Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway

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***RAD53* and *MEC1* are essential genes required for the transcriptional and cell cycle responses to DNA damage and DNA replication blocks. We have examined the essential function of these genes and found that their lethality but not their checkpoint defects can be suppressed by increased expression of genes encoding ribonucleotide reductase. Analysis of viable null alleles revealed that Mec1 plays a greater role in response to inhibition of DNA synthesis than Rad53. The loss of survival in *mec1* and *rad53* null or point mutants in response to transient inhibition of DNA synthesis is not a result of inappropriate anaphase entry but primarily to an inability to complete chromosome replication. We propose that this checkpoint pathway plays an important role in the maintenance of DNA synthetic capabilities when DNA replication is stressed.**

[*Key Words*: DNA replication; S-phase; checkpoint pathway; ribonucleotide reductase; nucleotide levels]

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The fidelity of DNA replication is critical to the proper duplication of a cell. Not only must cells replicate chromosomes, they must do so with great accuracy; without stretches of unreplicated DNA, without gaps, without replicational slippage in repetitive regions, without recombination causing rearrangements, and without breaks. S phase, the period of the cell cycle during which DNA is replicated, is a period of great vulnerability for a cell. Many complicated processes are undertaken during S phase, including the complete unwinding and replication of enormously complex DNA molecules, and chances for cataclysmic error are high. Interference with DNA replication by DNA damage, nucleotide depletion or imbalance, or polymerase malfunction can lead to a number of deleterious events, including increased mutagenesis, chromosome instability, gene amplification, microsatellite instability, and hyper-recombination (Loeb and Kunkel 1982). Each of these events can have severe consequences for an organism, including cell death, birth defects, and cancer. A number of factors cooperate to ensure the fidelity of DNA replication. These include processivity factors, proofreading functions, mismatch repair proteins, a variety of DNA repair activities, and regulatory pathways that sense DNA damage and replicational stress (Loeb and Kunkel 1982). For example, in response to DNA damage and DNA replicational interference, cells induce the transcription of genes that enhance repair capacities and arrest cell cycle progression to provide time for these repair processes to occur (for review, see Elledge 1996). This ensures that DNA replication and segregation—the critical events that allow genetic damage to become irreversibly inherited—are delayed until optimal repair can be achieved. In eukaryotes, these regulatory pathways are called checkpoints.

Checkpoint pathways ensure the proper order and timing of cell cycle events, and compromising these pathways contributes to genomic instability and cancer. The outline of the DNA damage response checkpoint pathway in mammals is emerging. *ATM* (ataxia telangiectasia mutated), a central player, is a member of the lipid kinase family of proteins and is likely a transducer of a DNA damage signal (for review, see Elledge 1996). *ATM* controls the timely activation of p53, a transcription factor that activates transcription of the cdk inhibitor p21 (Kastan et al. 1992). Cells defective for any of these genes show a defect in G_1 arrest in response to DNA damage, and *ATM* mutants are also defective in G_2 arrest and display radioresistant DNA synthesis. The roles of *p53* and *ATM* in tumorigenesis underscore the importance of the DNA damage response to organismal homeostasis. In the case of *ATM*, there are additional phenotypes that include specific neural degeneration (Friedberg et al. 1995; Meyn 1995). Recently, an additional mammalian checkpoint gene encoding a protein kinase, Chk1, has been identified (Flaggs et al. 1997; Sanchez et al. 1997). Mammalian Chk1 is phosphorylated in response to DNA damage and is capable of phosphorylating Cdc25C on an inhibitory serine residue (Peng et al. 1997; Sanchez et al. 1997). The fission yeast Chk1 homolog acts downstream of the *ATM* homolog Rad3 (Walworth et al. 1993; Ford et al. 1994; Carr et al. 1995, Walworth and Bernards 1996; Furnari et al. 1997).

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In the budding yeast *Saccharomyces ceriviseae* a number of genes have been identified that control the ability of cells to arrest the cell cycle and/or activate the transcriptional response. Upstream regulators involved in early steps in this pathway include *RAD9*, *RAD17*, *RAD24*, and *MEC3*, which are required for cell cycle arrest in G_1 and G_2 in response to DNA damage. *POL2*, encoding DNA polymerase 2, *DPB11*, and *RFC5* are upstream components of the cell cycle arrest and transcription pathways that respond to replication blocks (Elledge 1996). Checkpoint signal transducers include *MEC1* and *RAD53*, which are required for the S-phase checkpoint as well as the transcriptional and G_1 and G_2 arrest responses to DNA damage (Allen et al. 1994; Kato and Ogawa 1994; Weinert et al. 1994). *DUN1*, which encodes a protein kinase that is activated in response to DNA damage and replication blocks in a *MEC1*- and *RAD53*-dependent manner (Allen et al. 1994), is necessary for the transcriptional response (Zhou and Elledge 1993) and plays a partial role in the G_2 arrest in response to DNA damage (Pati et al. 1997). *MEC1* belongs to the same subfamily of proteins as *ATM*, underscoring the evolutionary conservation of this pathway (Greenwell et al. 1995; Morrow et al. 1995). *MEC1* and *TEL1* regulate the phosphorylation of the Rad53p kinase in response to DNA damage and replication blocks (Sanchez et al. 1996; Sun et al. 1996).

Whereas *MEC1* and *RAD53* control both the transcriptional and cell cycle responses to DNA damage and replication blocks, it is not clear whether these are the only roles these proteins carry out or whether these proteins play equivalent roles in these responses. In addition, the issue of whether these genes coordinate DNA replication and mitosis in an unperturbed cycle or only in response to replicational stress remains to be resolved. Both genes are essential for viability, perhaps suggesting a role for the checkpoint in each cell cycle, but to date their essential roles have remained obscure. In this study we sought to determine the essential functions of *RAD53* and *MEC1* by isolation of dosage suppressors of the null allele of *rad53*. We have discovered that increasing dNTP synthetic capacity can suppress both *rad53* and *mec1* null alleles. Furthermore, the primary lethal defect in these mutant strains in response to nucleotide depletion is not mitotic entry but a profound defect in the ability to finish chromosomal replication. We propose that one of the roles of this checkpoint pathway is the stabilization of replication structures under conditions of replication inhibition.

Results

RNR1 *overexpression suppresses* Δrad53 *and* Δmec1 *lethality*

To investigate the essential function of the S-phase checkpoint, we selected dosage suppressors of the lethality associated with a deletion of *RAD53*. A *TRP1* 2μ *S. cerevisiae* cDNA library under control of the *GAL1* promoter (Mulligan and Elledge 1994) was constructed in λTRP, converted to plasmid form by *cre–lox* automatic subcloning (Elledge et al. 1991) and used to transform a *rad53* null strain, Y324, being kept alive by *RAD53* on a *URA3 CEN* plasmid, pJA92 (Allen et al. 1994). Transformants were selected on synthetic complete medium lacking tryptophan (SC − Trp), with galactose as a carbon source to induce cDNA expression, and replica plated onto the same medium containing 5-fluoro-orotic acid (5-FOA) to select for strains able to grow in the absence of pJA92. We subsequently examined the ability of these 5-FOA^r transformants to grow with glucose as the carbon source. Because *GAL*-driven *RAD53* is capable of sustaining cell growth under repressed conditions (glucose), choosing only clones that exhibited partial galactose dependence eliminated both the *RAD53* background and any plasmid-independent extragenic suppressors. Twelve clones were at least partially dependent on galactose for suppression of Δ*rad53*. These plasmids were sequenced and the identities of the encoded genes are listed in Table 1, along with the efficiency with which they suppress the growth defect of *rad53* deletion mutants. We called those genes *SRL*, for suppressors of *rad53* lethality. A variety of genes are capable of suppressing Δ*rad53* to varying extents, including a number of transcription factors, both positive and negative. Those suppressors are likely to rescue the lethality indirectly, through effects on the transcription of other genes. Two suppressors are putative 26S proteasome components and are also likely to be indirect suppressors that act by changing the stability of other proteins that suppress the lethality of the *rad53* deletion. Other suppressors consist of a protein kinase (*MCK1*), a putative chaperone (*PDR13*), and the regulatory subunit of ribonucleoside diphosphate reductase (*RNR1*). The remainder, designated *SRL1*, *SRL2*, and *SRL3*, show no similarity to other proteins in the database.

RNR1 *overexpression suppresses* mec1, *indicating a common essential function for* RAD53 *and* MEC1

Because *RAD53* and *MEC1* operate in the same checkpoint pathway (Sanchez et al. 1996; Sun et al. 1996), it is possible that they are essential for the same reason. In an effort to determine whether these genes have the same essential function, we examined the *SRL* genes for their ability to suppress Δ*mec1* lethality. Most of the suppressors were capable of suppressing the *mec1* deletion mutant, albeit poorly. Only one plasmid was able to efficiently suppress both the *rad53* and *mec1* deletion mutants (Table 1). This plasmid contained the *RNR1* gene encoding a predicted protein product starting with amino acid 22 of Rnr1 and continuing to the end of the 888-amino-acid protein. *RNR1* was also shown to suppress the lethality of the Δ*mec1*Δ*tel1* and Δ*mec1*Δ*rad53* double mutants (data not shown). *RNR1* is an essential gene that encodes the large subunit of ribonucleoside diphosphate reductase (RNR), the rate-limiting enzyme of deoxyribonucleotide synthesis and the target of the DNA synthesis inhibitor hydroxyurea (HU). *RNR1* is both inducible by DNA damage and tightly cell cycle

