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Interaction Between the *MEC1*-Dependent DNA Synthesis Checkpoint and G1 Cyclin Function in *Saccharomyces cerevisiae*

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ABSTRACT

The completion of DNA synthesis in yeast is monitored by a checkpoint that requires *MEC1* and *RAD53*. Here we show that deletion of the *Saccharomyces cerevisiae* G1 cyclins *CLN1* and *CLN2* suppressed the essential requirement for *MEC1* function. Wild-type levels of *CLN1* and *CLN2*, or overexpression of *CLN1*, *CLN2*, or *CLB5*, but not *CLN3*, killed *mec1* strains. We identified *RNR1*, which encodes a subunit of ribonucleotide reductase, as a high-copy suppressor of the lethality of *mec1 GAL1-CLN1*. Northern analysis demonstrated that *RNR1* expression is reduced by *CLN1* or *CLN2* overexpression. Because limiting *RNR1* expression would be expected to decrease dNTP pools, *CLN1* and *CLN2* may cause lethality in *mec1* strains by causing initiation of DNA replication with inadequate dNTPs. In contrast to *mec1* mutants, *MEC1* strains with low dNTPs would be able to delay S phase and thereby remain viable. We propose that the essential function for *MEC1* may be the same as its checkpoint function during hydroxyurea treatment, namely, to slow S phase when nucleotides are limiting. In a *cln1 cln2* background, a prolonged period of expression of genes turned on at the G1-S border, such as *RNR1*, has been observed. Thus deletion of *CLN1* and *CLN2* could function similarly to overexpression of *RNR1* in suppressing *mec1* lethality.

CYCLINS and cyclin-dependent kinases (CDKs) have been shown to play important roles in many eukaryotic cell cycle transitions. In the yeast *Saccharomyces cerevisiae*, the cyclins that normally control the G1 to S phase transition (START) are *CLN1*, *CLN2*, and *CLN3*. The B-type cyclin, *CLB5*, can functionally substitute for the *CLN*s if it is overexpressed (Epstein and Cross 1992; Schwob and Nasmyth 1993), or if the B-type cyclin inhibitor, *SIC1*, is deleted (Schneider *et al.* 1996; Tyers 1996). The Cln proteins, when complexed with the CDK encoded by *CDC28*, activate a number of pathways, including activation of B-type cyclins (*CLB*s), DNA replication, bud emergence, and microtubule organizing center duplication (see Lew *et al.* 1997 for a recent review). Although *CLN*s are redundant for viability in an otherwise wild-type strain, there are significant and qualitative differences between the *CLN*s as evidenced by their *in vitro* kinase activities, requirements for other gene products, and ability to activate transcription of other genes (Benton *et al.* 1993; Cvrcková and Nasmyth 1993; Tyers *et al.* 1993; Vallen and Cross 1995; Levine *et al.* 1996). One specific difference between *CLN1* and *CLN2* compared to *CLN3* is *CLN3*'s ability to act as a strong transcriptional activator of cell cycle-regulated genes containing promoter elements regulated by the transcription factors SBF and MBF (Tyers *et al.* 1993; Dirick *et al.* 1995; Stuart and Wittenberg 1995). It is likely that the predominant role of Cln3 in the cell is the activation of transcription of these gene classes. *CLN3* appears to be less potent an activator of most of the other pathways that are initiated at START (Levine *et al.* 1996). Thus, in a wild-type *CLN* strain, the three different cyclins complexed with Cdc28p may act together leading to the coordinate activation of transcription and other START-associated processes.

A number of genes required directly for DNA replication have transcript levels that peak at or near the G1 to S phase transition. These genes are regulated by MBF, having MCB (*Mlu*I cell cycle box) elements upstream of their coding region (McIntosh 1993). One such gene is *RNR1*, which shows about a 15-fold fluctuation in RNA levels across the cell cycle (Elledge and Davis 1990). *RNR1* and a related gene, *RNR3*, encode the large α subunit of ribonucleotide reductase (Elledge and Davis 1990). Ribonucleotide reductase is a tetrameric enzyme of the structure $\alpha_2\beta_2$, which catalyzes the formation of deoxyribonucleotides from ribonucleotides. The small β subunits are encoded by *RNR2* and *RNR4* (Elledge and Davis 1987; Hurd *et al.* 1987; Huang and Elledge 1997; Wang *et al.* 1997). Enzymatic activity of the complex has been demonstrated to be cell cycle regulated, peaking in early S phase (Lowden and Vitols 1973). Because RNA levels of the small subunits vary only approximately twofold or less during the cell cycle and *RNR3* is not essential for viability, it is likely that Rnr1 levels are rate limiting for enzymatic activity (Elledge and Davis 1990; Huang and Elledge

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1997). Strong evidence supporting this conclusion comes from recent analysis of ribonucleotide reductase activity in yeast extracts, which demonstrates that the addition of Rnr1p increases enzymatic activity *in vitro* (Wang *et al.* 1997). Furthermore, deletion of *SML1*, which encodes a protein that binds Rnr1, increases the dNTP levels in cells (Zhao *et al.* 1998). Inhibition of ribonucleotide reductase activity by hydroxyurea (HU) leads to depletion of dNTP pools (Yarbro 1992) and results in cell cycle arrest in S phase in wild-type eukaryotic cells.

HU causes cell cycle arrest because there is a signaling pathway, or S phase checkpoint (Weinert and Hartwell 1989; Weinert *et al.* 1994), that monitors the completion of DNA replication and prevents mitosis until replication is completed. In *S. cerevisiae*, the incomplete replication and stalled replication forks caused by depletion of deoxyribonucleotide pools are likely sensed by DNA polymerase ε, Dpb11p, or Rfc5p (Araki *et al.* 1995; Navas *et al.* 1995; Sugimoto *et al.* 1996, 1997). The signal transduction pathway activated by HU and required for cell cycle arrest and the transcriptional induction of genes required for DNA synthesis and damage repair requires the kinases Mec1p, Rad53p, and Dun1p (Allen *et al.* 1994; Kiser and Weinert 1996; Pati *et al.* 1997). Activation of replication checkpoints by HU or DNA polymerase α mutants induces phosphorylation of Rad53p that is *MEC1* dependent (Sanchez *et al.* 1996; Sun *et al.* 1996). This, coupled with the observations that *MEC1* is required for the damage-induced transcription of some genes that do not require *RAD53* for transcriptional induction (Kiser and Weinert 1996), and that deletion of *MEC1* is suppressed by overexpression of *RAD53* (Sanchez *et al.* 1996), suggests that Mec1p functions upstream of Rad53p.

Although checkpoint genes were originally hypothesized to be required only in cells subjected to perturbation, both *MEC1* and *RAD53* genes are required for wild-type cell division in *S. cerevisiae* (Zheng *et al.* 1993; Paulovich *et al.* 1997; Zhao *et al.* 1998). On the basis of the requirements for *RAD53* and *MEC1*, it may be that *S. cerevisiae* cells need to actively inhibit progression through the cell cycle until the end of DNA replication in most cell cycles. In contrast, the homologs found in *Schizosaccharomyces pombe*, *CDS1* and *RAD3*, respectively, are not required for viability (Jimenez *et al.* 1992; Seaton *et al.* 1992; Murakami and Okayama 1995; Bentley *et al.* 1996).

Here we report that the essential requirement for *MEC1* can be suppressed by deletion of the G1 cyclins *CLN1* and *CLN2*. *mec1-1* and *mec1*Δ mutant cells deleted for *cln1* and *cln2* are killed by expression of *CLN1*, *CLN2*, or *CLB5*, but not by *CLN3*, from the strong, inducible *GAL1* promoter. Wild-type levels of either *CLN1* or *CLN2* also cause severe growth defects in *mec1-1* strain; the presence of wild-type levels of both *CLN1* and *CLN2* in *mec1-1* strains may be lethal, consistent with previously reported results (Paulovich *et al.* 1997; Zhao *et al.* 1998). Isolation and characterization of multicopy suppressors of the *mec1-1 GAL1-CLN1* lethality suggests that deoxyribonucleotide pools may be limiting during replication, with lethal consequences to *mec1* mutant strains that cannot pause the cell cycle.

