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Mutations in *RAD27* Define a Potential Link between G_1 Cyclins and DNA Replication

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The yeast *Saccharomyces cerevisiae* has three G_1 cyclin (*CLN*) genes with overlapping functions. To analyze the functions of the various *CLN* genes, we examined mutations that result in lethality in conjunction with loss of *cln1* and *cln2*. We have isolated alleles of *RAD27*/*ERC11*/*YKL510*, the yeast homolog of the gene encoding flap endonuclease 1, *FEN-1*. *cln1 cln2 rad27*/*erc11* cells arrest in S phase; this cell cycle arrest is suppressed by the expression of *CLN1* or *CLN2* but not by that of *CLN3* or the hyperactive *CLN3-2*. *rad27*/*erc11* mutants are also defective in DNA damage repair, as determined by their increased sensitivity to a DNA-damaging agent, increased mitotic recombination rates, and increased spontaneous mutation rates. Unlike the block in cell cycle progression, these phenotypes are not suppressed by *CLN1* or *CLN2*. *CLN1* and *CLN2* may activate an *RAD27*/*ERC11*-independent pathway specific for DNA synthesis that *CLN3* is incapable of activating. Alternatively, *CLN1* and *CLN2* may be capable of overriding a checkpoint response which otherwise causes *cln1 cln2 rad27*/*erc11* cells to arrest. These results imply that *CLN1* and *CLN2* have a role in the regulation of DNA replication. Consistent with this, *GAL-CLN1* expression in checkpoint-deficient, *mec1-1* mutant cells results in both cell death and increased chromosome loss among survivors, suggesting that *CLN1* overexpression either activates defective DNA replication or leads to insensitivity to DNA damage.

In *Saccharomyces cerevisiae*, the major control point in the cell cycle is the G_1-to-S transition known as Start. After passage through Start, cells begin a division program that includes bud emergence, DNA synthesis, and microtubule-organizing center duplication (42). Like many cell cycle transitions, passage through Start is controlled by a cyclin-dependent kinase (Cdc28)–cyclin complex (reviewed in reference 38).

Any one of the three G_1 cyclin genes, *CLN1*, *CLN2*, or *CLN3*, is sufficient for transition through Start (9, 44). However, it is likely that *CLN3*'s role in Start may be qualitatively different from the roles of *CLN1* and *CLN2*. This is based on a variety of observations, including structural homology, regulation of expression, in vivo activities, and in vitro kinase activities (5, 12, 21, 25, 39, 57, 63). From these data, it has been suggested that the substrates of Cln3 may be different from the substrates of Cln1 and Cln2 and that the primary function of Cln3 might be to activate the transcription of other genes, including *CLN1* and *CLN2* (57).

After progression through Start, cells are committed to DNA replication. The mechanistic details by which the Cdc28-Cln kinase commits cells to replication are not yet well understood. One likely role for the Cdc28-Cln protein kinase is in the activation of the transcription factor, MBF (3, 57). MBF can then induce the transcription of a number of genes required for DNA replication (reviewed in reference 35). The B-type cyclin genes, *CLB5* and *CLB6*, are expressed at about the time of Start and promote transit through S phase (17, 51, 57). The Cdc28-Cln kinase may affect Cdc28-Clb5,6 kinase activity both by activating the transcription of *CLB5,6* (57) and by leading to the degradation of the *CLB*-specific inhibitor, $p40^{SIC1}$ (50).

In addition to having a role as an activator of DNA synthesis after Start, the Cdc28 kinase is a likely target for the inhibition of cell cycle progression by proteins involved in checkpoint pathways. A checkpoint ensures that cells have completed an early event with sufficient fidelity to proceed to a later event (24, 59). If an upstream event has not been completed, the cell cycle must be arrested to allow completion or repair of the incompletely assembled structure. Both DNA damage and incomplete DNA replication result in checkpoint-mediated cell cycle arrest (59, 61).

In this work, we describe mutations in *RAD27*/*ERC11*/*YKL510*, the yeast homolog of the gene encoding FEN-1, a mammalian structure-specific endonuclease (23). Mutant alleles of *rad27*/*erc11* result in defects associated with DNA replication and repair. In the absence of both *CLN1* and *CLN2*, strains with *rad27*/*erc11* point mutations are inviable at 38°C and arrest as large-budded cells; expression of *CLN1* or *CLN2* suppresses the inviability and Cdc^- arrest in the mutants, apparently without significantly affecting the levels of DNA damage. *CLN1* and *CLN2* may either activate another pathway for DNA synthesis without affecting DNA repair or, alternatively, override a checkpoint block caused by the DNA damage found in *erc11* mutant strains. Consistent with *CLN1* altering regulation of DNA replication, *CLN1* overexpression is lethal in cells defective for *MEC1*, a DNA damage checkpoint component.

