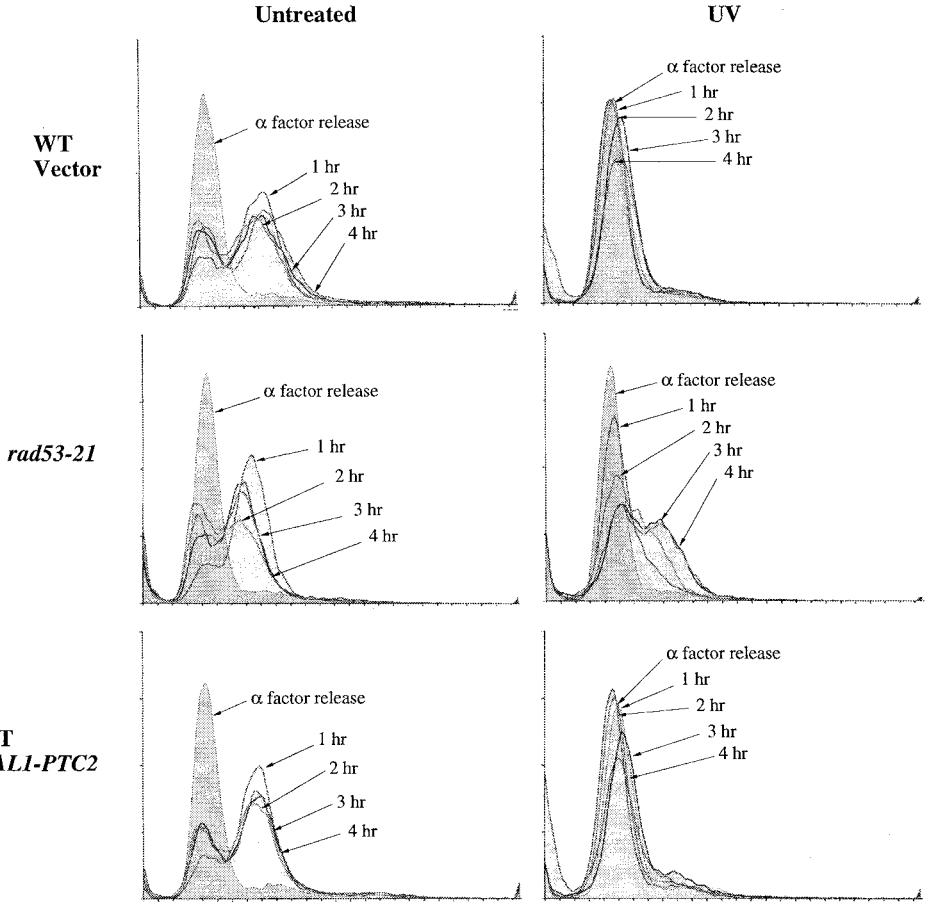

Figure 4.—*PTC2*-overexpressing cells are not defective for checkpoint arrest after UV irradiation. Wild-type (WT, Y300) cells containing either the empty vector pRS316 (Sikorski and Hieter 1989) or the *GAL1-PTC2* construct, as well as *rad53-21* mutant cells (Y301), were grown to log phase in YPGal+Raf and blocked in G1 phase by α-factor treatment for 3 hr. The cultures were divided into two aliquots, one of which was UV irradiated at 80 J/m^2 while the other one was left untreated. The cells were resuspended in YPGal + Raf and allowed to grow for an additional 4 hr. Progress through the cell cycle was monitored by FACS analysis at the indicated time points.

MEC1 on a *URA3* plasmid (Desany *et al.* 1998). Independent transformants were tested for their ability to lose the wild-type copies of *RAD53* or *MEC1* and to grow on 5-fluoroorotic acid (5-FOA)-containing plates. Deletion of *PTC2* significantly suppresses *mec1*Δ lethality, as 20 times as many 5-FOA-resistant clones were recovered from *mec1*Δ *ptc2*Δ double mutants than from *mec1*Δ single mutants, but less strongly than the overexpression of *RNR1* (Figure 6B; Desany *et al.* 1998). No suppression of *rad53*Δ lethality by *ptc2*Δ could be observed (data not shown).