MATERIALS AND METHODS

Strains and media: Media and genetic methods are as described elsewhere (Ausubel *et al.* 1987; Rose *et al.* 1990). The strains used in this study are listed in Table 1. All yeast strains were isogenic with BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*) unless otherwise noted. Mutant *cln1*, *cln2*, and *cln3* alleles, and the *GAL1-CLN1*, *GAL1-CLN2*, *GAL1-CLN3*, *GAL1-CLB5* cassettes have been described previously (Richardson *et al.* 1989; Cross 1990; Cross and Tinkelenberg 1991; Epstein 1992; Cross and Blake 1993; Oehlen and Cross 1994). The *mec1-1* allele (Weinert *et al.* 1994), *rad53::HIS3* disruption (Zheng *et al.* 1993), and *tel1*::*URA3* disruption (Greenwell *et al.* 1995) were backcrossed multiple times to BF264-15D strains as indicated in the strain list. The *mec1-1* mutant spores in the fifth and sixth backcrosses were uniform in size, and spore viability in the 48 tetrads analyzed in the fifth backcross was 86% for both the *mec1-1* and *MEC1* spores. In the 48 tetrads examined in the sixth backcross, the spore viability was 94% for the *mec1-1* spores and 95% for *MEC1* spores. Similarly, strains containing the *tel1* deletion allele were uniform in size, and viability of the *tel1* spores was 98% in the 48 tetrads analyzed in the fourth backcross.

A disruption of *mec1*, referred to as *mec1*Δ, deleting all but the first 98 and last 124 nucleotides of the 7107-nucleotide *MEC1* gene and inserting *URA3*, was constructed and integrated into a *cln1 cln2* diploid strain in the BF264-15D background (R. Gardner and T. Weinert, personal communication). Spores from the diploid were analyzed; the viability for *mec1*Δ spores was 100% in the 23 tetrads analyzed. The *URA3* marker disrupting *mec1* was swapped to *LEU2* or *TRP1* (Cross 1997) before transformation with *URA3* plasmids. A disruption of *sic1* (the gift of M. Mendenhall) was also integrated into *cln1 cln2* diploid strains in the BF264-15D background.

The *rad53* mutant spores were kept covered by the checkpoint defective *spk1-1* allele of *rad53* on a plasmid that was the gift of D. Stern (Fay *et al.* 1997). Those *rad53::HIS3* mutant spores that did not contain the *spk1-1* plasmid were uniform in size, although much smaller than wild-type *RAD53* spores or *rad53::HIS3* spores containing the *spk1-1* plasmid.

For all analyses using *mec1-1*, *mec1*Δ, *rad53::HIS3*, and *tel1::URA3*, a few different strains were examined for all phenotypes and they always behaved similarly. Representative experiments are shown.

Hydroxyurea (Sigma Chemical, St. Louis) was used in solid media at 0.2 m.

Plating efficiency assays: Tenfold serial dilutions in water were made from fresh stationary-phase cultures, and 5 μl from each dilution was plated. Plates were incubated for 2–4 days at 30°.

Northern (RNA) analysis: RNA was isolated, probes were labeled, and Northern blots were performed as described elsewhere (McKinney *et al.* 1993; Oehlen and Cross 1994). Quantification of mRNA was performed by using a Molecular Dynamics (Sunnyvale, CA) phosphorimager and ImageQuant software, and mRNA loading was normalized by using *TCM1* as a loading control. Probe fragments *CLN1*, *CLN2*, *UBI4*, *H2A*, and *CLB5* are as described elsewhere (Cross and Tinkelenberg 1991; Epstein and Cross 1992; Kiser and Wein-

TABLE 1

Yeast strains

Strain	Genotype
RGY48UT1	*MAT***a**/α *cln1/cln1 cln2/cln2 CLN3/CLN3 leu2/leu2::GAL1-CLN1::LEU2 MEC1/mec1*Δ*::ura3::TRP1*
0015 2C	*MAT*α *cln1 cln2 CLN3 mec1*Δ*::ura3::TRP1*
0016 2D	*cln1 cln2 CLN3 mec1-1 tel1::LEU2 GAL-CLN2* multicopy *RNR1*
0016 18B	*cln1 cln2 CLN3 mec1-1 tel1::LEU2 GAL-CLN2* multicopy *RNR1*
0016 19D	*cln1 cln2 CLN3 mec1-1 tel1::LEU2* multicopy *RNR1*
0018 4D	*cln1 cln2 CLN3 mec1-1 tel1::LEU2* YEp24
0018 8B	*cln1 cln2 CLN3 mec1-1 tel1::LEU2 GAL-CLN2* YEp24
0018 11B	*cln1 cln2 CLN3 mec1-1 tel1::LEU2 GAL-CLN2* YEp24
0020 1B	*MAT***a** *cln1 cln2 mec1*Δ*::ura3::TRP1 tel1::URA3*
0020 3A	*MAT***a** *cln1 cln2 mec1*Δ*::ura3::TRP1 leu2:GAL1-CLN1::LEU2*
0020 3D	*MAT***a** *cln1 cln2 mec1*Δ*::ura3::TRP1 tel1:URA3*
0020 5C	*MAT***a** *cln1 cln2 mec1*Δ*::ura3::TRP1 tel1:URA3 leu2:GAL1-CLN1::LEU2*
0020 7D	*MAT***a** *cln1 cln2 mec1*Δ*::ura3::TRP1 tel1:URA3 leu2:GAL1-CLN1::LEU2*
0020 11D	*MAT***a** *cln1 cln2 mec1*Δ*::ura3::TRP1*
1227 2C	*MAT***a** *cln1 CLN2 cln3*
1238 11A	*MAT***a** *cln1 cln2 CLN3 bar1*
1238 16B	*MAT*α *cln1 cln2 CLN3*
1255 5C-1	*MAT***a** *CLN1 CLN2 CLN3 bar1 HIS2*
2507 5B	*MAT*α *cln1 cln2 CLN3 leu2::GAL1-CLN1::LEU2*
2507 5D	*MAT***a** *cln1 cln2 CLN3*
2618 5B	*MAT*α *cln1 cln2 CLN3 mec1-1*(5× backcross)
2619 1B	*MAT*α *cln1 cln2 CLN3 mec1-1 leu2::GAL1-CLN1::LEU2* (5× backcross)
2620 12C	*MAT***a** *cln1 cln2 CLN3 mec1-1*(5× backcross)
2623 11D	*MAT*α *cln1 cln2 CLN3 mec1-1 leu2::GAL1-CLN1::LEU2* (5× backcross)
2662 20C	*MAT*α *cln1 cln2 CLN3 mec1-1 leu2::GAL1-CLN1::LEU2 his3* (6× backcross)
2665 13A	*MAT*α *cln1 cln2 CLN3 mec1-1 trp1::GAL1-CLN2::TRP1* (6× backcross)
2670 2D	*MAT*α *cln1 cln2 CLN3 mec1-1 leu2::GAL1-CLN3::LEU2* (6× backcross)
2670 8A	*MAT*α *cln1 cln2 CLN3 leu2::GAL1-CLN3::LEU2*
2671 5A	*MAT*α *cln1 cln2 CLN3 mec1-1* (6× backcross)
2671 5B	*MAT*α *cln1 cln2 CLN3 leu2::GAL1-CLN2::LEU2*
2671 11B	*MAT*α *cln1 cln2 CLN3 mec1-1 leu2::GAL1-CLN2::LEU2* (6× backcross)
2673 4C	*MAT***a** *cln1 cln2 CLN3 leu2::GAL1-CLN1::LEU2 HIS2 his3*
2673 5C	*MAT***a** *cln1 cln2 CLN3 HIS2 his3*
2673 6A	*MAT***a** *cln1 cln2 CLN3 rad53::HIS3 HIS2 his3* (4× backcross)
2673 6C	*MAT***a** *cln1 cln2 CLN3 rad53::HIS3 leu2::GAL1-CLN1::LEU2 HIS2 his3* (4× backcross)
2673 8A	*MAT***a** *cln1 cln2 CLN3 rad53::HIS3 leu2::GAL1-CLN1::LEU2 HIS2 his3* (4× backcross)
2673 9C	*MAT***a** *cln1 cln2 CLN3 rad53::HIS3 HIS2 his3* (4× backcross)
2687 26D	*MAT*α *cln1 cln2 CLN3 rad53::HIS3 leu2::GAL1-CLN1::LEU2 HIS2 his3* (5× backcross)
2687 28C	*MAT***a** *cln1 cln2 CLN3 RAD53 leu2::GAL1-CLN1::LEU2 HIS2 his3* multicopy *RNR1*
2687 30A	*MAT***a** *cln1 cln2 CLN3 rad53::HIS3 HIS2 his3* multicopy *RNR1* (5× backcross)
2687 30B	*MAT*α *cln1 cln2 CLN3 rad53::HIS3 leu2::GAL1-CLN1::LEU2 HIS2 his3* multicopy *RNR1* (5× backcross)
2687 34A	*MAT***a** *cln1 cln2 CLN3 rad53::HIS3 HIS2 his3 rad53* (5× backcross)
2688 26A	*MAT***a** *cln1 cln2 CLN3 RAD53 HIS2 his3* multicopy *RNR1*

All yeast strains were isogenic with BF264–15D (*trp1-1 leu2-3,112 ura3 ade1 his2*) and are *bar1* unless otherwise noted. The *rad53* and *mec1-1* mutations were backcrossed the indicated number of times into this background. Some strains were made *HIS2* by transformation; the *his3* allele was brought into the BF264–15D background by >11 backcrosses.

ert 1996). The *RNR1* probe was a 2.3-kb *Bst*EII-*Xba*I fragment purified from LB77, a plasmid from the YEp24 genomic library (Carlson and Botstein 1982) isolated in the course of this work as described below. The *RNR3* probe was made by PCR amplification of a 1300-bp fragment using primers of the sequence CTGCAAGCTATAATTTCGAGAG and GGTCTTAA TACATACTAACG.