Table 1. *Summary of* rad53 *and* mec1 *deletion suppressors*

ORF name	Gene name	Function	Strength of suppression[a] of		ORF size (nt)	Portion cloned (nt)[b]
			rad53	*mec1*		
YBR112c	*SSN6/CYC8/CRT8*	transcriptional repressor	weak	poor	2898	1–600
YDR173c	*ARGR3/ARG82*	transcriptional repressor/activator	good	weak	1065	entire
YER070w	*RNR1/CRT7*	ribonucleoside diphosphate reductase	strong	strong	2664	66–end
YHR064c	*PDR13*	drug resistance, Hsp70 family	weak	weak	1716	82–end
YJL110c	*GZF3/NIL2*	transcriptional repressor	weak	poor	1653	637–end
YKR091w	*SRL3*		weak	poor	456	entire
YLR082c	*SRL2*		good	strong	1176	entire
YNL307c	*MCK1*	meiotic protein kinase	poor	poor	1125	entire
YOR247w	*SRL1*		weak	poor	630	entire
YOR259c	*RPT4/SUG2*	SPB duplication, 26S proteasome	good	poor	1311	35–end
YOR261c	*RPN8*	26S proteasome	good	poor	1014	76–880
YPL129w	*ANC1/TFG3*	transcription factor	poor	weak	731	161–end

[a]Strength of suppression was judged qualitatively by the ability to grow on 5-FOA after allowing the wild-type *RAD53 URA3* or *MEC1 URA3* plasmid to be segregated.
[b]The extent of each ORF that was contained on each library plasmid was approximated using the sequence of the 5′ end of the insert and the size of the insert, which was approximated by gel electrophoresis. (nt) Nucleotide.

regulated (Elledge and Davis 1990). A gene encoding an alternative large subunit of Rnr, *RNR3*, is a target gene of the DNA damage and replication interference response pathways and is 80% identical to *RNR1* at the amino acid level. We found that full-length *RNR1* and *RNR3* are both able to efficiently suppress *Δrad53* and *Δmec1* when expressed from the constitutive *GAP* promoter on a 2μ plasmid (p*GAP–RNR1*, p*GAP–RNR3*; Fig. 1A).

Low levels of ectopic RNR1 *can suppress lethality*

To examine whether up-regulation of *RNR1* or *RNR3* was the mechanism through which the other suppressors functioned, Northern analysis was performed on total RNA isolated from asynchronously growing cultures of each suppressed *Δrad53* strain. There were no large increases in either *RNR1* or *RNR3* mRNA levels between wild-type cells and the suppressors (Fig. 1B–D), with the exception of Ssn6. There is a three-fold increase in *RNR3* expression when truncated Ssn6 protein is expressed. *RNR3* is negatively regulated by *SSN6* (Zhou and Elledge 1992); therefore, this truncated Ssn6 might be acting as a dominant-negative mutant. The more general failure to detect strong differences in *RNR* transcription does not completely rule out altered *RNR* expression as a mechanism of suppression because very low amounts of exogenously supplied Rnr1 are still capable of suppressing *Δrad53*. For example, *RNR1* under *GAL1* control can still suppress when grown on glucose (data not shown). Additional support comes from the fact that one additional copy of the *RNR1* gene under its own promoter is capable of efficient suppression, indicating that a twofold increase in *RNR1* gene dosage is sufficient for suppression.

Mec1 has a greater role than Rad53 in response to genotoxic stress

Mec1 and Rad53 are both required for the transcriptional and cell cycle arrest responses to DNA damage and replication blocks. However, it was unclear whether they were equivalent in these functions because only hypomorphic alleles could be compared because of their essential nature. Having common suppressors of *mec1* and *rad53* null mutations allowed us to examine the phenotypes associated with a complete loss of function. In addition to defects in cell cycle arrest and transcriptional responses, previously isolated point mutants of *RAD53* and *MEC1* show a high degree of sensitivity to UV and ionizing radiation, radiomimetic drugs, and HU. *Δrad53* + p*GAP–RNR1* cells show the same degree of sensitivity to HU and UV irradiation as *rad53-21* point mutants (Fig. 2A,B, circles). In addition, analysis of spindle elongation in α-factor-synchronized *rad53-21* and *Δrad53* cells released into media containing HU indicated that both of these alleles confer equivalent defects in the S-phase checkpoint (Fig. 2C,D, circles). The *rad53* null mutant actually exhibits a slower rate of accumulation of anaphase-like spindles, but this parallels the slower rate of budding that is also observed under these conditions (Fig. 2C, circles). Thus, although *RNR1* suppresses the lethality of *Δrad53*, it is unable to suppress the checkpoint and DNA damage sensitivity associated with loss of Rad53 function. This suggests that *RNR1* overexpression allows *rad53* (and *mec1*) null cells to tolerate an altered cellular physiology, rather than restoring function to the *MEC1 RAD53* pathway.

Δmec1 + p*GAP–RNR1* cells are also defective in the response to DNA damage and replication blocks but more so than the *mec1-21* point mutant, suggesting that *mec1-21* is still partially competent in some of its responses. When the *mec1* and *rad53* null strains are compared, it is clear that the *Δmec1* mutant is significantly more UV- and HU-sensitive (Fig. 2A,B). This indicates that *MEC1* has a greater role in response to DNA damage than does *RAD53*, which is consistent with the fact that Rad53 is downstream of Mec1 in the pathway and indi-

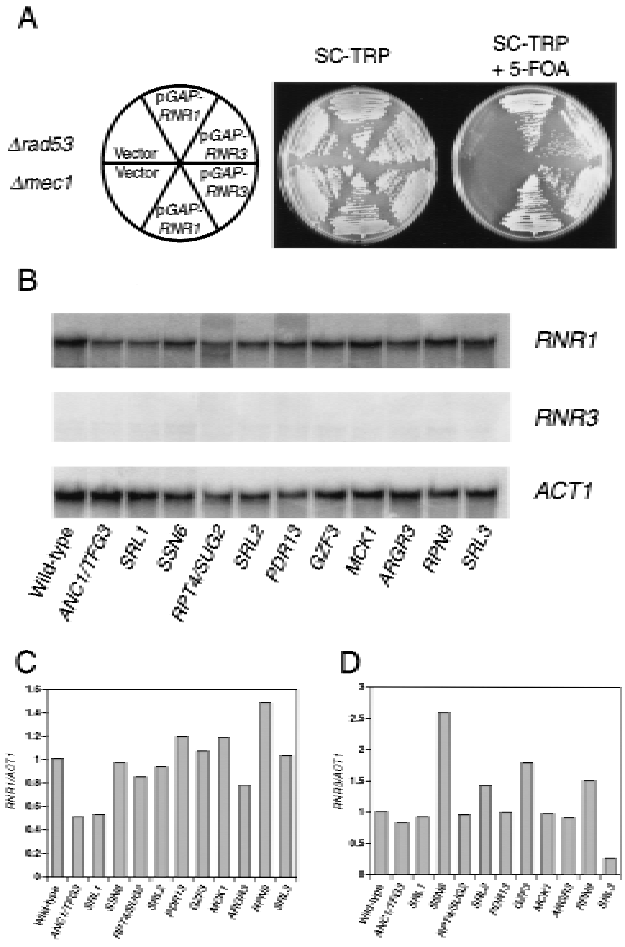

Figure 1. Suppression of null alleles of *rad53* and *mec1* by overproduction of *RNR1* and other genes. (*A*) *RNR* suppression of *Δrad53* and *Δmec1*. Y601, a *Δrad53* mutant containing a wild-type copy of *RAD53* on a *URA3* plasmid, and Y602, a *Δmec1* mutant containing a wild-type copy of *MEC1* on a *URA3* plasmid, were transformed with a *TRP* plasmid carrying *GAP*-controlled *RNR1* (pBAD70) or *RNR3* (pBAD79), or empty vector (pBAD54) as indicated. These transformants were struck onto SC − Trp and SC − Trp + 5-FOA to assess the ability of the null alleles to grow in the presence of the RNR expression plasmids. (*B*) *RNR1* and *RNR3* levels in suppressed *Δrad53* strains. Y81 (wild-type) and Y324 (*Δrad53*) strains containing the indicated suppressors were grown to log phase in YPGal at 30°C. Total RNA was prepared and Northern blot analysis was performed using *RNR1* (*top*)-, *RNR3* (*middle*)-, or *ACT1* (*bottom*)-specific probes (see Materials and Methods). (*C*, *D*) PhosphorImager quantitation of the Northern blots presented in *B*. The amount of *RNR1* (*C*) and *RNR3* (*D*) transcript was first normalized to the amount of *ACT1* transcript present in each strain and then to the amount of *RNR1* or *RNR3* present in wild-type cells.

cates that *MEC1* has functions in addition to its regulation of Rad53. However, inappropriate spindle elongation in the presence of HU by the *mec1* null mutant occurs to the same extent as the *rad53* null mutant (Fig. 2D). This suggests that the greater degree of lethality experienced by the *mec1* null mutant in HU may be independent of the defect in preventing anaphase entry. The possibility that it is an event other than aberrant spindle elongation that commits checkpoint-defective cells to death is addressed further below.