Rad53p is probably not a substrate for Ptc2p: Since phosphorylation of Rad53p is thought to increase its protein kinase activity, the simplest hypothesis for Ptc2p action was that it acts as a Rad53p phosphatase. We therefore tested whether Rad53p was a substrate for Ptc2p by analyzing Rad53p phosphorylation in strains overexpressing *PTC2* in the presence of HU. We found that Rad53p was not phosphorylated in the wild-type strain in the absence of HU, whether *PTC2* was overexpressed or not (Figure 7, lanes 1 and 2). Rad53p became phosphorylated after HU treatment and exhibited the same apparent phosphorylation patterns in the strains containing or lacking the *GAL1-PTC2* construct (Figure 7, lanes 3 and 4). In fact, the bands corresponding to the phosphorylated forms of Rad53p were even more intense in the cells overexpressing *PTC2*, suggesting that *PTC2* overexpression could indirectly increase Rad53p phosphorylation. This also suggests that Ptc2p itself does not dephosphorylate Rad53p. We performed the same experiment in *dun1*Δ cells to test whether the lethality of *PTC2* overexpression in these cells was correlated with a specific pattern of Rad53p phosphorylation (Figure 7, lanes 5–8). Even in the absence of HU, Rad53p was phosphorylated in both strains containing the empty vector or the *GAL1-PTC2* construct (Figure 7, lanes 5 and 6), and the patterns were very similar, closely resembling the phosphorylation pattern of Rad53p in HU-treated wild-type cells (lane 4). This result can be explained by the fact that *dun1*Δ cells are partially deficient for the transcription of the *RNR* genes and the resulting desoxyribonucleotide depletion could activate the checkpoint pathway. Rad53p completely shifted to slower migrating forms after the treatment with HU (Figure 7, lanes 7 and 8), and again the phosphorylation patterns were identical whether the *dun1*Δ mutant overexpressed *PTC2* or not. The phosphorylation patterns of Rad53p were also examined in wild-type cells grown in asynchronous log-phase cultures and irradiated by UV light at 40, 80, or 160 J/m^2: no obvious differences in Rad53p phosphorylation could be observed between the cells containing the empty vector and the *GAL1-*