MATERIALS AND METHODS

Strains and media. Media and genetic methods were as described elsewhere (4, 47). All yeast strains were isogenic with BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*). Mutant *cln1*, *cln2*, and *cln3* alleles, *CDC28*::*HA*, and the *GAL-CLN1* and *GAL-CLB5* cassettes have been described previously (10, 11, 16, 44). A derivative of the *LEU2*::*GAL1*::*CLN1* cassette in which *LEU2* was inactivated with *URA3* was constructed by transforming a *leu2*::*LEU2*::*GAL1*::*CLN1* strain with an *Hpa*I-*Sal*I fragment of pLU23 (9a). pLU23 contains the *LEU2* gene disrupted by introduction of the *URA3*/Kan^R cassette from JA53-delP (derived from JA53, obtained from S. Elledge) into the *Eco*RV site of *LEU2*. This converted *leu2*::*LEU2*::*GAL1-CLN1* to *leu2*::*URA3*::*GAL1-CLN1*.

Disruption of *RAD9* was accomplished by integrating pTW032 (*rad9*::*TRP1*) (60) into strain 1242-8B. Disruption of *RAD2* was accomplished by integrating KM55 (*rad2*::*URA3*) (obtained from L. Prakash) into strain 2507-5B. In both cases, transformants were scored for radiation sensitivity basically as described

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elsewhere (60). Other strains containing the *rad9* or *rad2* disruption were generated by crossing to the original transformants. The *mec1-1* allele (62) was backcrossed five times to BF264-15D strains; multiple strains were examined for all phenotypes.

The isolation of *erc11-1* and *erc11-2* has been described previously (5). Because of a bookkeeping error (4a), strains containing these alleles were incorrectly reported to have a non-Cdc$^-$ arrest phenotype (5), whereas in fact they had not been tested. We report here the first characterization of the *erc11* arrest; both *erc11* alleles result in first-cycle Cdc$^-$ arrest.

Cloning of *RAD27*/*ERC11*/*YKL510*. Strain 1983-5B (*cln1 cln2 CLN3 GAL1-CLN1 erc11-2*) was transformed with YCp50- and CE101-based genomic libraries (8, 17), and transformants were screened for their ability to grow at 38°C on YPD plates (47). Complementing plasmids were recovered by transformation into *Escherichia coli*, analyzed by restriction digestion and Southern blotting, and mapped by hybridization to a chromoblot and lambda phage grid.

To demonstrate that we had cloned *ERC11*, plasmid LB46 was digested with *Bam*HI and used to transform strain 2507-5D (*MAT***a** *cln1 cln2 CLN3 ura3*). Integration was confirmed by Southern blotting, two independent transformants were crossed to 2522-23B (*MAT*α *cln1 cln2 CLN3 leu2*::*GAL-CLN1*::*LEU2 ura3 erc11-2*), and tetrads were analyzed.

Plasmid constructions. Plasmid pLB16, recovered from the YCp50-based genomic library, was digested with *Bam*HI, treated with Klenow fragment, and religated to form plasmid pLB50.

A 3.8-kb *Eco*RI fragment from the YCp50-based genomic library plasmid pLB17 was subcloned into pRS316 (54) to form pLB44. A 4.4-kb *Eco*RI fragment from LB16 was subcloned into RS316 to form pLB39. pLB17 was digested with *Bam*HI and religated, which deleted a 3.5-kb fragment and resulted in LB37. The 3.5-kb *Bam*HI fragment was cloned into pRS316 to form pLB36.

pLB44 was digested with *Cla*I and religated to form pLB52 and pLB53. In pLB44, there are two *Cla*I sites present in the genomic DNA insert, as well as one site in the vector sequences; pLB53 contains a deletion of all the genomic DNA to the second *Cla*I site. pLB52 is most probably the result of a partial digest and contains a deletion of the genomic DNA to the first *Cla*I site.

