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 CLNs 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 (CLBs), DNA replication, bud emergence, and microtubule organizing center duplication (see Lew et al. 1997 for a recent review). Although CLNs are redundant for viability in an otherwise wild-type strain, there are significant and qualitative differences between the CLNs 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 MCM (Mlu1 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 α2β2, 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 Virols 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...
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 (Yarrow 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 ε 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 mec1Δ mutant cells deleted for cn1 and cn2 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 (trpl-1a leu2-3,112 ura3 ade1 his2) unless otherwise noted. Mutant cn1, cn2, and cn3 alleles, and the GAL1-CLN1, GAL1-CLN2, GAL1-CLN3, GAL1-CLBS cassettes have been described previously (Richardson et al. 1989; Cross 1990; Cross and Tinkel enberg 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 mec1Δ, 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 cn1 cn2 diploid strain in the BF264-15D background (R. Gardner and T. Weinert, personal communication). Spores from the diploid were analyzed; the viability for mec1Δ 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 cn1 cn2 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, mec1Δ, 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°C.

**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 TC1 as a loading control. Probe fragments CLN1, CLN2, UB14, H2A, and CLB5 were as described elsewhere (Cross and Tinkel enberg 1991; Epstein and Cross 1992; Kiser and Weinert 1997).
TABLE 1

Yeast strains

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<th>Genotype</th>
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<td>MAT α dn1/cln2 CLN3 mec1::ura3::TRP1</td>
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<tr>
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<td>2678 26A</td>
<td>MATα cln1 cln2 CLN3 RAD3::HIS3 HIS2 his3 multicopy RNR1</td>
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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.

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 SCGal-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 the sequence CTGCAAGCTATAATTTCGAG and GGTCTTAA TACATACTAACG.
Figure 1.—mec1 mutant cells die when CLN1, CLN2, or CLB5 is overexpressed. Strains 2507 SD (dn1 cln2 MEC1), 2620 12C (dn1 cln2 mec1), and 218UL-9 (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°C. Dex, dextrose (glucose); Gal, galactose.

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

<table>
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<th>Relevant genotype</th>
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</table>

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°C 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 mec1α 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 crosses were scored for mec1-1 by the slow-growth phenotype observed with some mec1-1 strains. Hence, 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 smll. Here, in SM1 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 mec1Δ 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 mec1-1 sic1::URA3 strains were also examined. Deletion of the G1 cyclin 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 (Kisler and Weinert 1996; Sanchez et al. 1996; Sun et al. 1996). RAD53 is an essential gene (Zhang 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 \(\frac{1}{10}\) 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 TEL1. 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 \(\frac{1}{10}\) to \(\frac{1}{100}\) 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 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 \(\times\) compared to the vector controls (data not shown). This was similar to the plating efficiencies found with MEC1 plasmids; however, the
the mec1Δ 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. Thirty spores that were mec1-1 and contained the RNR1 plasmid were recovered. All spores containing the RNR1 plasmid formed colonies similar in size to those in the MEC1 strains; 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 plasmid was crossed to a cln1 cln2 CLN3 RAD53 strain. Diploids that had lost the plasmid were transformed with the multicopy URA3-based RNR1 plasmid and sporulated, and the resulting tetrad was dissected. Tetrad 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 increase 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 resulting from 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 mec1Δ 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 tetrad were dissected. Doubly colony size of the mec1-1 GAL-CLN1 strains with the multicopy RNR1 plasmid was somewhat smaller at early times of incubation than that of the mec1-1 GAL-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