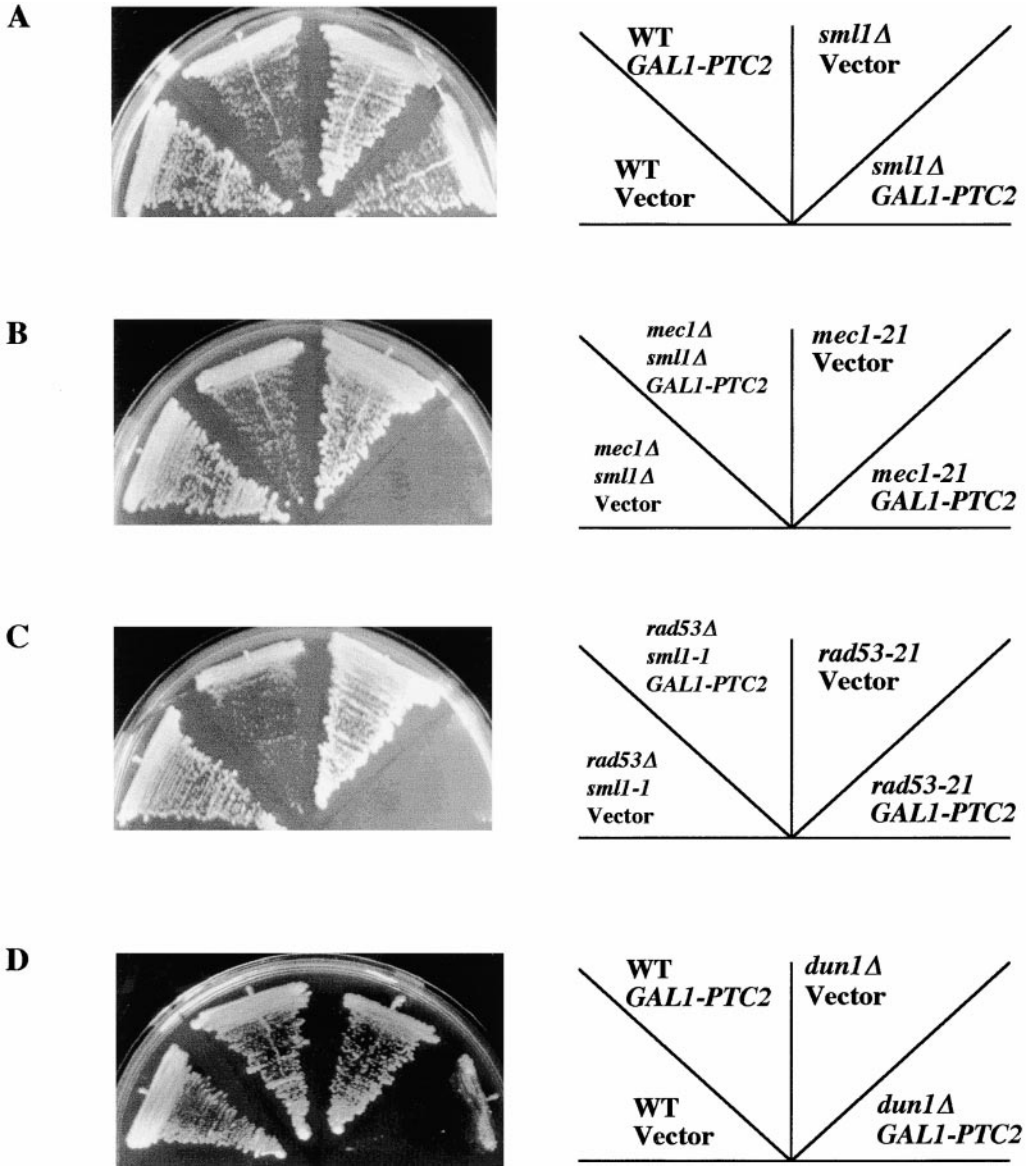

Figure 5.—Lethality of *PTC2* overexpression in *rad53*, *mec1*, and *dun1* strains. Strains with the indicated phenotype containing either the empty vector pRS316 (Sikorski and Hieter 1989) or the *GAL1-PTC2* construct were grown onto SRaf-URA plates and then streaked on SGal-URA plates. (A–C) WT (wild type), W1588-4C; *sml1*Δ, U952-3B; *mec1*Δ *sml1*Δ, U953-61A; *mec1-21*, Y306; *rad53*Δ *sml1-1*, U960-5C; and *rad53-21*, Y301. (D) WT Y300, and *dun1*Δ, MCM123.

PTC2 construct (data not shown). We conclude that Rad53p is probably not a Ptc2p substrate.

DISCUSSION

To identify new elements of the DNA checkpoint pathways acting downstream of Rad53p, we took advantage of a hyperactive form of Rad53p, Rad53-GFP, whose expression is lethal to wild-type cells. We isolated genes whose overexpression suppresses the toxicity caused by RAD53-GFP and whose products are candidates for negative regulators of *RAD53*-controlled pathways. The relevance of this strategy was demonstrated by the isolation of *CRT1* as a suppressor of RAD53-GFP lethality. Crt1p is an inhibitor of *RNR* gene transcription whose activity is regulated in a *MEC1*- and *RAD53*-dependent manner in response to DNA damage or replication blocks (Huang *et al.* 1998). Overexpression of the *RNR* genes is unlikely to explain the toxicity of RAD53-GFP because increased ribonucleotide reductase activity is not toxic to yeast cells (Desany *et al.* 1998; Huang *et al.* 1998; Zhao *et al.* 1998). Thus, the suppressor activity of *CRT1* suggests either that Crt1p overexpression sequesters Rad53-GFP and prevents the hyperactivation of other effectors or that Crt1p represses the transcription of unidentified genes whose hyperactivation is toxic.