A 2.0-kb *Bgl*II fragment from pLB17 was subcloned into pRS306 and pRS316 digested with *Bam*HI, resulting in pLB46 and pLB48. pLB46 was cut with *Xho*I and *Eco*RI, treated with Klenow fragment, and religated to delete the *Cla*I site within the polylinker sequences. The resulting plasmid was digested with *Cla*I and *Bam*HI to delete a portion of the *RAD27*/*ERC11* coding sequence (from bp −60 to +756 relative to the ATG start codon), which was replaced by a TRP1/Kan^r cassette from pJA52 (obtained from S. Elledge) to form pLB68.

To disrupt *RAD27*/*ERC11*, diploid 2536-2A/2D was transformed with plasmid pLB68. The occurrence of the transplacement events was confirmed by Southern blot analysis of the structure of the genomic DNA, and two independently transformed diploids were sporulated and analyzed.

Sequence analysis. Sequencing of DNAs from plasmids LB37 and LB36 was performed by using Sequenase version 2.0 according to the manufacturer's instructions.

Determination of DNA content by fluorescence-activated cell sorter (FACS). Flow cytometric DNA quantitation was performed as described elsewhere (17).

Pulsed-field gel electrophoresis assay. Yeast strains were grown to early log phase in YPGal at 30 or 38°C. Glucose was added to half of each culture, and incubation was continued for 6 h. For control samples, hydroxyurea (added directly; final concentration, 0.1 M) or nocodazole (added from a 10-mg/ml stock solution in dimethyl sulfoxide; final concentration, 15 μg/ml) was added. The *cdc9* mutant strain, H9C1A1 (obtained from L. Hartwell), was grown to early log phase at 23°C. The culture was split and incubated at 23 or 38°C for 6 h. Chromosomal DNA samples were prepared from strains by a modification of the method of Schwartz and Cantor (49) as described elsewhere (47), applied to a 1% agarose gel, and electrophoresed in 0.5× TBE (Tris-borate-EDTA) at 15 to 18°C for 36 h at 170 V (CHEF-DRII pulsed-field system; Bio-Rad) with an initial switching time of 70 s ramped to 120 s by a Pulsewave 760 Switcher (Bio-Rad).

Immunofluorescent microscopy. Immunofluorescent staining of yeast cells was performed by a modification of the methods of Adams and Pringle (1) and Kilmartin and Adams (30) as described elsewhere (46). Rat monoclonal antiserum against α-tubulin (YOL1/34 [31]) was from Accurate Chemical and Scientific Corp. (Westbury, N.Y.). Secondary antibodies were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The fluorescent DNA-specific dye DAPI (4′,6-diamidino-2-phenylindole) was used to visualize yeast nuclei.

Northern (RNA) analysis. To analyze the cell cycle regulation of *ERC11* transcription, RNA was isolated from cells arrested by *cdc15-2* block release and *cln* block release protocols as described previously (36, 39). *CLN2*, *Pr1*, *CLB5*, and *H2A* probes were as described previously (17, 39). A 1.6-kb *Hin*dIII-*Hin*dIII fragment was used as an *ERC11* probe, and a 0.7-kb *Nco*I-*Nco*I fragment was used as a *CDC9* probe. The *PCL1* probe was made from pBA674 (the gift of B. Andrews).

To analyze the induction of *ERC11* transcription as a result of DNA damage, *cln1 cln2 CLN3 GAL-CLN1* cells were grown to log phase in media containing glucose or galactose. To prearrest cells with pheromone before addition of methyl methane sulfonate (MMS), *MAT***a** bar$^-$ cell cultures were preincubated with α-factor (Sigma) added to a final concentration of 0.1 μM for 2.5 h. MMS was added to a final concentration of 0.05%, and cells were harvested at 1-h intervals.

RNA was isolated, probes were labelled, and Northern blots and cell-budding assays were performed as described elsewhere (36, 39). Quantification of mRNA levels was performed by using a Molecular Dynamics phosphoimager, and mRNA loading was normalized by using *TCM1* as a loading control.