Probing the essential function of Rad53 and Mec1

To examine the possibility that the lethal defect in *Δrad53* and *Δmec1* mutants during an otherwise normal cell cycle is low or aberrant *RNR1* expression, we measured the accumulation of endogenous *RNR1* mRNA after release from an α-factor block in strains deleted for *mec1* containing additional *RNR1* under *GAP1* control (*TRP1::GAP–RNR1*) (see Materials and Methods). To specifically detect endogenous *RNR1* mRNA, we used a probe specific for the 3′-untranslated region of the *RNR1* gene that was absent in the *TRP1::GAP–RNR1* expression cassette. Endogenous *RNR1* expression in a population of *Δmec1 TRP1::GAP–RNR1* cells synchronously moving through the cell cycle was compared with that of a *MEC1 TRP1::GAP–RNR1* strain. Although the mutant accumulates appreciable amounts of *RNR1* transcript, that accumulation is delayed and occurs at a slower rate than that of wild type (Fig. 3B). By the time *RNR1* levels start to decline in *Δmec1 TRP1::GAP–RNR1*, there is approximately a 15 to 20 minute difference between it and wild type. A similar phenomenon is observed in *Δrad53 TRP1::GAP–RNR1* cells (Fig. 3A). To determine whether these differences were due to a defect in *RNR1* expression in the mutants or to a general cell cycle perturbation, we examined three other indicators of cell cycle progression. Figure 3C shows the expression profile of *CLN2* mRNA out of α-factor arrest. Like *RNR1*, *CLN2* expression in the *Δmec1 TRP1::GAP–RNR1* strain is delayed relative to *MEC1 TRP1::GAP–RNR1* cells, with the peak occurring ~15 minutes later. The budding profile of the *mec1* null mutant also shows a delay (Figs. 3D and 2C), indicating a delayed passage through start after α-factor arrest. Finally, the FACS profiles (Fig. 3E) clearly show that the mutant cells enter S phase later than, and persist in S phase longer than, the control cells. These results demonstrate that the *MEC1* pathway plays a complex role in the cell cycle, affecting several aspects of cell cycle regulation. However, whereas the regulation of *RNR1* is altered, it appears to be a secondary effect of a general cell cycle perturbation and not a specific target of the *MEC1/RAD53* pathway. If the apparent delay and reduced expression of *RNR1* was not an artifact of general cell cycle perturbation, then *RNR1* levels should also be lower in asynchronous cultures. *RNR1* appears to be expressed at wild-type levels in asynchronous cultures of *rad53* and *mec1* null mutants kept alive with *RNR3* (Fig. 3F), supporting the notion that the altered *RNR1* expression in the synchrony experiment is simply a reflection of the slower kinetics of cell cycle progression.

RNR1 *overproduction does not enhance the rate of DNA replication*

Because low levels of additional *RNR1* expression are

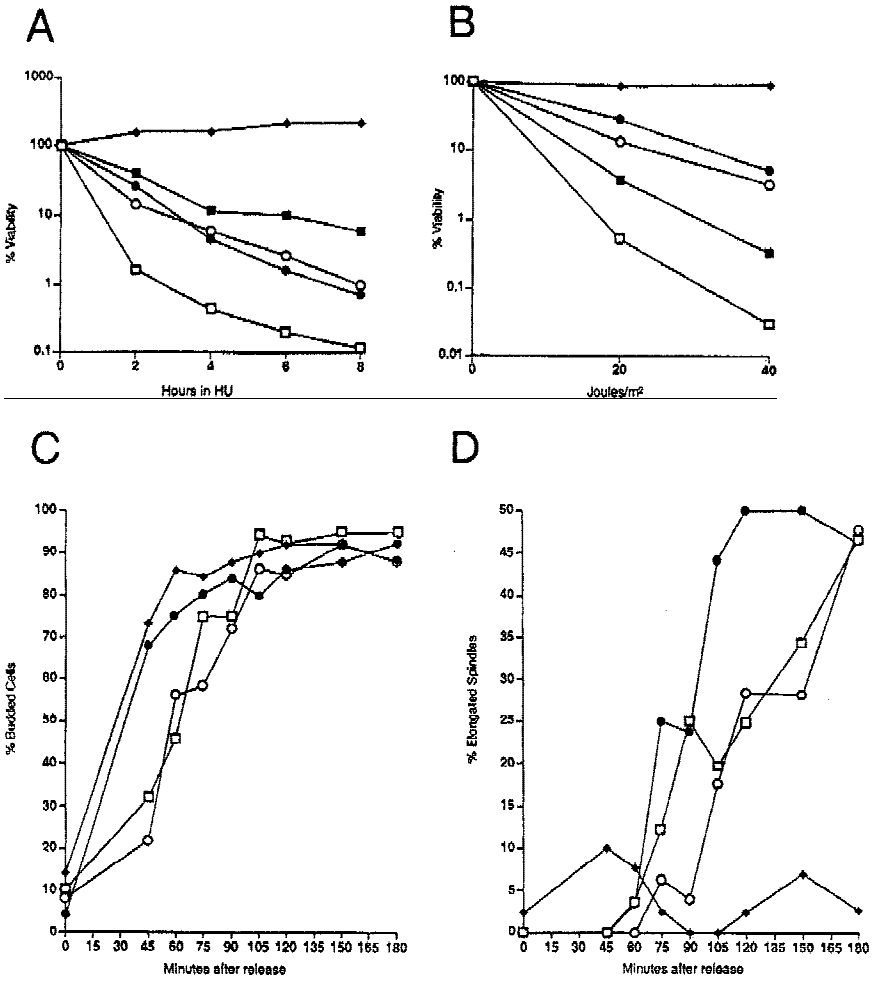

Figure 2. Characterization of checkpoint deficiency of *rad53* and *mec1* null mutants. (*A*) Viability in HU of *mec1* and *rad53* null mutants compared to point mutants. Asynchronously growing log phase cultures were treated with 0.2 M HU. Aliquots were removed at timed intervals to determine cell number and to score for viable colony-forming units on YPD plates. The strains used were Y80 (wild type, ♦), Y301 (*rad53-21*, ●), Y603 (*Δrad53 + pGAP–RNR1*, ○), Y604 (*mec1-21*, ■), and Y605 (*Δmec1 + pGAP–RNR1*, □). (*B*) UV sensitivity of *mec1* and *rad53* null mutants compared to point mutants. The same strains as in *A* were grown asynchronously to log phase at 30°C and plated onto YPD. The plates were irradiated at 0, 20, or 40 J/m^2, and surviving colony-forming units were calculated. (*C*) Budding profiles of checkpoint mutants in HU following release from an α-factor block. Log-phase yeast cultures were incubated at 30°C in YPD supplemented with 10 μg/ml α-factor for 3 hr. To release from the block, cultures were washed into YPD lacking α-factor but containing 0.2 M HU, and aliquots were removed at timed intervals and scored for the presence of a bud. The strains used were Y580 (*RAD$^+$ MEC$^+$ TRP1::GAP–RNR1*, ♦), Y301 (*rad53-21*, ●), Y606 (*Δrad53 TRP1::GAP–RNR1*, ○), and Y581 (*Δmec1 TRP1::GAP-RNR1*, □). (*D*) Kinetics of spindle elongation of checkpoint mutants in HU following release from α-factor. Samples were taken from the same experiment as in *C* and stained with anti-α-tubulin antibodies. Cells were scored for the presence of an elongated mitotic spindle by indirect immunofluorescence.

capable of suppressing the lethality of *mec1* and *rad53* mutants, we entertained two general hypotheses for how this suppression might work. The first is based on the assumption that because *MEC1* and *RAD53* coordinate S-phase completion and mitosis under certain circumstances, their loss may allow S phase and mitosis to occur based on their natural timing, akin to a race between S phase completion and mitotic onset. Thus, by adding additional nucleotides S phase may be shortened to the point where it is completed prior to a lethal mitosis. The second hypothesis is that the *MEC1/RAD53* pathway provides a function other than cell cycle coordination, such that the loss of Rad53 and Mec1 creates a special nucleotide stress or a greater sensitivity to normal nucleotide levels—levels that may be suboptimal for DNA polymerization or fork stability. Because *rad53* mutants are sensitive to low nucleotide levels, we know that nucleotide depletion is toxic. Although the HU sensitivity is generally assumed to be due to inappropriate mitotic entry, this has not been rigorously demonstrated and other explanations exist. For example, nucleotide depletion sensitivity could result from the occasional disassembly of a paused replication complex searching for nucleotides, and *MEC1/RAD53* might help to restore the function of these (transiently) nucleotide-starved complexes. Providing additional nucleotides in the form of *RNR* overexpression might prevent this stress from occurring. In both hypotheses, *RNR1* overexpression suppresses by providing extra dNTPs; in the first case, the dNTPs would suppress by accelerating the rate of S-phase completion, whereas in the second case they would suppress by preventing a cataclysmic response to perceived nucleotide depletion by reversing that depletion.

To test the first hypothesis, we examined whether S phase was shorter in wild-type cells overproducing *RNR1* under *GAP* control. Cells were arrested in G_1 with α-factor, released from the block, and their DNA content was measured by FACS analysis at 2 min intervals. Although a very small effect cannot be ruled out, the overexpression of *RNR1* had no apparent effect on the timing of S-phase completion or the overall rate of DNA synthesis (Fig. 4).