Isolation and characterization of multicopy plasmid suppressors of *GAL1-CLN1 mec1-1*: Strain 2619 1B (*mec1-1 GAL1-CLN1*) was transformed with a YEp24 genomic library (Carlson and Botstein 1982). Transformants were screened for their ability to grow on SCGal-Ura plates. Putative Gal^+ colonies were picked from SCDex-Ura plates, purified, and retested. Plasmids were recovered from Gal^+ strains (Hoffman and Winston 1987) and plasmid linkage of the Gal^+ phenotype was tested after retransformation. Plasmids were analyzed by restriction mapping and Southern blotting.

For the *RNR1*-containing plasmids, the region required for suppression was identified by the isolation and analysis of transposon insertions into the plasmid (Huisman *et al.* 1987). The ends of the genomic DNA insert were sequenced using primers complementary to the region flanking the *Bam*HI site in YEp24. The location of transposon insertion was determined by restriction digestion analysis and sequence analysis

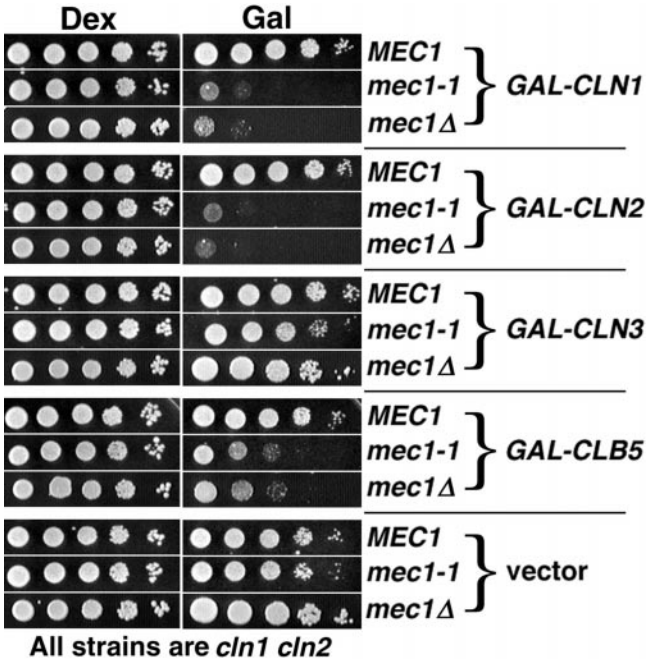

Figure 1.—*mec1* mutant cells die when *CLN1*, *CLN2*, or *CLB5* is overexpressed. Strains 2507 5D (*cln1 cln2 MEC1*), 2620 12C (*cln1 cln2 mec1-1*), and 218UL-9 (*cln1 cln2 mec1*Δ) were transformed with the indicated *CEN*-based plasmids. Colonies were picked and grown to stationary phase in selective media containing 2% dextrose. Tenfold serial dilutions were made from fresh stationary phase cultures of the strains indicated. Five microliter volumes were plated and incubated for 3–4 days at 30°. Dex, dextrose (glucose); Gal, galactose.

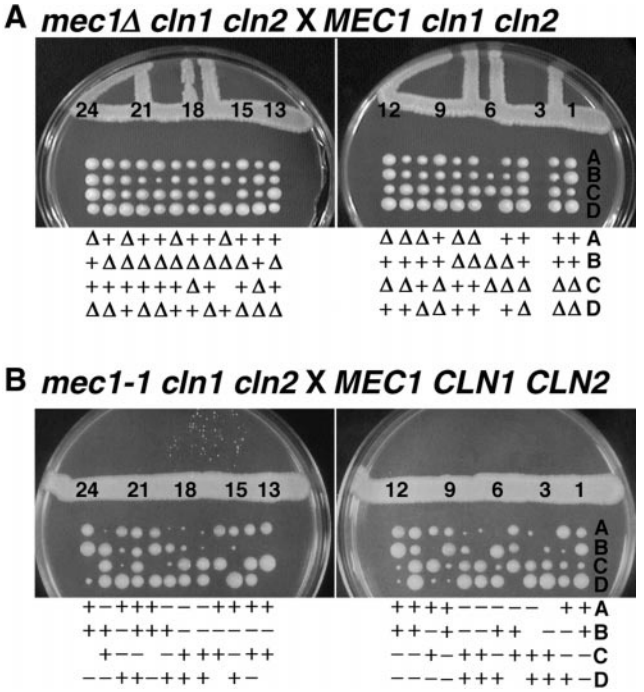

Figure 2.—(A) *mec1 cln1 cln2* mutant cells are viable. Spores from a diploid strain (RGY48UT1) formed by disruption of *mec1*Δ in a *cln1 cln2* homozygous diploid were dissected and incubated at 30° for 3 days. The *mec1*Δ genotype was assigned to spores on the basis of testing for hydroxyurea sensitivity and scoring the *TRP1* marker used to mark the *mec1*Δ allele. The *MEC1* genotype of each spore colony is noted below the tetrad plates (Δ, *mec1*Δ; +, *MEC1*). (B) Wild-type levels of *CLN1* and *CLN2* cause slow growth of *mec1-1* mutant cells. Spores from a diploid strain formed by crossing *CLN1 CLN2 CLN3 MEC1* (1255 5C-1) and *cln1 cln2 CLN3 mec1-1* (2662 20C) were dissected and incubated at 30° for 3 days. The *mec1-1* genotype was assigned to spores on the basis of testing for hydroxyurea sensitivity. The *CLN1* and *CLN2* genotypes were assigned by Northern blot analysis. The *mec1* genotype of each spore colony is noted below the tetrad plates (−, *mec1-1*; +, *MEC1*).

from primers complementary to the transposon ends. Dideoxy sequencing with Sequenase 2.0 (United States Biochemical, Cleveland) was performed according to the manufacturer's instructions). Sequences were then compared to the genomic DNA sequences in the *S. cerevisiae* database using a BLAST search (http://genome-www2.stanford.edu:5555/cgi-bin/nph-blastsgd/).

RESULTS

Lethality of *mec1-1* and *CLN1*, *CLN2*, and *CLB5* overexpression: We have shown previously that *mec1-1 cln1 cln2* strains are viable and are killed when *GAL1-CLN1* is expressed (Vallen and Cross 1995; see also Figure 1). Expression of *GAL1-CLN2*, and to a somewhat lesser extent, *GAL1-CLB5*, is also lethal to *cln1 cln2 mec1-1* mutant cells (Figure 1). In all cases, there is about a 1000- to 10,000-fold decrease in plating efficiency of strains containing *GAL1-CLN1*, *GAL1-CLN2*, or *GAL1-CLB5* compared to control *mec1-1* mutant strains transformed with vector on galactose-containing media. Similar results were seen with strains containing a deletion of *MEC1* (Figure 1). Overexpression of *CLN1*, *CLN2*, or *CLB5* had no effect on the plating efficiency of the *MEC1* strains. In contrast to the results with *CLN1*, *CLN2*, and *CLB5*, overexpression of *CLN3* or the dominant activating allele of *CLN3*, *CLN3-2*, from the *GAL1* promoter does not kill the *mec1-1* or *mec1*Δ strains (Figure 1 and data not shown). Colonies grow up slightly more slowly than the vector controls, but the plating efficiency of transformants is similar in the presence and absence of *CLN3* overexpression and comparable to that of the control strains with no *GAL1-CLN* construct. In addition, it is critical to point out that there are no obvious differences between the *mec1-1 cln1 cln2 CLN3*, *mec1*Δ *cln1 cln2 CLN3*, and *MEC1 cln1 cln2 CLN3* strains on galactose media when strains are transformed with the vector, or between any of the strains on dextrose where the *CLN*s are not overexpressed (Figures 1 and 2A). *mec1-1 cln1 cln2* and *MEC1 cln1 cln2* strains also had similar doubling times in liquid media as measured by the optical density of logarithmically growing cultures (T. Brenner and E. Vallen, unpublished results). In contrast to the results with *CLN1*, *CLN2*, and *CLB5*, *GAL1-CLB2* slowed cell growth and decreased plating efficiency similarly in both *MEC1* and *mec1-1* strains (data not shown).