PTC2, another suppressor of RAD53-GFP toxicity, encodes a protein that is highly similar to the serine/threonine phosphatases of the PP2C family. PP2C-like enzymes play multiple roles in regulating a number of signal transduction pathways in eukaryotes (Fukunaga *et al.* 1993; Leung *et al.* 1994; Chin-Sang and Spence 1996). The *S. cerevisiae* genome encodes six PP2C-related enzymes (Stark 1996), three of whose physiological roles are unknown. Ptc1p and Ptc3p are implicated in the downregulation of the HOG osmosensing signal

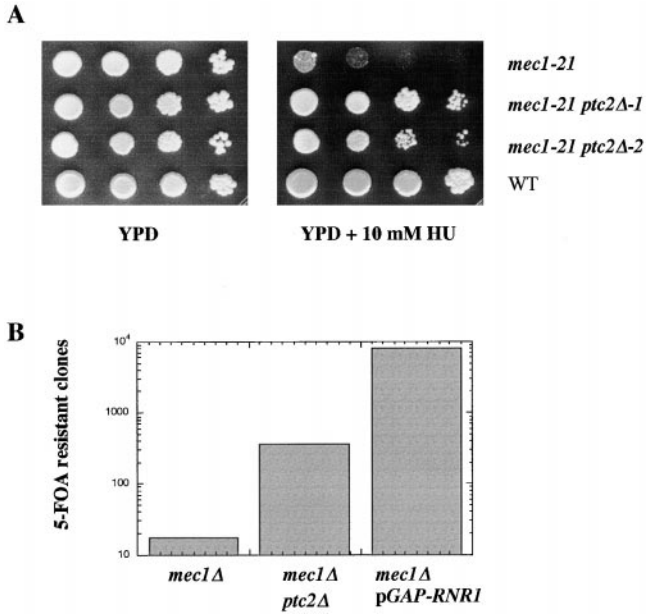

Figure 6.—(A) *ptc2*Δ suppresses the HU sensitivity of *mec1-21* cells. Wild-type (WT, Y300), *mec1-21* (Y306), and *mec1-21 ptc2*Δ (MCM134 and MCM135, two independent disruptants) cells were grown to mid-log phase in YPD. Tenfold serial dilutions were then spotted on YPD plates with or without HU. The plates were then incubated at 30° and examined after 3 days. (B) *ptc2*Δ partially suppresses the lethality of *mec1*Δ. A *mec1*Δ mutant containing a wild-type copy of *MEC1* on a *URA3* plasmid (*mec1*Δ, Y602) was either transformed with pBAD70 (Desany *et al.* 1998), a *TRP1* plasmid harboring the p*GAP-RNR1* construct (*mec1*Δ + p*GAP-RNR1*), or deleted for the *PTC2* gene (*mec1*Δ *ptc2*Δ*-2* and *mec1*Δ *ptc2*Δ*-3*, corresponding to two independent disruptants, MCM190 and MCM191). Independent clones were grown overnight in YPD (*mec1*Δ, *mec1*Δ *ptc2*Δ*-2*, and *mec1*Δ *ptc2*Δ*-3*) or in SD-TRP (*mec1*Δ + p*GAP-RNR1*) to stationary phase, and 10^6 cells were plated onto 5-FOA-containing plates. The values reported on the graph correspond to the number of 5-FOA-resistant clones determined after a 3-day incubation at 30°. The assays were carried out in triplicate and averaged. The standard error was ~20%.

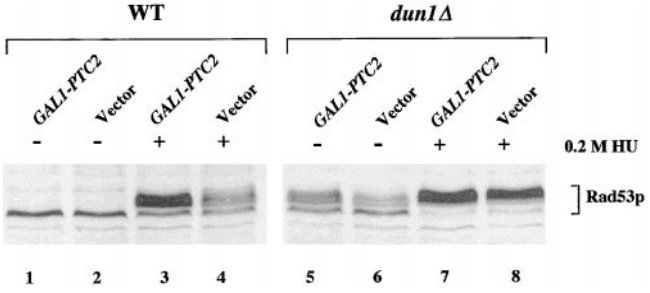

Figure 7.—*PTC2* overexpression does not affect qualitatively the phosphorylation patterns of Rad53p. Western blot analysis of total proteins from asynchronous log-phase cultures of wild-type or *dun1*Δ cells containing the empty vector pRS316 or *GAL1-PTC2*, either untreated or HU treated. Wild-type (WT, Y300) and *dun1*Δ (MCM123) cells were grown overnight in SRaf-URA medium and diluted to an OD of 0.4 in the same medium, to which galactose was added at a final concentration of 2%. The cultures were grown for an additional 2.5 hr and split into two, whereupon one-half was treated with HU (0.2 m final) and the other half was left untreated. All cells were then allowed to grow for an additional 2 hr before extraction.

transduction pathway (Maeda *et al.* 1994). Ptc1p is also involved in many other pathways, including cell separation, mitochondrial inheritance, and tRNA splicing (Robinson *et al.* 1994; Roeder *et al.* 1998). Ptc2p has so far only been shown to negatively regulate the unfolded protein response by dephosphorylating the Ire1p kinase (Welihinda *et al.* 1998). We have presented evidence suggesting a role for Ptc2p in the DNA checkpoint pathways.