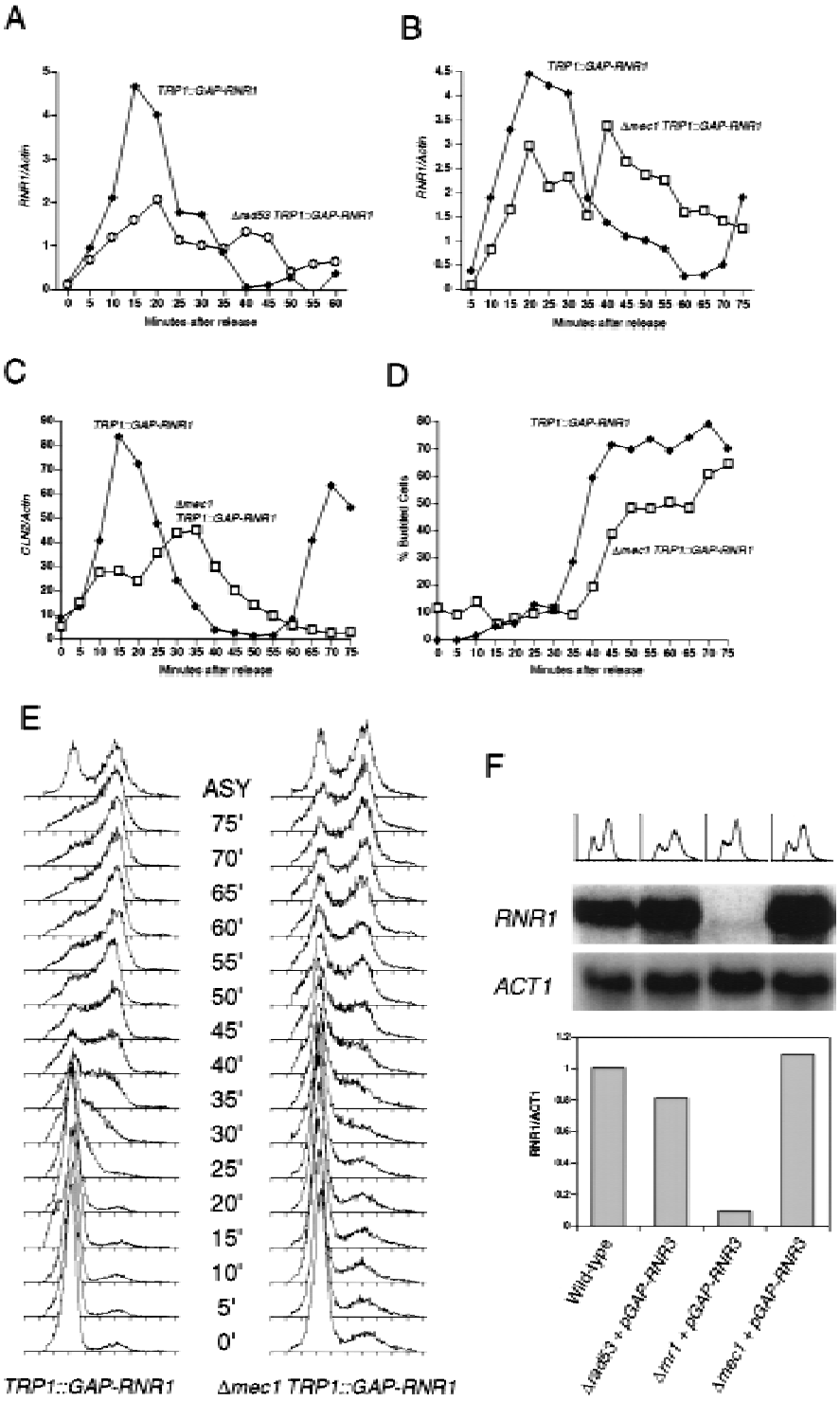

Figure 3. Kinetics of cell cycle events in *rad53* and *mec1* deletion mutants. (*A*) Accumulation of endogenous *RNR1* mRNA in a *rad53* deletion mutant. Y607 (*RAD*$^+$ *TRP1::GAP–RNR1*, ♦) and Y606 (*Δrad53 TRP1::GAP–RNR1*, ○) were grown at 30°C to log phase and arrested with 10 μg/ml α-factor for 3 hr. Upon release into YPD, aliquots were taken and total RNA was prepared and blotted. The blot was probed with DNA specific to the endogenous *RNR1* transcript and also to *ACT1* for normalizing to the total amount of RNA in each lane (see Materials and Methods). Quantitation was performed using ImageQuant and the values obtained for each time point were plotted as a function of minutes after α-factor release. (*B–E*) The data presented in parts *B–E* all come from the same experiment and employed strains Y580 (*MEC*$^+$ *TRP1::GAP–RNR1*, ♦) and Y581 (*Δmec1 TRP1::GAP–RNR1*, □). (*B*) Accumulation of endogenous *RNR1* mRNA in the *mec1* null. RNA was harvested, blotted, and probed and quantitated as in *A*. (*C*) Accumulation of *CLN2* mRNA in the *mec1* deletion mutant. The blot used in *B* was stripped and reprobed with DNA specific to the *CLN2* transcript (see Materials and Methods). (*D*) Budding profile of the *mec1* null mutant. A small aliquot of the cells used in *B* and *C* was retained for visual analysis of bud growth. The data are represented as the percentage of the total cells that have elaborated a bud at the indicated times. (*E*) DNA content of *Δmec1* cells as they progress through the cell cycle upon release from an α-factor block. A portion of each aliquot used in parts (*B–D*) was stained with propidium iodide and analyzed by flow cytometry (see Materials and Methods). (*F*) Overall *RNR1* mRNA levels in asynchronously growing *rad53* and *mec1* null mutants suppressed by high copy *RNR3*. Strains were grown to log phase at 30°C in YPD. Total RNA was purified from harvested cells, blotted, and probed with DNA specific for *RNR1* and *ACT1*, as noted. Abundance of *RNR1* transcript was calculated as noted in *A* and *B* and is represented in the bar graph below the autoradiograms. Above each lane in the autoradiograms the FACS profile of each strain is placed at the time the cells were harvested, indicating that there is a similar cell cycle distribution between them and validating the comparison of mRNA levels. The strains employed were Y692 (*TRP*$^+$ *MEC*$^+$), Y608 (*Δrad53* + p*GAP–RNR3*), Y609 (*Δrnr1* + p*GAP–RNR3*), and Y610 (*Δmec1* + p*GAP–RNR3*). Y609 is a deletion of *RNR1* that is suppressed by overexpression of *RNR3*. This provides a control for the specificity of the *RNR1* probe used in this experiment.

Delaying mitosis cannot rescue the lethality of mec1 *and* rad53 *null mutants*

If the outcome of a race between S phase and mitosis determines lethality, the result could be influenced not only by making S phase happen faster but also by delaying mitosis. To test this we examined the effects of agents capable of delaying mitosis on *mec1* and *rad53*

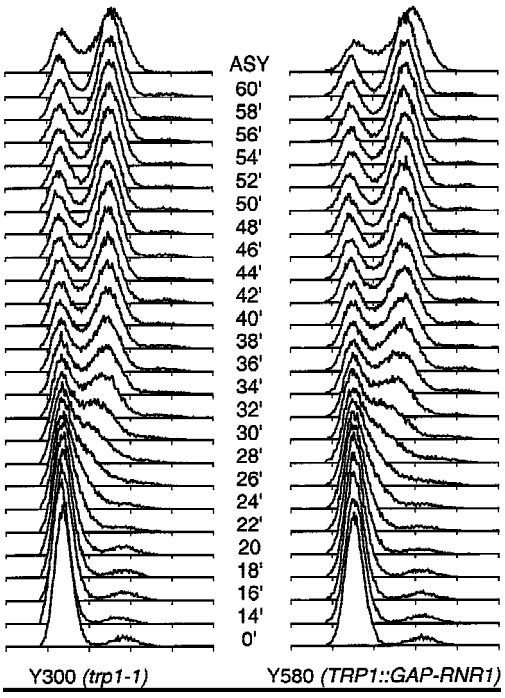

Figure 4. *RNR1* overproduction does not accelerate progression though S phase. DNA replication timing of Y300 (wild type, *trp1-1*) and Y580 (*TRP1::GAP–RNR1*) strains is shown. Cells were grown to log phase at 30°C and arrested with 10 μg/ml α-factor for 3 hr. Upon release from the α-factor block into YPD, samples were taken at close intervals and stained with propidium iodide and analyzed by flow cytometry for the purpose of detecting subtle differences in the rate of replication due to *RNR1* overproduction.

mutants. We germinated spores from a *Δrad53::HIS3/RAD53* heterozygous diploid on media containing sublethal amounts of benomyl (15 μg/ml), which delays mitosis through activation of the mitotic spindle assembly checkpoint (Elledge 1996). No His^+ colonies were viable under these conditions. We also streaked *rad53* null cells containing *RAD53* on a *URA3 CEN* plasmid (pJA92) onto media containing 5-FOA and 15 μg/ml benomyl but observed no increase in the appearance of 5-FOA^r colonies relative to the absence of benomyl. *mec1* and *rad53* null mutants are extremely sensitive to low HU levels on plates. We identified the minimal concentration of HU that blocked growth on plates (5 mM) and attempted, unsuccessfully, to suppress the lethality of either mutant with 15 μg/ml benomyl.

Because survival was measured as growth on plates in previous experiments, the concentration of microtubule inhibitors employed was necessarily not sufficient to completely block mitosis, and low levels of suppression might be obscured as a result. To examine this more thoroughly, we tested the ability of a sustained mitotic block to allow *rad53* mutants to recover from a transient HU block (Fig. 5A). *rad53-21* mutants were released from a G_1 block into media containing 0.25 M HU. After 30 min, the HU was washed out and the cells were resuspended in media containing 80 μg/ml benomyl with no HU, and viability was measured over time. Blocking mitosis with benomyl was unable to restore any measure of viability. The inability of benomyl to rescue either the lethality of the null, or the sensitivity of either the null or the point mutant to HU, suggests that the lethal event may be the same in each case (the consequence of nucleotide depletion) and unrelated to whether or not cells are allowed to proceed into mitosis. This is consistent with the fact that in *rad53* and *mec1* null mutants, loss of viability in HU does not correlate with the degree of spindle elongation (Fig. 2A,D).

rad53 *mutants fail to complete DNA replication after a transient replication block*

As the cause of lethality in null mutants and HU-treated null and point mutants does not appear to be solely due to the relative timing of S phase and mitosis, it is likely that in *rad53* and *mec1* null cells a lethal event is occur-