TABLE 2

The *mec1-1* mutation causes a growth defect in strains containing *CLN1* and/or *CLN2*

Relevant genotype	Fast growing	Slow growing
mec1-1 cln1 cln2 CLN3	16	1
mec1-1 CLN1 cln2 cln3	0	9
mec1-1 CLN1 cln2 CLN3	0	6
mec1-1 cln1 CLN2 cln3	0	6
mec1-1 cln1 CLN2 CLN3	0	3

Spores from a diploid strain formed by crossing either *CLN1 cln2 cln3 MEC1* (1239 18A) or *cln1 CLN2 cln3 MEC1* (1227 2C) and *cln1 cln2 CLN3 mec1-1* (2623 11D) were dissected and incubated at 30° for 3 days. Fast growing and slow growing refer to spore colony size as can be seen in Figure 1B. The *mec1-1* genotype was assigned to spores on the basis of testing for hydroxyurea sensitivity. The *CLN1* and *CLN2* genotypes were assigned by Northern blot analysis.

To examine the phenotype of *mec1-1* cells with wild-type levels of the G1 cyclins, we crossed *mec1-1 cln1 cln2 CLN3* strains to *MEC1 CLN1 CLN2 CLN3* strains (Figure 2). In crosses when *mec1-1* or *mec1*Δ was segregating in a *cln1 cln2 CLN3* background, it was difficult to distinguish the *mec1* mutant spore colonies by colony size (Figure 2A and data not shown). Some colonies in the cross between the *mec1 cln1 cln2 CLN3* and *MEC1 cln1 cln2 CLN3* strains were slightly smaller than others but this did not correlate with the *MEC1* genotype (Figure 2A). These spores were usually *MAT***a**, and the slight growth defect may be due to the fact that the strains are *bar1*$^-$ and are therefore very sensitive to mating pheromone.

In contrast to the fairly homogenous colony size in the crosses when *cln1* and *cln2* were homozygous, in crosses when *mec1-1* and *CLN1* and *CLN2* were segregating, many of the spore colonies ranged in size from small to tiny (Figure 2B). When tetrads from the *CLN1 CLN2 CLN3* cross were scored for *mec1-1* by HU sensitivity, the small and tiny colonies were always HU sensitive, demonstrating that they contained *mec1-1*. A subset of the colonies was scored for the presence of *CLN1* and *CLN2* by Northern blotting. In 7/7 cases when the *mec1-1* strains were scored as fast growing (1D, 5A, 11C, 11D, 14D, 20D, 23D), the spore was *cln1 cln2 CLN3*. Furthermore, in 6/7 cases when the *mec1-1* strains were scored as slow growing (3B, 9C, 10D, 15B, 22B, 24D), the spore was *CLN1 cln2 CLN3* or *cln1 CLN2 CLN3*. In 1/7 cases, the slow-growing spore was *CLN1 CLN2 CLN3* (19C).

As all spores described in the crosses above were *CLN3*, we wished to determine whether the slow-growth phenotype observed with some *mec1-1* spore colonies was due to an increase in cyclin dosage or specifically due to the presence of *CLN1* or *CLN2*. We crossed *CLN1 cln2 cln3 MEC1* strains with *cln1 cln2 CLN3 mec1-1* strains and, similarly, crossed *cln1 CLN2 cln3 MEC1* with *cln1 cln2 CLN3 mec1-1* strains. Spore colonies were scored for size, HU sensitivity, and *CLN* genes as described above. In almost every case, small colony size correlated with the presence of *CLN1* or *CLN2* and the *mec1-1* mutation (Table 2). Strains that had *CLN3* in addition to *CLN1* or *CLN2* did not give significantly different colony sizes than those strains that had only *CLN1* or *CLN2*.

These results demonstrate that *MEC1* is required for normal growth rates in cells with wild-type levels of *CLN1* and/or *CLN2* and that its essential function can be suppressed by deletion of *CLN1* and *CLN2*. Although *MEC1* was originally reported to be necessary only in cells suffering from DNA damage (Weinert *et al.* 1994), these data demonstrate that *MEC1* is essential for normal growth of *CLN* cells. This is consistent with the observations of Paulovich *et al.* (1997) and Zhao *et al.* (1998) suggesting that *mec1-1* mutant strains are inviable in the A364a background in the absence of the suppressor locus *sml1*. Here, in *SML1* cells, the essential requirement for *MEC1* function is suppressed by deletion of *CLN1* and *CLN2*. The requirement for *MEC1* function in the DNA damage checkpoint is not suppressed; strains containing *cln1 cln2 mec1-1* or *cln1 cln2 mec1*Δ are still sensitive to HU.

To analyze the effects of increasing the amount of *CLB5* kinase activity on the *mec1* mutant strains, crosses between *cln1 cln2 CLN3 mec1-1* and *cln1 cln2 CLN3 sic1::URA3* strains were also examined. Deletion of the cyclin B kinase inhibitor *sic1* should result in increased and earlier activity of B-type cyclins, including *CLB5* (Schwob *et al.* 1994; Dirick *et al.* 1995). Tetrad analysis demonstrated that the *MEC1 SIC1*, *mec1-1 SIC1*, and *MEC1 sic1* spore colonies were all similar in size. In contrast, all 33 of the viable *mec1-1 sic1* spore colonies were significantly smaller than the other spore colonies (data not shown), consistent with the decreased plating efficiency of the *mec1 cln1 cln2 GAL-CLB5* strains. The viability of the *sic1 mec1* double mutants was 79%, comparable to the viability of the *sic1* single mutants (73%).

***rad53* and *mec1 tel1* mutants are not completely suppressed by loss of *CLN1* and *CLN2*:** On the basis of genetic and biochemical data, it has been suggested that *MEC1* functions upstream of *RAD53* and the kinase activity of Mec1p is required to activate Rad53p (Kiser and Weinert 1996; Sanchez *et al.* 1996; Sun *et al.* 1996). *RAD53* is an essential gene (Zheng *et al.* 1993). If *RAD53*'s only role is transducing a signal from *MEC1*, and loss of *CLN1* and *CLN2* suppress loss of *MEC1*, loss of *CLN1* and *CLN2* should also suppress the essential role of *RAD53*.

We backcrossed *rad53::HIS3* strains against *cln1 cln2 CLN3* strains multiple times. To cover the *rad53* lethality, the checkpoint-defective *rad53* allele, *spk1-1*, was present on a *URA3*-containing plasmid. In contrast to the results seen with *mec1*, deletion of *CLN1* and *CLN2* did not completely suppress the requirement for *RAD53*; all the

spore colonies that were His^+Ura^- (*i.e.*, *rad53::HIS3*) were significantly smaller than His^- or His^+Ura^+ spore colonies. Cultures of the *cln1 cln2 rad53* mutants grew to about $1/10$ the density of *cln1 cln2 RAD53* strains in rich liquid medium even after long times of incubation at 30° (Figure 3A). When cells from these cultures were plated on dextrose, the *rad53::HIS3* strains formed colonies that were smaller than wild type. We assayed strains containing *GAL1-CLN1 rad53::HIS3* on galactose and found that the presence of *GAL1-CLN1* decreases plating efficiency less severely for them than it did for the *mec1* strains. There was an ~10- to 100-fold decrease in plating efficiency of *rad53 GAL1-CLN1* strains compared to *rad53::HIS3* strains without *GAL1-CLN1* (Figure 3A). These results were obtained using *rad53* strains that had been backcrossed into the BF264-15D strain background four times; similar results were observed using strains that had been additionally backcrossed into this strain background (data not shown). Strains containing *rad53::HIS3* and the checkpoint-defective *rad53* allele *spk1-1* on a plasmid were not killed by expression of *CLN1* from the *GAL1* promoter (data not shown). As the growth defect of the *rad53* mutants was not fully suppressed by *cln1 cln2*, as the growth defect is in the *mec1* mutants, it appears that *rad53* has some *MEC1*-independent functions.