It is difficult to link the suppression of RAD53-GFP lethality by the overexpression of *PTC2* to a precise target, as the specific causes of RAD53-GFP toxicity have not been elucidated. We presume that RAD53-GFP constitutively activates several pathways normally triggered in response to DNA damage or replication blocks. The simplest explanation is that Ptc2p acts as a Rad53p phosphatase but Ptc2p does not appear to be a major regulator of Rad53p overall phosphorylation.

Overexpression of *PTC2* is lethal to wild-type cells growing in the presence of 20 mm hydroxyurea, and to *rad53-21*, *mec1-21*, and *dun1*Δ, but not to *rad53*Δ *sml1-1* nor to *mec1*Δ *sml1*Δ mutants. A common property of all the cells hypersensitive to *PTC2* overexpression is an impairment of their ribonucleotide reductase activity. A simple interpretation of these results is that Ptc2p is involved in a pathway related to ribonucleotide reductase function. It could be the ribonucleotide reductase activity itself or a process depending on or related to this activity. We favor the latter hypothesis for two reasons. First, although a post-transcriptional regulation of the ribonucleotide reductase activity cannot be ruled out, *PTC2* overexpression does not affect the transcriptional activation of a *RNR3-lacZ* reporter gene after a treatment with HU (data not shown), and so it is unlikely to act directly on *RNR* gene transcription. Second, overexpression of *PTC2* in wild-type cells does not trigger the phosphorylation of Rad53p, in contrast to the presence of HU or the disruption of *DUN1*, which both impede ribonucleotide reductase activity.

DNA replication is a process dependent on ribonucleotide reductase activity in which Ptc2p could be involved. Recent articles have demonstrated that the firing of replication origins is closely related to ribonucleotide reductase activity and DNA checkpoint functions (Desany *et al.* 1998; Santocanale and Diffley 1998; Shirahige *et al.* 1998; Dohrmann *et al.* 1999). In particular, Rad53p and Mec1p act as negative regulators of the firing of replication origins (Santocanale and Diffley 1998; Shirahige *et al.* 1998). Besides, *mec1*Δ, but not *rad53*Δ, is suppressible by mutations in the Dbf4p/Cdc7p protein kinase complex that is required for origin initiation (Jackson *et al.* 1993; Desany *et al.* 1998), and the *mec1-21* mutation, but not the *rad53-21* mutation,

can suppress the temperature sensitivity of the origin-firing mutant *orc2-1* (Liang *et al.* 1995; Desany *et al.* 1998). Similarly, the deletion of *PTC2* partially suppresses the lethality of *mec1*Δ, but not of *rad53*Δ, and the HU sensitivity of *mec1* mutants, but not of *rad53* nor of *dun1* mutants. If *PTC2* played a part in DNA replication, we would expect that its deletion would impair this process and affect cell growth. However, wild-type cells deleted for the *PTC2* gene have no obvious defect in DNA replication nor in their response to HU treatment or to UV irradiation. It is possible that Ptc2p activity is redundant. Ptc2p belongs to the PP2C family of protein serine/threonine phosphatases, which numbers five other members in *S. cerevisiae*: Ptc1p, Ptc3p, Ybr125p, Yor090p, and Ycr079p (Stark 1996). In particular, *PTC2* and *PTC3* belong to two blocks of genes originating from the duplication of the yeast genome (Wolfe and Shields 1997) and they are 62% identical at the amino acid level. We found that disrupting both *PTC2* and *PTC3* had no effect on the HU sensitivity or the UV resistance of yeast cells (data not shown). However, the possibility remains that other members of the PP2C family could carry out some functions of Ptc2p in *ptc2*Δ strains.

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