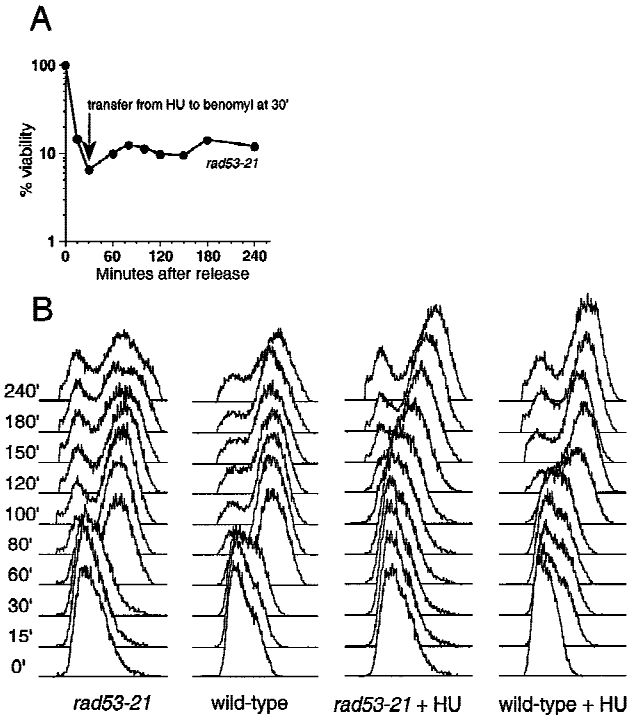

Figure 5. Inability of a microtubule inhibitor to suppress the lethality of *rad53* mutants transiently exposed to HU. (*A*) Sensitivity of *rad53-21* to HU in the presence of benomyl. A *rad53-21* strain, Y301, was released from α-factor arrest into 0.25 M HU for 30 min. Following this transient incubation the culture was maintained in 80 μg/ml benomyl, and timed aliquots were plated onto YPD for measurement of viable colony-forming units. (*B*) FACS analysis of Y301 (*rad53-21*) and Y300 (wild-type) cultures that had been transiently treated with HU. Wild-type and *rad53-21* cultures were released from the G_1 block into either 0.25 M HU for 30 min or medium lacking HU, as indicated. At 30 min after α-factor release, cells were washed and transferred into YPD containing 80 μg/ml benomyl. Progress through S phase was monitored by FACS at the indicated time points.

ring that commits the cells to death regardless of the timing of the subsequent mitosis. As we described earlier, one such event could be defective DNA replication caused by a condition of nucleotide depletion. To determine whether mutant cells transiently arrested with HU did in fact have difficulty finishing DNA replication after removal of the replication block, we examined DNA content in *rad53-21* and wild-type cells under these conditions. Although the *rad53* mutant showed a delay in replicating its DNA relative to wild-type cells transiently treated with HU, it eventually accumulated with an approximately G_2 DNA content (Fig. 5B), indicating that it recovered the ability to produce sufficient dNTP levels to replicate a genome's worth of DNA.

Because FACS analysis cannot determine to what extent mitochondrial DNA contributes to the amount of G_2 DNA observed in this experiment, we performed a similar experiment in ρ^0 *rad53-21* mutant strains (Fig. 6A). ρ^0 *rad53-21* mutants were released from α-factor into 0.2 M HU and 10 μg/ml nocodazole, the HU was washed away after 1 hr, and samples were analyzed for DNA content for up to 3 hr (Fig. 6B, bottom). Under transient HU-treatment conditions that resulted in 75% lethality (Fig. 6A), we observed the same accumulation of apparent G_2 DNA content as in the ρ^+ strains (cf. Figs. 6B and 5B). The control experiment in the absence of HU (Figs. 5B and 6B, top) indicates that the effect is specific to HU. The observed delay in replication in *rad53-21* mutants was not unexpected because *rad53* mutants are unable to induce expression of the *RNR1*, *RNR2*, *RNR3*, and *RNR4* genes to quickly increase nucleotide biosynthetic capacity (Allen et al. 1994; Huang and Elledge 1997). Alternatively, the delay could be due to the presence of lesions that occur in the transiently nucleotide-starved cells (e.g., stalled replication complexes or abandoned replication forks) that persist and impede the function of the active replication complexes that subsequently encounter them. These data confirm that *rad53-*

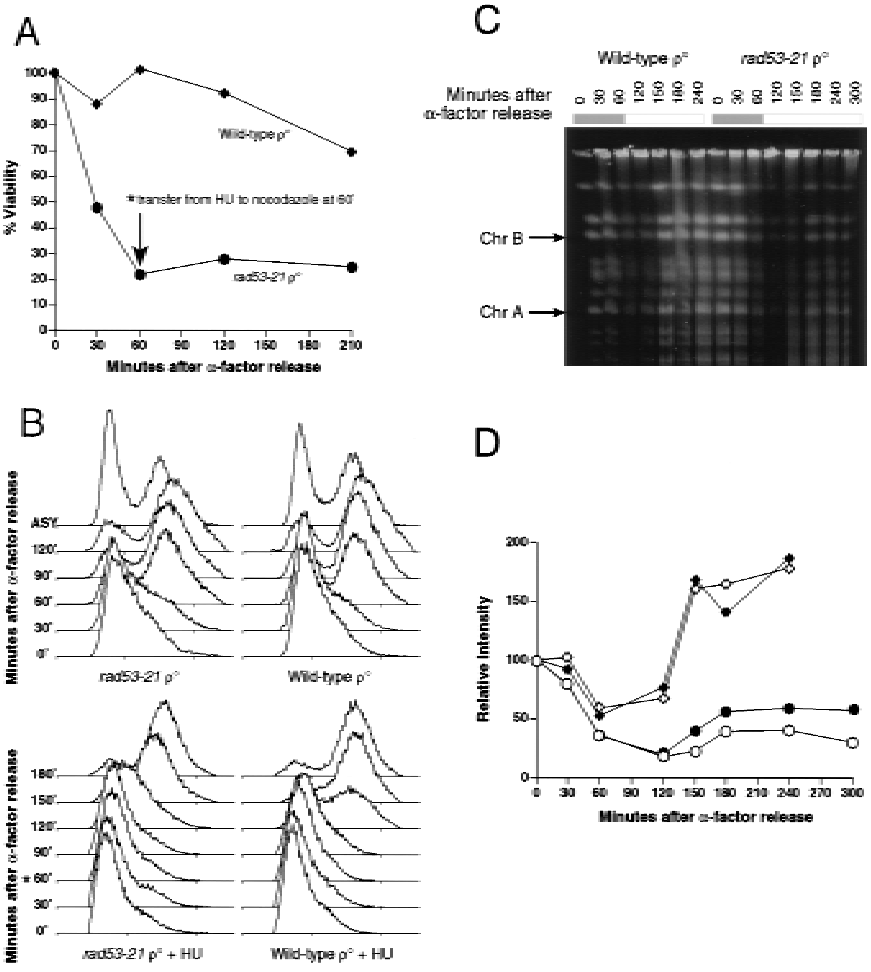

Figure 6. Inability of ρ^0 *rad53-21* mutants to complete chromosomal replication after a transient HU treatment. Y623 (wild-type ρ^0) and Y624 (*rad53-21* ρ^0) cells were arrested in α-factor for 3 hr and washed into YPD media containing either 10 μg/ml nocodazole or 0.2 M HU and nocodazole. After a 60-min incubation, cells were washed and resuspended into YPD medium containing 10 μg/ml nocodazole only and monitored for viability (*A*), DNA content (*B*), and chromosome integrity (*C*,*D*). (*A*) Sensitivity of *rad53-21* (●) to transient HU treatment in the presence of nocodazole. Wild-type (♦) is shown for comparison. (*B*) Flow cytometric analysis of the DNA content of wild-type and *rad53-21* strains. Transiently HU-treated cultures are shown at *bottom*, with the asterisk (*) indicating the time at which the cells were washed out of HU. (*Top*) Cultures released from α-factor into nocodazole only. (*C*) CHEF gel of chromosomes from wild-type (*left*) and *rad53-21* (*right*) strains transiently treated with HU. The vertical bar over each lane indicates time points at which HU was present (shaded bars) or had been washed out (open bars). The two chromosomes that were used in part (*D*) are indicated. (*D*) Quantitation of replication of chromosomes from wild-type [ρ^0 Chr A (♦) and ρ^0 Chr B (◇)] and *rad53-21* [rad53-21 ρ^0 Chr A (●) and *rad53-21* ρ^0 Chr B (○)] cultures that had been transiently treated with HU and resolved by CHEF in *C*. The two chromosomes examined are indicated in *C*. The amount of fully duplicated chromosomes in the *rad53* mutants precisely correlates with the percentage survival. Intensities of the bands were quantitated using NIH Image software and plotted as a function of time after release from α-factor and plotted in arbitrary units.

21 cells are delayed but not deficient in restoring DNA synthetic capability after transient HU treatment. However, the cells are clearly dying, and forestalling mitosis with microtubule destabilizing drugs has no effect on this.

FACS analysis measures only bulk DNA content, and it cannot determine whether a small percentage of the DNA is unreplicated or, in the case of the previous experiment, whether the apparently replicated chromosomes at the end of the experiment are intact. To examine the integrity of chromosome structure, we employed pulsed-field gel electrophoretic (PFGE) analysis. Incompletely replicated chromosomes fail to enter a pulsed-field gel because of the presence of forks and replication bubbles that impede migration (Hennessy et al. 1991). Chromosomal DNA was prepared from the cultures of wild-type ρ^0 and *rad53-21* ρ^0 mutant cells that had been treated transiently with HU and kept in the presence of nocodazole. At timed intervals, DNA from these cells was prepared and examined by PFGE (Fig. 6C) and quantitated densitometrically (Fig. 6D) (see Materials and Methods). Transient HU treatment delayed the re-entry of chromosomes from wild-type cells, consistent with the kinetics observed by FACS analysis. In contrast, chromosomes from the *rad53* mutant never re-entered the gel, even during a 6-hr mitotic block. Similar results were obtained with *mec1* mutants (data not shown). Quantitation of the intensities of two chromosome bands, designated A and B, shows that wild-type chromosomes double in intensity from 150 min, indicating completed replication. *rad53* chromosomes reappear at 180 min at half the original intensity, indicating that a quarter of the population has properly completed DNA synthesis, consistent with the survival data. This indicates that in addition to experiencing a significant delay in the recovery of bulk DNA synthetic capacity, when the *rad53* mutant's chromosomes do eventually become apparently fully replicated (by FACS analysis), they have a profoundly abnormal structure (by PFGE).