TEL1 has homology to *MEC1* and increased dosage of *TEL1* can suppress some *mec1* mutant phenotypes (Greenwell *et al.* 1995; Morrow *et al.* 1995). Rad53p may function downstream of both Mec1p and Tel1p. To determine if the *rad53* phenotypes were similar to the phenotypes observed with loss of *MEC1* and *TEL1*, we first generated *cln1 cln2 tel1* strains. *cln1 cln2 tel1* mutants displayed no growth defect and their plating efficiency was not affected by overexpression of *CLN1* or *CLN2* (data not shown). We then crossed *cln1 cln2 mec1-1* and *cln1 cln2 tel1::URA3* strains and analyzed spores resulting from the diploids. The spore viability of the *mec1-1 tel1* double mutants was high (93% in 95 tetrads), although the *mec1 tel1* double mutant spore colonies were always smaller than the other spore colonies. Like the *rad53* mutants, the *cln1 cln2 mec1 tel1* strains grew to about $1/10$ to $1/100$ the density of *cln1 cln2 MEC1* or *cln1 cln2 TEL1* cultures (Figure 3B and data not shown). The lethality in the *mec1 tel1* mutants caused by expression of *CLN1* or *CLN2* from the *GAL* promoter was similar to that seen with *mec1* mutants alone and is more severe than the lethality seen with the *rad53* strains (Figure 3B and data not shown). On the basis of these data and previous genetic analysis, the simplest interpretation of the similar growth defects seen with *cln1 cln2 rad53* and *cln1 cln2 mec1 tel1* strains is that *RAD53* functions downstream of both *MEC1* and *TEL1*. The decreased viability seen with overexpression of *CLN1* or *CLN2* in *mec1* or *mec1 tel1* strains compared to *rad53* strains is consistent with previous observations that

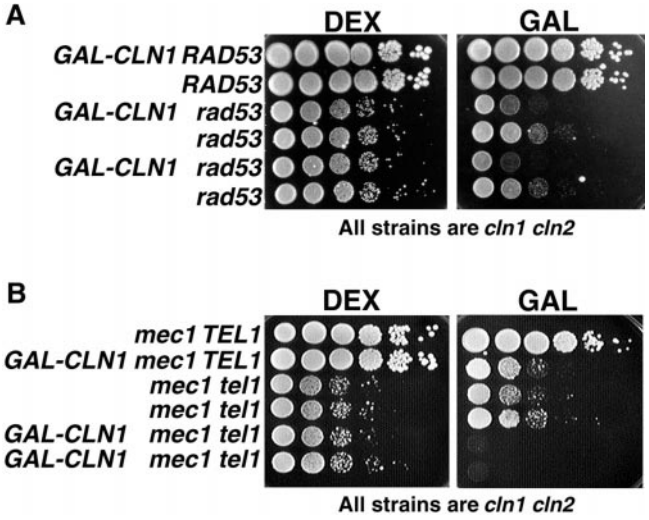

Figure 3.—(A) *rad53* mutant cells are only partly suppressed by *cln1 cln2* and are less sensitive to *GAL1-CLN1* expression than *mec1* mutants. Tenfold serial dilutions were made from fresh stationary phase cultures in YPD of strains with the indicated genotypes (*GAL-CLN1 cln1 cln2 RAD53*; 2673 4C; *cln1 cln2 RAD53*, 2673 5C; *GAL-CLN1 cln1 cln2 rad53*, 2673 6C and 2673 8A; *cln1 cln2 rad53*, 2673 6A and 2673 9C). Five microliter volumes were plated and incubated for 2–3 days at 30°. DEX, dextrose (glucose); GAL, galactose. (B) *mec1*Δ *tel1*Δ mutant cells have a growth defect and are sensitive to overexpression of *CLN1*. Tenfold serial dilutions were made from fresh stationary phase cultures in YPD of strains with the indicated genotypes (*cln1 cln2 mec1*Δ *TEL1*, 0020 11D; *GAL-CLN1 cln1 cln2 mec1*Δ *TEL1*, 0020 3A; *cln1 cln2 mec1*Δ *tel1*Δ, 0020 1B and 0020 3D; *GAL-CLN1 cln1 cln2 mec1*Δ *tel1*Δ, 0020 5C and 0020 7D. Five microliter volumes were plated and incubated for 2–3 days at 30°.

MEC1 has at least one *RAD53*-independent function (Kiser and Weinert 1996).

Multicopy *RNR1* suppresses the lethality of *mec1 CLN1* and *mec1 CLN2*: To understand more completely the cause of the inviability of *mec1-1 GAL1-CLN1* strains, we isolated multicopy plasmid suppressors of the lethal phenotype. Transformants (17,000) from a YEp24 library (Carlson and Botstein 1982) were screened for their ability to grow on galactose. The 13 strongest suppressors fell into three groups by restriction analysis and Southern blotting. Two plasmids contained *MEC1* and eight plasmids contained *TEL1*. Both of these classes were expected; the *mec1-1* mutation is known to be recessive to *MEC1*, and increased levels of *TEL1* have previously been shown to suppress other phenotypes associated with the *mec1-1* mutation (Morrow *et al.* 1995; Sanchez *et al.* 1996). The three remaining plasmids contained the *RNR1* gene. Transposon mutagenesis (Huisman *et al.* 1987) of the plasmid demonstrated that the suppression required an intact *RNR1* gene.

Multicopy *RNR1* suppressed the lethality of *mec1-1 GAL1-CLN1* strains about 1000× compared to the vector controls (data not shown). This was similar to the plating efficiencies found with *MEC1* plasmids; however, the

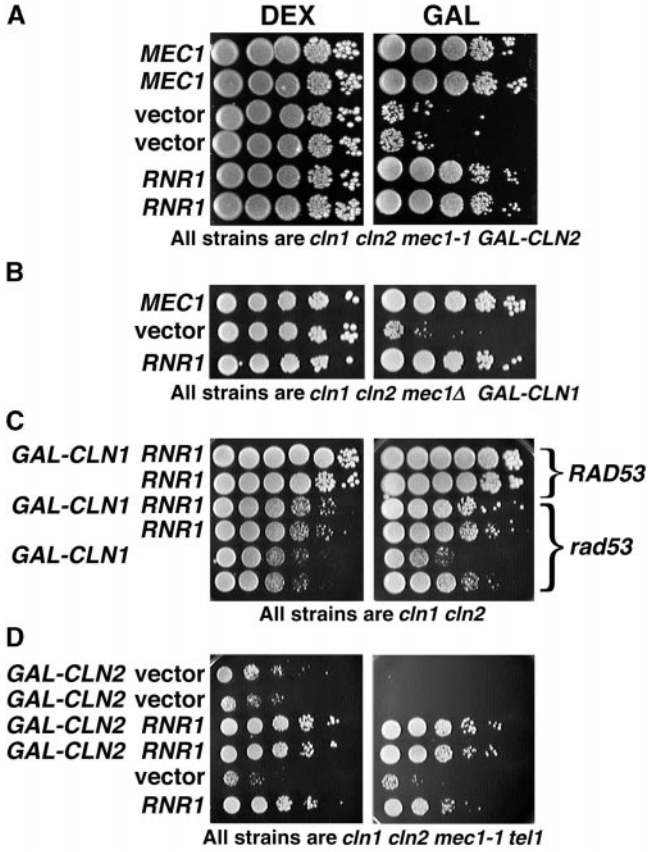

Figure 4.—Suppression by multicopy *RNR1*. (A and B) *mec1-1 GAL1-CLN2* and *mec1*Δ *GAL-CLN1* mutants are suppressed by multicopy *RNR1*. Strains 2665 13A (*mec1-1 GAL1-CLN2*) and 0015 2C (*mec1*Δ *GAL-CLN1*) were transformed with the indicated plasmids. Colonies were picked and grown to stationary phase in selective media containing 2% dextrose. Tenfold serial dilutions were made from fresh stationary phase cultures and 5 μl volumes were plated and incubated for 3–4 days at 30°. (C) Strains with the indicated genotypes (*RAD53 GAL-CLN1* multicopy *RNR1*, 2687 28C; *RAD53* multicopy *RNR1*, 2688 26A; *rad53 GAL-CLN1* multicopy *RNR1*, 2687 30B; *rad53* multicopy *RNR1*, 2687 30A; *rad53 GAL-CLN1*, 2687 26D; *rad53*, 2687 34A) were recovered after sporulation of a diploid containing the multicopy *RNR1* plasmid and analyzed. Cells were grown to stationary phase in YPD and 10-fold serial dilutions were made. Five microliter volumes were plated and incubated for 3–4 days at 30°. (D) Strains with the indicated genotypes (*mec1-1 tel1::LEU2 GAL-CLN2* YEp24, 0018 8B and 0018 11B; *mec1-1 tel1::LEU2 GAL-CLN2* multicopy *RNR1*, 0016 2D and 0016 18B; *mec1-1 tel1::LEU2 YEp24*, 0018 4D; *mec1-1 tel1::LEU2* multicopy *RNR1*, 0016 19D) were recovered after sporulation of a diploid heterozygous for *mec1-1* and *tel1::LEU2* that contained the multicopy *RNR1* plasmid. Cells were grown and plated as described for A and B. DEX, dextrose (glucose); GAL, galactose.

colony size of the *mec1-1 GAL1-CLN1* strains with the multicopy *RNR1* plasmid was somewhat smaller at early times of incubation than that of the *mec1-1 GAL1-CLN1* strains with the *MEC1* plasmid. The *RNR1* plasmid also suppressed the lethality caused by overexpression of *CLN2* (Figure 4A) or *CLB5* (data not shown) in a *mec1-1* strain. Similar results were seen with strains containing the *mec1*Δ allele, demonstrating that multicopy *RNR1* bypasses the requirement for *MEC1* function (Figure 4B).