Figure 4.—Suppression by multicopy RNR1. (A and B) mec1-1 GAL1-CLN2 and mec1Δ GAL1-CLN1 mutants are suppressed by multicopy RNR1. Strains 2665 3A (mec1-1 GAL1-CLN2) and 0015 2C (mec1Δ GAL1-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 26C; RAD53 multicopy RNR1, 2687 26A; rad53 GAL-CLN1 multicopy RNR1, 2687 30B; rad53 multicopy RNR1, 2687 30A; rad53 GAL-CLN1, 2687 26D; rad53, 2687 30B) 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 8A; mec1-1 tel1::LEU2 GAL-CLN2 multicopy RNR1, 0016 2D and 0016 3B; 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.
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 Channelizer (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 phosphorimagery and ImageQuant software. Data from two different experiments 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 (2618 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 CLN5 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 affects 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, weel mutant fission yeast, which converts from a G2/M to a G1/S size control (Fant 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 viable or else form tiny colonies in tetrad analysis, in contrast to the large colonies formed by cln1 cln2 mec1-1 and cln1 cln2 mec1Δ strains. In addition, when ssk1-1, 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 \textit{dn1 cln2 rad53} cells. Other work has also suggested that \textit{RAD53} may have some roles that are \textit{MEC1}-independent as temperature-sensitive defects in a component of the replication factor C complex, \textit{rfc5-1}, can be suppressed by increased expression of \textit{RAD53} and \textit{TEL1}, but not by \textit{MEC1} (Sugimoto et al. 1997). Furthermore, the ability of \textit{RAD53} overexpression to suppress the \textit{rfc5-1} defect is dependent on \textit{TEL1} function (Sugimoto et al. 1997).

Additional evidence that \textit{RAD53} may have \textit{MEC1}-independent functions is that \textit{rad53 rad16} double mutants show increased sensitivity to UV irradiation compared to either single mutant, while \textit{mecl rad16} double mutants do not show this synthetic phenotype (Kiser and Wittenberg 1996). Although interpretation of the UV sensitivity is complicated by the fact that the \textit{mecl} and \textit{rad53} mutations analyzed were point mutations, rather than null alleles, and also that the \textit{sm1} suppressor may be present only in the \textit{mecl} mutant strains, these data, as well as the data presented here, are consistent with the model that \textit{Rad53p} is regulated by proteins in addition to \textit{MEC1}.

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

\textit{CLN1} and \textit{CLN2} function may lead to dNTP limitation and a requirement for the \textit{Mecl} checkpoint: \textit{dn1 dn2 mecl-1} strains overexpressing \textit{Cln1p} (from the \textit{GAL1-CLN1} construct) are inviable (Vallen and Cross 1995). \textit{RNR1}, encoding the limiting subunit of ribonucleotide reductase, is an efficient high-copy plasmid suppressor of this inviability. We found that overexpression of either \textit{CLN1} or \textit{CLN2} lowered \textit{RNR1} expression (similarly in \textit{mecl-1} and \textit{MEC1} backgrounds). These results combined to lead us to the following hypothesis to explain \textit{mecl} \textit{GAL1-CLN1} lethality: if \textit{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 \textit{MEC1} background, this should result in the characterized \textit{mecl}-dependent slowing of S phase, consistent with full viability; but in a \textit{mecl} 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 \textit{mecl-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 \textit{RNR1} to suppress the essential requirement for \textit{MEC1} is that cells require \textit{MEC1} to inhibit or slow S phase until adequate pools of dNTPs have accumulated. Overexpression of \textit{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 \textit{CLN} genes, but much less of the cell cycle is taken up by G1 because cells go through \textit{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 \textit{GAL-CLN1} or \textit{GAL-CLN2} are delayed in S phase in a \textit{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 \textit{CLN1} overexpression prolonged S phase, variability between strains in this analysis prevents drawing definitive conclusions from these experiments. Additionally, because \textit{mecl} mutant cells fail to arrest in HU, it is not possible to measure the length of S phase in \textit{mecl} strains by this method. Another prediction of the model is that \textit{GAL-CLN1} strains containing multicopy \textit{RNR1} would have a shorter S phase. Although FACS analysis of cells containing the high-copy \textit{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 \textit{CLN1 CLN2 CLN3 mecl-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 \textit{Mecl} 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 \textit{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.

\textbf{Deletion of \textit{CLN1} and \textit{CLN2} may result in an unbalanced cell cycle with excess time for preparation for DNA synthesis, suppressing the \textit{Mecl} requirement:} Cln3p has been proposed to be specialized for transcriptional activation of SCB- and MCB-regulated genes at the G1-S border; \textit{RNR1} is one such gene (Tyers et al. 1993; Koch and Nasmyth 1994; Dirick et al. 1995; Stuart and Wittetemberg 1995; Levine et al. 1996). Cln1 and Cln2, in contrast, directly trigger cell cycle \textit{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 semi-invasive 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 CLN3s), 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 Sm1 protein, which inhibits ribonucleotide reductase (Zhao et al. 1998). Deletion of sm1, 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 Sm1p 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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