Genetic interactions between the checkpoint and origin initiation machinery

We have described defective DNA replication as a consequence of transient nucleotide depletion in checkpoint mutants. Because checkpoint null mutants can be suppressed by increasing nucleotide biosynthetic capacity, it is likely that the null mutants experience a nucleotide depletion and die for the same reason as hypomorphic mutants that experience a transient nucleotide depletion. Therefore, an important issue is the nature of the perceived nucleotide depletion in checkpoint null cells. These mutants could be sensitive to the normal dNTP levels present in each cell cycle, or alternatively, the absence of the checkpoint could create a nucleotide depletion to which the cells cannot subsequently respond. In the latter case, the mechanism could be a direct failure to up-regulate RNR activity or an indirect consequence of a failure to properly regulate the nucleotide consumption of other cellular machinery. While investigating the genetic interactions between checkpoint mutants and origin-firing mutants, we have uncovered support for the idea that timing of origin firing may contribute to the nucleotide depletion that kills checkpoint null mutants. The temperature-sensitive origin firing mutant *orc2-1* (Liang et al. 1995) displays an extended duration of S phase upon release from an α-factor arrest, even at the permissive temperature (data not shown). To determine whether this might be mimicking the effect that HU has on S phase, we constructed *orc2-1 mec1-21* and *orc2-1 rad53-21* double mutants. Surprisingly, both double mutants are viable, suggesting that the lengthened S phase in *orc2-1* is a qualitatively different phenomenon than that caused by HU treatment, which kills these checkpoint mutants. Even more startling is the fact that the *mec1-21* mutation, but not the *rad53-21* mutation, can suppress the temperature sensitivity of *orc2-1* (Fig. 7A) at 30°C. This observation suggests that the checkpoint pathway is acting antagonistically to the origin-firing defect of *orc2-1*.

The suppression of *orc2* by a *mec1* mutation bears on the essential function of the DNA replication checkpoint because if there is an antagonistic interplay between checkpoint genes and origin-firing genes at the level of origin firing, then it could be that inappropriate origin firing in checkpoint null mutants creates a nucleotide depletion that commits the cells to lethality. If true, then origin firing mutants might be expected to abrogate this effect and suppress the lethality of check-

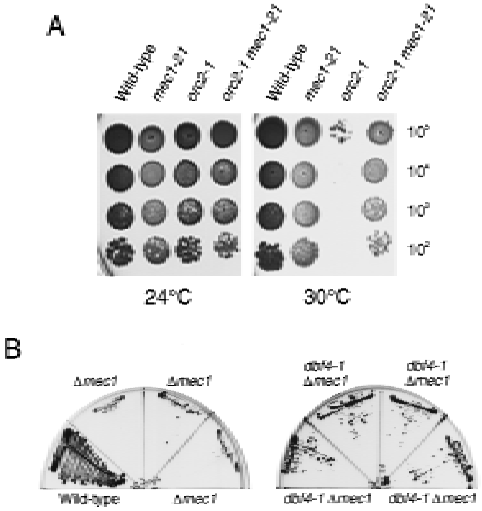

Figure 7. Genetic interactions between *mec1* mutants and origin-firing mutants. (*A*) Suppression of *orc2-1* by the *mec1-21* mutation. Y300 (wild-type), Y604 (*mec1-21*), Y611 (*orc2-1*), and Y612 (*orc2-1 mec1-21*) cultures were grown to log phase in YPD at 24°C. Serial dilutions of 10^5, 10^4, 10^3, and 10^2 cells were spotted onto YPD plates at either 24°C (*left*) or 30°C (*right*). (*B*) Suppression of *Δmec1* by *dbf4-1*. Representative *Δmec1 dbf4-1* double mutants (Y613–Y616) containing *MEC1* on a *URA3 CEN* plasmid (pBAD45) were struck to 5-FOA plates to identify suppressors of the *mec1* null mutation. The wild-type and *Δmec1* controls that were used in this experiment were isolated from the same cross as the double mutants.

point null mutants. The concept that the checkpoint and the origin-firing machinery specifically interact with each other is further supported by recent work (Santocanale and Diffley 1998, and pers. comm.) indicating that the timing of origin firing is negatively regulated by the DNA replication checkpoint pathway. To further explore this idea we examined interactions between the checkpoint pathway and the Dbf4/Cdc7 complex, a protein kinase that is required for origin initiation (Jackson et al. 1993). We tested *dbf4-1* and *cdc7-1* mutants for suppression of *Δrad53* and *Δmec1* by isolating double mutants that contained the wild-type alleles of *RAD53* or *MEC1* on a *URA3* plasmid. These strains were struck onto plates containing 5-FOA to assess their ability to grow in the absence of checkpoint gene product. We found that *Δmec1* but not *Δrad53* was suppressible by *dbf4-1* and *cdc7-1* (Fig. 7B, data not shown), supporting the plausibility of this idea. Why *mec1* and not *rad53* mutants would exhibit these interactions with origin firing mutants is not clear, but the explanation may lie in the additional functions of Mec1 somehow impinging on these events or in a more complex relationship between origin firing and checkpoint function, as detailed in the Discussion.

Discussion

Cell cycle checkpoints have been thought of primarily as surveillance mechanisms that respond to aberrations in cellular structures, such as DNA damage or replication blocks, and prevent catastrophic cell cycle transitions. Unlike the checkpoint genes specific for DNA damage, those involved in the DNA replication checkpoint are essential for viability. The fact that all known replication interference checkpoint genes in *S. cerevisiae* are essential is an indication either that events occurring during the course of a normal cell cycle require the coordinating activities of this checkpoint or that the DNA replication checkpoint genes have activities in addition to the cell cycle coordination traditionally thought to be their primary function (Weinert and Hartwell 1988). We investigated this poorly understood aspect of checkpoint function by performing a high copy suppressor screen of the lethal *rad53* null mutation. We found that overproduction of *RNR1* eliminated the requirement for both *MEC1* and *RAD53*, indicating an interaction between nucleotide levels and checkpoint function even in the absence of nucleotide-depleting drugs. We also determined that lethality caused by nucleotide stress in checkpoint-deficient cells can be attributed to failure of replication structures to completely recover from the immediate effects of nucleotide depletion, suggesting that replicational stress due to suboptimal nucleotide levels may occur during a normal cell cycle.

Functional distinction between MEC1 *and* RAD53

The *mec1* and *rad53* alleles that were previously available for study were necessarily hypomorphic and not complete loss-of-function alleles. This has made determination of the relative roles played by each in the checkpoint pathway impossible to definitively establish. The existence of a common suppressor allows a direct comparison of the two null mutants with existing hypomorphic alleles and with each other. The UV and HU sensitivities of the *mec1-21* mutant are much less severe than the *mec1* null mutant, indicating that the *mec1-21* allele retains significant residual function. The UV and HU sensitivities of the *rad53-21* and *rad53* null mutants are very similar. Furthermore, the kinetics and extent of spindle elongation in HU-treated *rad53-21* mutants are essentially indistinguishable from that of both *rad53* and *mec1* null mutants after general cell cycle perturbations are taken into account, indicating that *rad53-21* can be considered to be nearly completely defective for the cell cycle delay function.

The major point of similarity between the *mec1* and *rad53* null strains is the fact that even moderate *RNR1* overproduction can efficiently suppress them both. Furthermore, a *rad53 mec1* double null mutant is also easily suppressible by *RNR1* (data not shown). This indicates that the essential functions of both genes are the same. Moreover, using the common suppressor approach we can state unequivocally that there is a functional distinction between *RAD53* and *MEC1* observable at the level of sensitivity to UV irradiation and HU treatment, with *MEC1* contributing more to resistance than *RAD53*. Given that the kinetics of anaphase entry of *rad53* and *mec1* null mutants in the presence of HU are very similar to each other, we believe that the actual cell cycle regulatory functions of the two gene products are therefore also similar but that *MEC1* has additional roles required for recovery from replicational stress. This is also consistent with the fact that *MEC1* acts upstream of *RAD53* in the checkpoint pathway and is required for its phosphorylation in response to DNA damage and replication blocks.

What is the essential function of the S-phase checkpoint?

Whereas *RAD53* and *MEC1* are essential genes in *S. cerevisiae*, their homologs in *Schizosaccharomyces pombe*, *cds1*$^+$ and *rad3*$^+$, respectively, are not (Al-Khodairy et al. 1994; Murakami and Okayama 1995). The *MEC1*-related gene *ATM* is also dispensable for cell growth in humans and mice (Barlow et al. 1996; Elson et al. 1996; Xu et al. 1996). This suggests that the essential natures of *MEC1* and *RAD53* are reflections of a checkpoint requirement that manifests in every cell cycle in *S. cerevisiae*. Our findings that *RNR1* and *RNR3*, the rate-limiting regulatory subunits of ribonucleotide reductase, are dosage suppressors of the lethality of the *mec1* and *rad53* null mutations support this idea and indicate that the essential function of these genes involves maintaining an adequate nucleotide supply, as opposed to responding to some kind of DNA damage. The fact that low amounts of exogenously supplied *RNR1* can efficiently suppress lethality suggests that the defect responsible for lethality is just below the threshold for survival. However, *RNR1*

can do little to overcome the effects of exposure to the RNR inhibitor HU, which requires full activation of the checkpoint for a prolonged period of time.