To determine whether multicopy *RNR1* could suppress the growth defects caused by wild-type levels of *CLN1* and *CLN2* in a *mec1* strain, *mec1-1 cln1 cln2 CLN3* strains were crossed to *MEC1 CLN1 CLN2 CLN3* strains containing the multicopy *RNR1* plasmid. Diploids were sporulated and tetrads were dissected and scored as described above. Thirteen spores that were *mec1-1* and contained the *RNR1* plasmid were recovered. All spores containing the *RNR1* plasmid formed colonies similar in size to the *MEC1* spores; seven of the colonies were *CLN1* and/or *CLN2*. Furthermore, spore colonies that were *cln1 cln2 mec1-1* were able to lose the *URA3*-based *RNR1* plasmid as determined by their ability to grow on media containing 5-FOA while colonies that were *mec1-1 CLN1* and/or *CLN2* were unable to lose the plasmid. Taken together, this demonstrates that increased *RNR1* dosage can suppress the growth defect caused by *CLN1* and *CLN2* in a *mec1* mutant strain and suggests that the defect caused by overexpression of *CLN1* or *CLN2* is qualitatively similar to that caused by wild-type levels of G1 cyclin dosage in a *mec1* mutant strain.

To determine whether the multicopy *RNR1* plasmid could suppress the growth defect caused by deletion of *rad53*, a *cln1 cln2 CLN3 rad53::HIS3* strain containing the *URA3*-based *spk1-1* plasmid was crossed to a *cln1 cln2 CLN3 RAD53* strain. Diploids that had lost the *spk1-1* plasmid were transformed with the multicopy *URA3*-based *RNR1* plasmid and sporulated, and the resulting tetrads were dissected. Tetrads contained two large His^- colonies and zero, one, or two very small His^+ colonies. Increased *RNR1* dosage did not affect the colony size; Ura^+ His^+ (*RNR1*-containing; *rad53*) and Ura^- His^+ (*rad53*) colonies appeared similarly small on the tetrad dissection plate (data not shown). However, quantitative plating efficiencies showed that *cln1 cln2 rad53* strains containing the multicopy *RNR1* plasmid grew to higher densities in liquid culture than similar strains lacking the plasmid, although they did not reach the density achieved by *RAD53* strains. When *rad53* mutants containing *GAL-CLN1* were analyzed on galactose, the presence of the *RNR1* plasmid suppressed the decrease in viability associated with overexpression of *CLN1* in the *rad53* strains (Figure 4C). The ability of multicopy *RNR1* to suppress the lethality caused by overexpression of *CLN1* in both *mec1* and *rad53* mutant strains is consistent with the lethality being caused by a similar mechanism in both cases. Furthermore, this experiment demonstrates that *RAD53* function is not likely to be required for *RNR1*'s suppression of *mec1 GAL-CLN1* lethality.

To determine whether the multicopy *RNR1* plasmid could suppress the growth defect caused by *mec1 tel1*, a *cln1 cln2 CLN3 tel1::LEU2* strain was crossed to a *cln1 cln2 CLN3 mec1-1* strain. Diploids were transformed with the multicopy *URA3*-based *RNR1* plasmid and sporulated, and the resulting tetrads were dissected. Doubly

mutant *mec1 tel1* spore colonies were smaller than the singly mutant or wild-type colonies. As described above for *rad53* strains, increased *RNR1* dosage did not appear to affect the colony size; Ura$^+$ (*RNR1*-containing) and Ura$^-$ *mec1 tel1* colonies appeared similar in size (data not shown). However, quantitative plating efficiencies showed that, similar to *rad53* strains, *cln1 cln2 mec1 tel1* strains containing the multicopy *RNR1* plasmid grew to higher densities in liquid culture than similar strains lacking the plasmid. When *mec1 tel1* mutants containing *GAL-CLN2* were analyzed on galactose, the presence of the *RNR1* plasmid suppressed the decrease in viability associated with overexpression of *CLN2* (Figure 4D). This demonstrates that suppression of *mec1 GAL-CLN2* by multicopy *RNR1* does not depend on *TEL1* function. However, the persistent growth defect seen in *rad53* and *mec1 tel1* strains even in the presence of increased *RNR1* demonstrates that it is unlikely that the observed growth defects are due to limiting nucleotide levels.

One way to suppress the *mec1 GAL-CLN1* and *GAL-CLN2* synthetic lethality might be inhibition of passage through the G1 to S phase transition (START). We consider this explanation unlikely for *RNR1*'s ability to suppress for a few reasons. First, inhibition of passage through START is not consistent with the known function of ribonucleotide reductase. Second, if *RNR1* were inhibiting passage through START, there should be an accumulation of cells with 1N DNA content. Using FACS analysis, we analyzed the cell cycle distribution of logarithmically growing cells containing *GAL-CLN1*, *GAL-CLN2*, or *GAL-CLN3* and either a multicopy *RNR1* plasmid or a multicopy plasmid with *RNR1* disrupted with a transposon insertion. No difference in the cell cycle distribution of these strains was observed (data not shown). Third, if *RNR1* were inhibiting passage through START without affecting cell growth, cell size would be expected to increase (Cross *et al.* 1989). Analysis of cell volume [using a Coulter Channelyzer (Coulter Corp., Hialeah, FL)] demonstrated that cells containing the *RNR1* plasmid were no bigger than cells found in the vector controls (data not shown). Taken together, these data suggest that it is unlikely that increased *RNR1* function is simply inhibiting passage through START.

***RNR1* transcription levels are decreased in *GAL1-CLN1* and *GAL1-CLN2* strains:** As multicopy *RNR1* suppressed the lethality of the *mec1-1 GAL1-CLN1* and *GAL1-CLN2* strains, we analyzed the levels of *RNR1* transcript in these strains. Levels of *RNR1* are about threefold lower in *mec1-1 GAL1-CLN1* or *mec1-1 GAL1-CLN2* strains than in *mec1-1* with vector controls (Figure 5, A and B). A similar decrease in *RNR1* transcription was found in *MEC1 GAL1-CLN1* and *MEC1 GAL1-CLN2* strains, dem-