dNTPs levels are highly regulated (for review, see Elledge et al. 1992). The mRNA for *RNR1* is tightly cell cycle regulated, the mRNAs for all four RNR genes are inducible in response to DNA damage and replication blocks, the substrate specificity of the reductase is modulated by particular dNTPs to ensure an equal supply of all four dNTPs, and dATP feedback inhibits the overall activity of the enzyme to prevent excessive build up of dNTPs. An important question is why the levels of dNTPs in *mec1* and *rad53* mutants are insufficient for survival. One possibility is that *mec1* and *rad53* cells are simply more sensitive to normal levels of nucleotides. Perhaps nucleotide levels are normally maintained at a level that is limiting for polymerase function. In vitro it has been shown that high nucleotide levels lead to increased misincorporation rates because proofreading mechanisms have less time to function before the next nucleotide is inserted (Fersht 1979). Thus, it is possible that normal in vivo nucleotide levels cause polymerase pausing in a state that is deleterious in the absence of the replication stress response pathway. A second possibility is that the checkpoint has a direct role in up-regulating dNTP synthesis during S phase such that the loss of checkpoint function would actually cause a nucleotide depletion to which it then would not be able to respond. *RAD53* does regulate the transcription of *RNR1*, *RNR2*, *RNR3*, and *RNR4* in response to HU treatment and DNA damage; however, the viable *rad53-21* allele is completely defective for this transcriptional regulation (Allen et al. 1994; Huang and Elledge 1997), suggesting that this function is not specifically lacking in null mutants. If up-regulation of nucleotide synthesis is regulated by the checkpoint, the defect is not at the level of *RNR1* accumulation because *RNR1* levels appear to be normal in the null mutants. Furthermore, overproduction of *RNR2* and *RNR4* fail to suppress *rad53* lethality (data not shown). A third possibility is that in the absence of the checkpoint, a secondary event causes a more rapid consumption of dNTPs such that their levels are lower than normal, mimicking HU treatment. This, together with an inability to respond to such a nucleotide depletion, however transient, could cause lethality.

Currently we cannot distinguish between the three models presented in the preceding paragraph. However, the third model, indirect nucleotide depletion as a secondary effect of checkpoint deficiency, has recently gained support. The firing of late replication origins is advanced in *rad53* and *mec1* mutants (Santocanale and Diffley 1998, and pers. comm.). Consistent with this observation, we found that the *mec1-21* point mutant suppresses the temperature sensitivity of mutations in *ORC2*, a gene required for origin recognition and firing. Normally at the G_1-S transition, up-regulation of ribonucleotide reductase and the triggering of replication origins occur by separate but parallel regulatory networks. Yet the activation of replication complexes and the dNTP supply must be coordinated because firing of origins with insufficient nucleotide levels would cause a condition of effective nucleotide deprivation. The S-phase checkpoint pathway may provide this coordination. Failure to do so would result in premature or excessive origin firing as observed in *mec1* and *rad53* mutants. The presence of more origins replicating DNA at the same time might consume nucleotides faster than they can be synthesized, leading to DNA replicative stress, a checkpoint requiring situation. *RNR1* overexpression could alleviate this problem without restoring checkpoint function. We tested this by artificially slowing down origin firing in checkpoint mutant backgrounds using temperature-sensitive *dbf4-1*, *cdc7-1*, and *orc2-1* mutants. Although these mutants were unable to suppress the lethality of *rad53* null mutants, we have found that mutations in *dbf4* and *cdc7* can suppress the *mec1* null mutant. The inability to suppress the *rad53* null mutation might indicate a novel role for *RAD53* relative to *MEC1*, or a possible redundancy in *RAD53* regulation. We have shown previously that *TEL1*, a *MEC1* homolog, can activate Rad53 to a limted degree (Sanchez et al. 1996). Thus, it is possible that a *rad53* null mutant could have a more severe defect than a *mec1* null mutant under certain circumstances. In addition, it is possible that *dbf4* mutants can suppress the lethality of *rad53* null mutations but that the double mutant then dies because of a condition unique to the *rad53* null mutation. In support of such a possibility we have observed that *dbf4-1 rad53-21* and *cdc7-1 rad53-21* double mutants are inviable (B.A. Desany and S.J. Elledge, unpubl.).

The genetic interactions between the checkpoint and origin initiation pathways support the notion that the *MEC1/RAD53* pathway is acting antagonistically to the origin firing machinery for the purpose of maintaining coordination between the initiation of DNA replication and the nucleotide supply. Furthermore, we believe that the simplest interpretation of our data is that in the absence of the checkpoint pathway, nucleotide levels become limiting either by increased consumption due to increased origin-firing or by an unknown mechanism, and this situation, together with the absence of the ability to properly respond to nucleotide depletion, results in lethality.

What is responsible for lethality in the presence of HU?

Replication checkpoint-defective cells die rapidly when exposed to HU, and inappropriate spindle elongation has been thought to be responsible for this lethality. However, microtubule-inhibiting drugs are incapable of rescuing either the lethality of the *mec1* and *rad53* null mutants or the HU sensitivity of the point mutants. Additionally, the spindle elongation defects of the *mec1* and *rad53* null mutants are similar to each other, whereas their sensitivities to HU are significantly different. We interpret this to indicate that spindle elongation, rather than being the sole lethal event in these cells, is being misregulated independently of another event that is irreversibly committing cells to death. This is similar

to the results obtained in *S. pombe* with mutations in *cds1*, the gene related most closely to *RAD53*. *cds1* mutants die in response to HU treatment but do not appear to enter mitosis prematurely (Murakami and Okayama 1995; Lindsay et al. 1998). Similar results were obtained with *hus1* mutants (Enoch et al. 1992). Although there was no attempt to artificially delay mitotic entry to rescue the lethality in those experiments, it is likely that these mutants are dying for the same reasons as *rad53* mutants in HU. Our experiments show that *rad53* mutant cells have a reduced ability to synthesize intact chromosomes following transient nucleotide depletion. This is not due to an inability to resume dNTP production because bulk DNA synthesis resumes after the block is removed, albeit with slower kinetics than wild type. Whether the structures that prevent chromosome migration in pulsed field gels are normal replicational intermediates that persist much longer than usual, such as replication forks, or are structurally aberrant in some way because of errors resulting from stalled polymerases is not clear. Stalled replication complexes could occasionally disintegrate and require checkpoint-mediated restoration. Alternatively, the collapse of complexes on converging forks could leave lethal gaps of unreplicated DNA. Aberrant DNA repair could also lead to defective chromosomal structure. Although it is not known whether the *MEC1/RAD53* pathway directly controls repair processes, it is clear that HU causes damage because *rad51* and *rad52* mutants are very sensitive to HU (Allen et al. 1994).

Taken together, our results suggest that inviability of *rad53* and *mec1* null mutations is not due to premature mitotic entry but to an inability to survive with the existing nucleotide levels present in those mutants. Furthermore, our results indicate that the lethality resulting from limiting nucleotides is not purely a cell cycle transition phenomenon but is due instead to the profound inability of these mutants to properly carry out chromosomal replication after transient nucleotide depletion. Although this defect could be caused by misregulation of an as yet unappreciated aspect of cell cycle coordination distinct from anaphase commitment, it is clearly not the onset of anaphase that is causing lethality in these mutants because preventing anaphase cannot restore viability after a transient replication block. We favor the model that the checkpoint pathway is more than a cell cycle response. The fact that *mec1* and *rad53* null mutants appear to be equally checkpoint defective but have significantly different sensitivities to DNA-damage and replication-blocking agents suggests that this pathway controls repair activities in addition to coordination of cell cycle transitions. In this light, these pathways should be considered to be DNA-damage and DNA replication-block stress-response pathways as opposed to solely concerning themselves with cell cycle transitions.

Materials and methods

Yeast growth conditions

Yeast cells were grown at 30°C unless indicated otherwise. Rich and SC medium was formulated according to Kaiser et al. (1994). The carbon source was glucose, unless indicated, in which case the glucose was replaced by galactose. Where indicated, 5-FOA was used at 0.1%, and benomyl in solid media was used at 15 μg/ml.

Isolation of SRL *genes*

Strain Y324 (see text and Table 2) was grown in YPD and transformed with a 2μ *TRP1 S. cerevisiae* cDNA library (ATTC nos. 87288 and 47059) using the lithium acetate method. Transformants were plated on SC − Trp GAL (containing galactose) and replica-plated to SC − Trp GAL supplemented with 5-FOA. Positive clones were tested for their ability to grow on SC − Trp supplemented with 100 mM HU. Negatives were then struck to either YPD or YPD with the glucose replaced by galactose (YP-Gal). Clones that displayed any degree of galactose-dependent growth were tested for repeatability by plasmid rescue and retransformation of Y324, followed by verification of 5-FOA resistance. These final positive clones were christened *SRL* genes.

RNA purification and Northern blotting

RNA purification and Northern blotting were performed as described (Navas et al. 1995). For detection of the endogenous *RNR1* transcript in the presence of exogenously provided *RNR1*, we used a *Hin*dIII–*Spe*I fragment as a probe corresponding to nucleotides 2642–3317 of the 3′ end of the *RNR1* transcript. These sequences are not present on the exogenous *RNR1* expression constructs. For detection of *CLN2* mRNA, we probed using a *Sty*I fragment of *CLN2* comprising nucleotides 460–1541 of the 1638 nucleotide ORF.

Quantitation of bands was performed by exposing the blots to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) and using ImageQuant software to quantitate the band intensities. In all cases, the lane background was subtracted from each band prior to normalization to the loading control (*ACT1*).

HU- and UV-killing assays

For HU killing, cultures were grown to log phase in YPD, whereupon the medium was replaced with YPD + 0.2 M HU (unless indicated otherwise), and aliquots were removed and plated on YPD at timed intervals and allowed to grow for several days at 30°C. For UV killing, cells were grown to log phase in YPD, plated on YPD, and irradiated (Stratagene UV Stratalinker 1800) with 0, 20, or 40 J/m^2 prior to incubation at 30°C.

Synchronization of cells in G_1 phase

Strains were grown to log phase in YPD (pH 3.9), treated with 10 μg/ml α-factor for 1.5 hr, and supplemented with an additional 5 μg/ml α-factor for another 1.5 hr. Cells were then centrifuged and resuspended in YPD containing the 0.2 M HU, 0.25 M HU, 80 μg/ml benomyl, and/or 10 μg/ml nocodazole as indicated in the individual experiments.