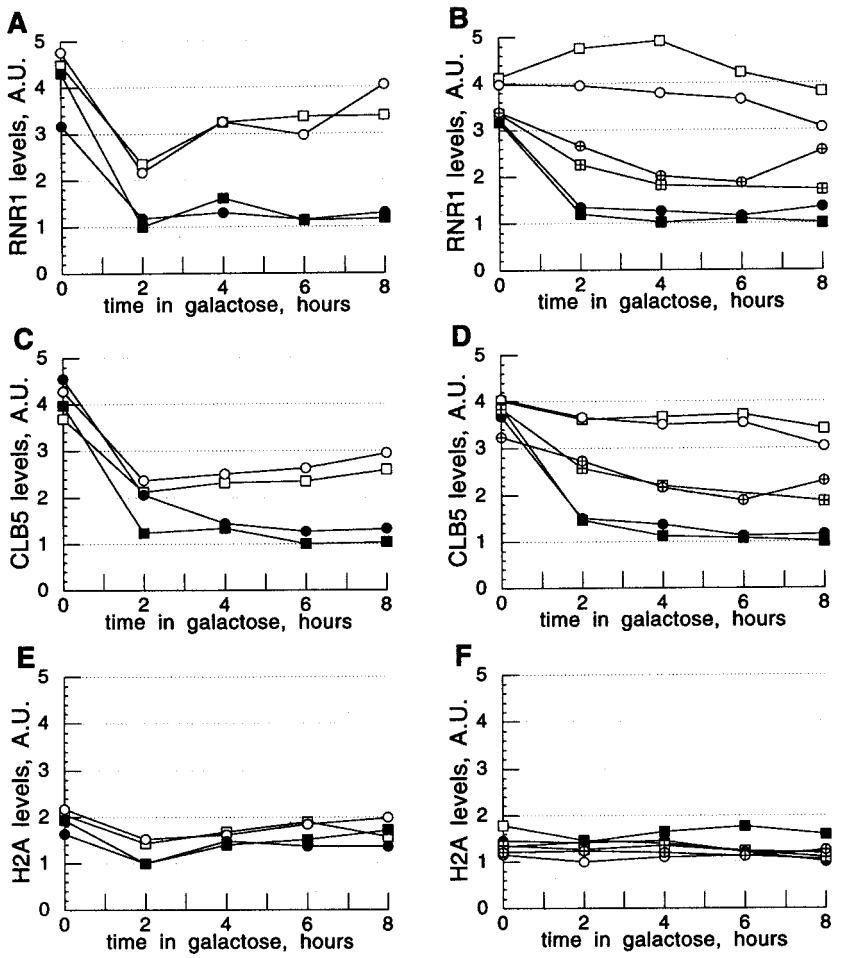

Figure 5.—Transcriptional regulation of MCB-containing genes *RNR1* and *CLB5* and of *H2A*. *cln1 cln2 CLN3 MEC1* and *cln1 cln2 CLN3 mec1-1* strains with the indicated *GAL1-CLN* construct were grown to log phase in YEP-3% raffinose at 30°. At time 0, galactose was added to the cultures to a final concentration of 3%. Samples were taken at 2-hr intervals and RNA was isolated. Blots were hybridized with *RNR1* (A and B), *CLB5* (C and D), *H2A* (E and F), and *TCM1* (used as a loading control). Quantification of mRNA was performed using a Molecular Dynamics phosphorimager and ImageQuant software. Data from two different experiments are shown; Northern blots were prepared and analyzed from samples five times with equivalent results. (A, C, and E) *MEC1*, open squares (1238 16B); *MEC1 GAL-CLN1*, solid squares (2507 5B); *mec1-1*, open circles (2618 5B); *mec1-1 GAL-CLN1*, solid circles (2623 11D). (B, D, and F) *MEC1*, open squares (1238 16B); *MEC1 GAL-CLN2*, solid squares (2671 5B); *MEC1 GAL-CLN3*, hatched squares (2670 8A); *mec1-1*, open circles (2671 5A); *mec1-1 GAL-CLN2*, solid circles (2671 11B); *mec1-1 GAL-CLN3*, hatched circles (2670 2D).

onstrating that the decrease in *RNR1* levels was not due to the *mec1-1* mutation (Figure 5, A and B). The decrease in *RNR1* transcription was evident in both *MEC1* and *mec1-1* cells, but has lethal consequences only in the *mec1-1* mutants. *GAL1-CLN3* decreased transcription of *RNR1* to a level intermediate between that of *GAL1-CLN1* or *GAL1-CLN2* and the vector control (Figure 5B).

RNR1 transcription has been previously shown to be cell cycle regulated (Elledge and Davis 1990) and the coding sequence is preceded by four MCB elements within the 500 nucleotides upstream of the AUG that starts the protein-coding region. To determine whether *GAL1-CLN1* and *GAL1-CLN2* affected other MCB-regulated genes, we analyzed the transcript levels of another MCB-containing gene, the B-type cyclin *CLB5*. *CLB5* levels also decreased as a consequence of *GAL1-CLN1* and *GAL1-CLN2* expression (Figure 5, C and D). It is likely that the decrease in *RNR1* and *CLB5* RNA levels seen upon induction of the *CLN* genes is due to a change in the amount of active MBF present in the population or to an alteration in the distribution of cells in the cell cycle, not to direct repression of *RNR1* and *CLB5* transcription.

To determine whether the transcription of other genes was also affected, we analyzed the expression of the histone *H2A*. In contrast to the results seen with the MCB-regulated *CLB5* and *RNR1* transcripts, *H2A* mRNA was not affected by the expression of *CLN1* or *CLN2* (Figure 5, E and F). H2A transcripts peak about 0.1 cell cycle units after the MCB-regulated genes and are subject to a different pathway of regulation (White *et al.* 1987). Although histone transcription is cell-cycle regulated, the steady-state levels of histone transcripts appear to be tightly coupled to the ongoing rate of DNA replication (Osley 1991; Muller 1994). The observation that H2A transcript levels do not decrease upon *CLN* overexpression suggests that DNA replication and cell division are occurring similarly in all strains.

Because *RNR1* is also regulated by DNA damage (Elledge and Davis 1990), we wished to determine whether high levels of expression of the *CLN* genes from the *GAL1* promoter affected DNA-damage-inducible genes. We analyzed the levels of two damage-inducible genes, *RNR3* and *UBI4*, in *mec1-1* and *MEC1* strains containing *GAL1-CLN* constructs. DNA damage induces *RNR3* transcription in a *MEC1*-dependent pathway and *UBI4* transcription in a *MEC1*-independent pathway (Kiser and Weinert 1996). The levels of these transcripts were not altered upon *GAL1-CLN* expression (data not shown). This demonstrates that high levels of *CLN* expression do not induce a DNA-damage response.

DISCUSSION

***MEC1* is required in unperturbed wild-type cells, but not in *cln1 cln2* cells:** Although the *mec1-1* mutation was originally identified as causing lethality specifically when DNA damage was induced or replication slowed (Weinert *et al.* 1994), our results clearly show that *MEC1* is required in normally cycling wild-type cells. This is consistent with the observation that a suppressor locus, *sml1*, was present in the previously characterized *mec1-1* strains (T. Weinert, personal communication; Paulovich *et al.* 1997; Zhao *et al.* 1998). However, we showed previously (Vallen and Cross 1995) and confirm here that in a *cln1 cln2* background, no additional suppressor in our strain background is required for full viability and wild-type growth of *mec1-1* strains.

Mec1p has been shown to be required for slowing of S phase in response to DNA damage (Paulovich and Hartwell 1995). The present results therefore suggest that some Mec1-dependent slowing of S phase may be required even in unperturbed wild-type cell cycles, but that this slowing is not required in *cln1 cln2* strains. In contrast to the case with *S. cerevisiae*, the *S. pombe MEC1* homolog, *rad3*, is not essential. One possibility is that because the two yeasts regulate their size control in different stages of the cell cycle (G1 for *S. cerevisiae*, G2 for *S. pombe*), they have different requirements for DNA synthesis checkpoints in unperturbed cell cycles (Elledge 1996). Consistent with this argument, *wee1* mutant fission yeast, which converts from a G2/M to a G1/S size control (Fantes and Nurse 1978), requires *rad3* for viability (Al-Khodairy and Carr 1992). It may be that in both yeasts, *MEC1*/*rad3* is required to ensure that there is sufficient time to prepare for and execute DNA synthesis but that this requirement is cryptic in *S. pombe* because the time spent in G2 usually results in adequate growth for the following S phase (Elledge 1996). The Mec1p requirement for the DNA replication checkpoint induced by hydroxyurea treatment is separate temporally from the cell cycle function and is not bypassed in *cln1 cln2* strains, as *cln1 cln2 mec1-1* strains are sensitive to hydroxyurea inhibition of DNA synthesis. Therefore, we conclude that deletion of *CLN1* and *CLN2* eliminates the Mec1p requirement specifically in the unperturbed cell cycle.

Rad53p cannot function solely downstream of Mec1p: *RAD53* is an essential gene that has been proposed to function in the same pathway as *MEC1*. Analysis of the transcriptional induction of DNA-damage-inducible genes suggests that *MEC1* is upstream of *RAD53* because it affects the transcription of more genes (Kiser and Weinert 1996). However, *cln1 cln2 rad53* strains are inviable or else form tiny colonies in tetrad analysis, in contrast to the large colonies formed by *cln1 cln2 mec1-1* and *cln1 cln2 mec1*Δ strains. In addition, when *spk1-1*, a checkpoint-deficient allele of *RAD53*, is used, full viability is observed, and *CLN1* overexpression does not affect this viability. These data suggest that Rad53p has at least one function that is not wholly dependent on Mec1p.

It is likely that *TEL1* modulates the *MEC1*-independent activity of *RAD53*. *TEL1* and *MEC1* are 48% similar and it has been shown that they have some overlap in function (Greenwell *et al.* 1995; Morrow *et al.* 1995). *cln1 cln2* cells deleted for both *MEC1* and *TEL1* have a

growth defect that appears similar to that of *cln1 cln2 rad53* cells. Other work has also suggested that *RAD53* may have some roles that are *MEC1*-independent as temperature-sensitive defects in a component of the replication factor C complex, *rfc5-1*, can be suppressed by increased expression of *RAD53* and *TEL1*, but not by *MEC1* (Sugimoto *et al.* 1997). Furthermore, the ability of *RAD53* overexpression to suppress the *rfc5-1* defect is dependent on *TEL1* function (Sugimoto *et al.* 1997). Additional evidence that *RAD53* may have *MEC1*-independent functions is that *rad53 rad16* double mutants show increased sensitivity to UV irradiation compared to either single mutant, while *mec1 rad16* double mutants do not show this synthetic phenotype (Kiser and Weinert 1996). Although interpretation of the UV sensitivity is complicated by the fact that the *mec1* and *rad53* mutations analyzed were point mutations, rather than null alleles, and also that the *sml1* suppressor may be present only in the *mec1* mutant strains, these data, as well as the data presented here, are consistent with the model that Rad53p is regulated by proteins in addition to Mec1p, such as Tel1p.