Staining of cells for microtubule visualization

Cells were fixed by the addition of 5% formaldehyde to growing cultures and allowed to stand for at least 4 hr at 4°C. Cells were washed in PBS, and microtubules were immunostained using the antitubulin antibody YOL1/34 and a FITC-conjugated secondary antibody as described (Allen et al. 1994).

Table 2. *Strains and plasmids used in this study*

Strain	Genotype	Source
Y81	*MAT*α *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	Allen et al. (1994)
Y300	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	Allen et al. (1994)
Y301	as Y300 *rad53-21*	Allen et al. (1994)
Y312	as Y323 *Δrad53::HIS3/RAD53*	Allen et al. (1994)
Y323	*MAT***a**/α *trp1-1/trp1-1 ura3-1/ura3-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 ade2-1/ade2-1 can 1-100/can 1-100*	Allen et al. (1994)
Y324	as Y81 *Δrad53::HIS3* + pJA92	Allen et al. (1994)
Y580	as Y300 *TRP1::GAP–RNR1*	this study
Y581	as Y300 *Δmec1::HIS3 TRP1::GAP–RNR1*	this study
Y601	as Y300 *Δrad53::HIS3* + pJA92	this study
Y602	as Y300 *Δmec1::HIS3* + pBAD45	this study
Y603	as Y300 *Δrad53::HIS3* + pBAD70	this study
Y604	as Y300 *mec1-21*	this study
Y605	as Y300 *Δmec1::HIS3* + pBAD70	this study
Y606	as Y300 *Δrad53::HIS3 TRP1::GAP–RNR1*	this study
Y607	as Y300 *TRP1::GAP–RNR1*	this study
Y608	as Y300 *Δrad53::HIS3* + pBAD79	this study
Y609	as Y300 *Δrnr::HIS3* + pBAD79	this study
Y610	as Y300 *Δmec1::HIS3* + pBAD79	this study
Y611	as Y300 *orc2-1*	this study
Y612	as Y300 *orc2-1 mec1-21*	this study
Y613–616	as Y300 *dbf4-1 Δmec1::HIS3* + pBAD45	this study
Y617	as Y323 *Δmec1::HIS3/MEC1*	this study
Y618	as Y323 *Δrad53::HIS3/RAD53 TRP1::GAP–RNR1/trp1-1*	this study
Y619	as Y323 *Δmec1::HIS3/MEC1 TRP1::GAP–RNR1/trp1-1*	this study
Y620	as Y81 *mec1-21*	this study
Y621	as Y323 *orc2-1/ORC2 mec1-21/MEC1*	this study
Y622	as Y323 *dbf4-1/DBF4 Δmec1::HIS3/MEC1*	this study
Y623	as Y300 ρ^0 *HIS3*	this study
Y624	as Y301 ρ^0 *HIS3*	this study
Y692	as Y300 TRP^+	this study
YCH266	as Y81 *dbf4-1*	C. Hardy (Washington University, St. Louis, MO)

Plasmid	Relevant markers	
pAB23BXN	Ap^r 2μ *URA3 GAP* promoter	T. Brake (Chiron Corporation, Emeryville, CA)
pTRP	Ap^r *TRP1* 2μ *GAL* promoter	Mulligan and Elledge (1994)
pJA50	Ap^r Kn^r *HIS3*	Allen and Elledge (1994)
pJA92	Ap^r *URA3 CEN4 RAD53*	Allen et al. (1994)
pSAD3-3B	Ap^r *CEN4 TRP1 MEC1*	this study
pWJ87	Ap^r *CEN4 TRP1 Δmec1::HIS3*	this study
pJR1267	Ap^r *URA3 orc2-1*	C. Fox and J. Rine (University of California, Berkeley)
pSE734	Ap^r *RNR3*	Elledge and Davis (1990)
pSE757	Ap^r 2μ *TRP1 RNR1*	Elledge and Davis (1990)
pBAD40	Ap^r *CEN4 URA3*	this study
pBAD45	Ap^r *URA3 CEN4 MEC1*	this study
pBAD49	Ap^r *RNR1* PCR product	this study
pBAD54	Ap^r *TRP1* 2μ *GAP* promoter	this study
pBAD58	Ap^r *RNR3* PCR product	this study
pBAD62	Ap^r *RNR1* ORF	this study
pBAD70	Ap^r *TRP1* 2μ *GAP–RNR1*	this study
pBAD74	Ap^r *RNR3* ORF	this study
pBAD79	Ap^r *TRP1* 2μ *GAP–RNR3*	this study
pBAD114	Ap^r *TRP1 GAP–RNR1*	this study

FACS analysis

The amount of 250 μl of cell culture (~1.5×10^6 to 4×10^6 cells) was added directly to 1 ml of ethanol and allowed to stand 1 hr for fixation. Cells were washed once with 70% ethanol and once with FACS buffer (0.2 M Tris at pH 7.5, 20 mM EDTA). In a volume of 100 μl of FACS buffer, cells were treated with 1 mg/ml RNase A at 37°C for 2 hr. Cells were then washed in PBS, treated with 5 μg/ml propidium iodide in a final volume of 1 ml of PBS, and analyzed for fluorescence content using a Coulter model Epics XL-MCL. The DNA content of ~30,000 cells was determined for each sample.

PFGE of replication intermediates

α-Factor-arrested ρ^0 strains were released into YPD containing 0.2 M HU and 10 μg/ml nocodazole for 60 min; cells were spun down, washed, and resuspended in YPD containing 10 μg/ml nocodazole. Cells from different time points during and after HU treatment were fixed in 70% ethanol overnight. These were subsequently resuspended in 0.5 M EDTA, 1.2 M sorbitol, and 1 M Tris (pH 7.5). Chromosome plugs were prepared following a rapid two-step protocol without use of proteinase K (Johnston 1994). Each 75 μl plug contained 4.5×10^6 cells. PFGE was carried out in a Bio-Rad DR II apparatus for 24 hr, at 200 V. Switching was done every 60 sec for the first 15 hr, and every 90 sec for the last 9 hr. Chromosomes were visualized with ethidium bromide. The gel was photographed and chromosome band intensities were quantitated using NIH Image software.

Strain and plasmid construction

The source of the *MEC1* gene was pSAD3-3B, which is a 9.5-kb fragment of the *MEC1* genomic locus cloned into pRS414 (Sikorski and Hieter 1989). pBAD45 contains the 7.7-kb *Sac*I *MEC1*-containing fragment from pSAD3-3B cloned into the *Sac*I site of pBAD40, which is a derivative of pRS416 (Sikorski and Hieter 1989) deleted between the *Not*I and *Sal*I sites. pBAD54 is a *GAP* promoter expression vector made by cloning the *GAP* expression cassette, containing the *GAP* promoter and *GAP* terminator flanking a multicloning site, as a *Bam*HI fragment from pAB23BXN into the *Bam*HI site of YEplac112 (Gietz and Sugino 1988).

The *RNR1* and *RNR3* ORFs were cloned by PCR and subcloned into pBS II KS(−) to make pBAD49 and pBAD58. The ends of each ORF were sequenced to verify lack of mutation, and the central parts of each ORF were replaced by the corresponding fragment from a functional genomic clone. For *RNR1* this was a *Bst*EII–*Xba*I fragment from pSE757 generating pBAD62, and for *RNR3* it was a *Bst*EII–*Hin*dIII fragment from pSE734 generating pBAD74. pBAD70 was made by subcloning the *RNR1* ORF as a *Xho*I–*Not*I fragment from pBAD62 into *Xho*I–*Not*I-digested pBAD54. pBAD79 was made by subcloning the *RNR3* ORF as a Psp1406I(T4-filled in)–*Not*I fragment from pBAD74 into pBAD54 that had been cut with *Xho*I and T4-filled in and subsequently cut again with *Not*I.

The *RAD53* gene knockout has been described previously (Allen et al. 1994). *MEC1* was knocked out by replacing a 7.5-kb *Bam*HI fragment from pSAD3-3B with the *Bam*HI fragment from pJA50 containing the *HIS3* gene and a kanamycin resistance gene from Tn*5* to form pWJ87. This removes all but the amino-terminal 33 amino acids from the *MEC1* ORF. The 4.4-kb *Sac*I fragment from pWJ87 containing the *Δmec1::HIS3* deletion construct was transformed into Y323 to generate a diploid heterozygous for the *mec1* knockout Y617.

The *TRP1*::*GAP–RNR1* expression cassette was created by subcloning a *Pst*I–*Sac*I fragment from pBAD70 into *Pst*I–*Sac*I-digested pRS404 to create pBAD114. *rad53* and *mec1* null mutants suppressed by this *GAP–RNR1* expression cassette were generated as follows. pBAD114 was linearized within the *TRP1* gene and transformed into Y312 and Y617 to create Y618 and Y619, and correct integration was confirmed by Southern blotting. Y618 was sporulated and Y606 and Y607 were recovered. Y619 was sporulated to obtain Y580 and Y581.

The temperature-sensitive *orc2-1* mutant Y611 was generated by looping the *orc2-1* allele from the *URA3*-integrating plasmid pJR1267 into Y300. We then selected transformants for 5-FOA resistance and screened them for temperature sensitivity. Y612 was made by crossing Y611 with Y620 and sporulating and dissecting the resulting diploid Y621. Y613, Y614, Y615, and Y616 are four spores of identical genotype that were isolated from the diploid Y622, which was in turn created by a mating between Y602 and YCH266.

Y623 and Y624, His^+ ρ^0 derivatives of Y300 and Y301, respectively, were generated by serial culturing in minimal media containing ethidium bromide, as described in Fox et al. (1991).

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