One difference between the *rad53* and *mec1 tel1* strains is their response to overexpression of *CLN*s; the growth defect in the *rad53* strains is not as exacerbated by *CLN1* or *CLN2* overexpression as the *mec1* or *mec1 tel1* mutant strains. *MEC1* and *TEL1* likely have some activity that is not mediated through *RAD53*. It is known, for example, that *MEC1* is required for the transcriptional activation of some genes that do not require *RAD53* (Kiser and Weinert 1996).

***CLN1* and *CLN2* function may lead to dNTP limitation and a requirement for the Mec1 checkpoint:** *cln1 cln2 mec1-1* strains overexpressing Cln1p (from the *GAL1-CLN1* construct) are inviable (Vallen and Cross 1995). *RNR1*, encoding the limiting subunit of ribonucleotide reductase, is an efficient high-copy plasmid suppressor of this inviability. We found that overexpression of either *CLN1* or *CLN2* lowered *RNR1* expression (similarly in *mec1-1* and *MEC1* backgrounds). These results combined to lead us to the following hypothesis to explain *mec1 GAL1-CLN1* lethality: if *CLN1* expression results in entry into S phase before a sufficient period for accumulation of Rnr1p, cells may enter S phase with inadequate dNTP pools. If this happens in a *MEC1* background, this should result in the characterized Mec1-dependent slowing of S phase, consistent with full viability; but in a *mec1* background this slowing of S phase would not occur, leading to mitosis without completion of replication and inviability of progeny. We showed previously that in diploid cells of the genotype *mec1-1 GAL-CLN1*, rare survivors showed signatures of DNA damage: 100-fold elevated chromosome loss and recombination frequencies, as would be expected from this hypothesis (Vallen and Cross 1995). The most likely explanation for the ability of multicopy *RNR1* to suppress the essential requirement for *MEC1* is that cells require *MEC1* to inhibit or slow S phase until adequate pools of dNTPs have accumulated. Overexpression of *RNR1* would be expected to increase the levels of dNTPs and might allow S phase to begin earlier or proceed more quickly.

It has been previously reported that cell cycle length or doubling time does not change much in the presence of overexpressed *CLN* genes, but much less of the cell cycle is taken up by G1 because cells go through START at a smaller size (Cross 1988; Nash *et al.* 1988). Because doubling time is constant, the cells must be delayed at some other cell cycle stage. It may be that cells containing *GAL-CLN1* or *GAL-CLN2* are delayed in S phase in a *MEC1*-dependent fashion. We attempted to perform execution point experiments to determine the length of S phase in wild-type cells and cells overexpressing the G1 cyclins; while the data suggested that *CLN1* overexpression prolonged S phase, variability between strains in this analysis prevents drawing definitive conclusions from these experiments. Additionally, because *mec1* mutant cells fail to arrest in HU, it is not possible to measure the length of S phase in *mec1* strains by this method. Another prediction of the model is that *GAL1-CLN1* strains containing multicopy *RNR1* would have a shorter S phase. Although FACS analysis of cells containing the high-copy *RNR1* plasmid demonstrated that the plasmid does not appear to affect the cell cycle distribution of strains, because of the breadth of the 1N and 2N peaks, and because the number of cells that are in S phase is small, it is impossible to tell whether the number of cells in S phase is reduced by this analysis.

A surprising consequence of the hypothesis that cells frequently enter S phase with inadequate dNTP pools, combined with the observation of semilethality or lethality of *CLN1 CLN2 CLN3 mec1-1* strains, is that preparation for DNA replication, including dNTP accumulation, in wild-type cells may be barely adequate for completion of S phase, resulting in a significant requirement for Mec1 function to restrain the rate of S phase progression. Wild-type cells may operate according to a "just-in-time" principle, *i.e.*, transit through START and entry into S phase may occur when there are usually just adequate materials for DNA replication. This would be highly efficient because it allows cells to enter the cell cycle with a minimum of preparatory time, thus giving rise to more progeny, but it could impose a requirement for safeguards in case of shortages.

Deletion of *CLN1* and *CLN2* may result in an unbalanced cell cycle with excess time for preparation for DNA synthesis, suppressing the Mec1 requirement: Cln3p has been proposed to be specialized for transcriptional activation of SCB- and MCB-regulated genes at the G1-S border; *RNR1* is one such gene (Tyers *et al.* 1993; Koch and Nasmyth 1994; Dirick *et al.* 1995; Stuart and Wittenberg 1995; Levine *et al.* 1996). Cln1 and Cln2, in contrast, directly trigger cell cycle START, and lead to DNA replication (at least in part by activation of Clb-Cdc28 kinase complexes; reviewed

by Cross 1995; Nasmyth 1996). Thus in a *cln1 cln2* background, a prolonged period of transcriptional activation of SCB- and MCB-dependent genes occurs before DNA synthesis and other START events (Dirick *et al.* 1995; Stuart and Wittenberg 1995). Deletion of *CLN1* and *CLN2* may suppress inviability due to *mec1* by providing a longer period for preparation for DNA synthesis, including dNTP accumulation [for which our results and others (Wang *et al.* 1997) suggest that *RNR1* may be limiting].

The results obtained with deletion of *CLN1* and *CLN2* may be due to qualitative functional differences between Cln3p and Cln1p or Cln2p, because the efficiency of cell cycle transit is lower in *cln2 cln3* strains than in *cln1 cln2* strains (as measured by cell volume; Lew *et al.* 1992) and yet the former, but not the latter, genotype is semiinviable in combination with *mec1-1*. Additionally, *mec1 cln1 cln2* cells expressing high levels of *CLN3* from the *GAL* promoter are viable; these cells transit through G1 more quickly than *CLN* strains. Taken together, this demonstrates that the requirement for *MEC1* is not simply correlated with cell volume. Intrinsic qualitative differences between Cln3 and Cln2 have been documented previously on other grounds (Levine *et al.* 1996); such differences can be attributed to differences in efficiency of transcriptional activation by Cln3p compared to Cln2p, consistent with the results here. It is likely that the *GAL-CLN3* strains have more transcriptional activation of SCB- and MCB-regulated genes relative to other START events than the *GAL-CLN1* and *GAL-CLN2* strains do.

The essential requirement of *MEC1* may be identical to its checkpoint function in HU-treated cells: Although deletion of *CLN1* and *CLN2* can suppress the essential function of *MEC1*, cells are still sensitive to HU. These data are consistent with a model suggesting that deletion of *CLN1* and *CLN2* does not directly substitute for *MEC1* function, but, instead, bypasses the essential requirement for *MEC1* by altering the timing of some cell cycle events. When cells are treated with the ribonucleotide reductase inhibitor HU, the delay in activation of DNA synthesis caused by deletion of *CLN1* and *CLN2* must no longer suffice. This would be expected as cells must pause for a longer time, and within S phase (after the B-type cyclins have already been activated by the *CLN*s), until they have accumulated enough nucleotides in the presence of HU to complete DNA synthesis. However, in both cases, the requirement for *MEC1* is identical: to restrain DNA replication and/or mitosis when nucleotides are limiting. Nucleotides may be limiting due to low levels of *RNR1*, the Rnr inhibitor HU, or the recently characterized Sml1 protein, which inhibits ribonucleotide reductase (Zhao *et al.* 1998). Deletion of *sml1*, like deletion of *cln1 cln2*, would function to increase the levels of active Rnr. In the presence of wild-type *SML1*, or *CLN1 CLN2*, *MEC1* function would restrain the cell cycle until there were adequate levels of dNTPs to complete S phase. Conversely, deletion of these genes would lead to an increase in Rnr activity and thereby bypass the essential requirement for *MEC1*. While Zhao *et al.* suggest the possibility that Mec1p may relieve Sml1p antagonism of Rnr1p, a simpler explanation, consistent with our results, is that Sml1p is a partial inhibitor of ribonucleotide reductase that is not regulated by Mec1p. The presence of Sml1p might then result in a borderline or insufficient level of deoxyribonucleotides for DNA replication, thus resulting in a Mec1p requirement for the same reason that Mec1p is required in HU-treated cells. This model is simpler in that it accounts for rescue of *mec1* lethality by high-copy *RNR1*, by deletion of *cln1* and *cln2* and by *sml1* mutation, and does not require Mec1p to have additional checkpoint functions unrelated to its essential role.

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