Immunoprecipitation, immunoblot analysis, and kinase assays. Cultures (100 ml) of yeast cells at 10^7 cells per ml were harvested by filtration, washed twice in LSHN buffer (50 mM NaCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5] per 10% glycerol), and resuspended in LSHNN buffer (LSHN plus 0.1% Nonidet P-40) with inhibitors (5% aprotinin, 10 mM $NaPP_i$, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 10 μg of phenylmethylsulfonyl fluoride per ml, 1 mM $NaVO_4$, 10 mM NaF, 25 mM β-glycerophosphate). Cells were broken by being vortexed with glass beads (three times for 2 min each), and the lysate was cleared by spinning the mixture for 2 min at 13,000 × *g*. The clarified supernatant was incubated with 1 μl of 12CA5 antibody (from mouse ascites fluid [Babco]) on ice for 1 h, reclarified by centrifugation, and then rotated in the presence of protein A beads for 1 h at 4°C. Beads were collected by centrifugation and washed four times with LSHNN buffer and once with kinase buffer (10 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol). After being resuspended in 60 μl of kinase buffer, the sample was split for kinase assays and Western blot (immunoblot) analysis.

One-quarter of each sample was transferred to a fresh tube for kinase assays. Kinase mix (1 μl of histone H1 [2 mg/ml], 2 μl of 50 μM ATP, 1 μl of [γ^{32}P]ATP [10 mCi/ml, 3,000 Ci/mmol], 1 μl of H_2O) was added, and samples were incubated at 30°C for 10 min with occasional mixing. Reactions were terminated by addition of 2× sample buffer, and mixtures were heated to 95°C for 5 min before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel. Protein gels were transferred to Immobilon-P membranes (Millipore) with a semidry-transfer apparatus (Hoefer), and membranes were exposed to film.

The beads from the remainder of the sample were aspirated dry, 60 μl of 2× sample buffer was added, and samples were treated as described above. Protein gels were transferred to Immobilon-P membranes with a wet-transfer apparatus (Hoefer), and proteins were detected by the ECL system (Amersham) according to the manufacturer's directions. Primary antibody (rabbit polyclonal anti-HA; Babco) was used at a dilution of 1:10,000, and secondary antibody was used at a dilution of 1:1,000.

Mutation rate analysis, mitotic recombination and chromosome loss assays, and sensitivity to MMS. Rate of mutation to Can^R was calculated by the Lea-Coulson method of the median (33). Single colonies from YPGal plates were grown in YPD or YPGal medium. Nine independent cultures of each genotype were analyzed in the first experiment, and 15 independent cultures of each genotype were analyzed in the second.

To analyze the amount of chromosome loss and mitotic recombination in *rad27*/*erc11* mutant strains, *ERC11*, *erc11-2*, and *erc11*::*TRP1* diploids (*leu2*::*LEU2*::*GAL1-CLN1*/*leu2*::*URA3*::*GAL1-CLN1*; note that *leu2* and *MAT* are on opposite arms of chromosome III) were purified and a single colony was used to inoculate SCGal-Ura-Leu medium (47). Cultures were grown to early stationary phase, diluted in YPGal or YPD, and grown for 12 h. Approximately 5×10^6 cells were mixed with an equal number of W3031a or W3031b cells and mated on filters for 3.5 h as described elsewhere (47). To determine the number of cells and mating events, cells were sonicated and then plated on SCGal-Ura-Leu and SCDex-Ura-Leu (to determine input number of viable cells) and mating events were selected on SCDex-Ade-His. To determine the number of chromosome loss events, the mating selection plates were replica plated to SCDex-Ade-His-Ura and SCDex-Ade-His-Leu. Chromosome loss events were scored as those which resulted in loss of both *MAT* and the linked *URA3* or *LEU2* marker.

For the *mec1-1* mutant strains, diploids (*MEC1* or *mec1-1* and *leu2*::*LEU2*::*GAL1-CLN1*/*leu2*::*URA3*::*GAL1-CLN1*) were purified and a single colony was used to inoculate SCDex-Ura-Leu medium. Cultures were grown to early stationary phase and diluted 1:100 in YPRaf and grown for 12 h. These cultures were then diluted in YPGal or YPD and grown for 12 h. Matings and selections were performed as described above.

To analyze the sensitivity of strains to MMS, exponential-phase cultures were grown in YPGal and spread on YPD or YPGal plates. Sterile paper discs (diameter, 0.635 cm, no. 740-E; Schleicher & Schuell, Keene, N.H.) containing 20 μl of dilutions of MMS (Sigma) were placed on the surface. Plates were incubated at 30°C for 2 days.

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 days.

RESULTS

Isolation and characterization of mutant alleles of *RAD27*/*ERC11*/*YKL510*. To identify potential targets of the Cdc28-Cln kinase required for cell cycle progression, we have analyzed mutations that result in lethality in a *cln1 cln2 CLN3* background at 38°C and are suppressed by expression of *CLN1* (see

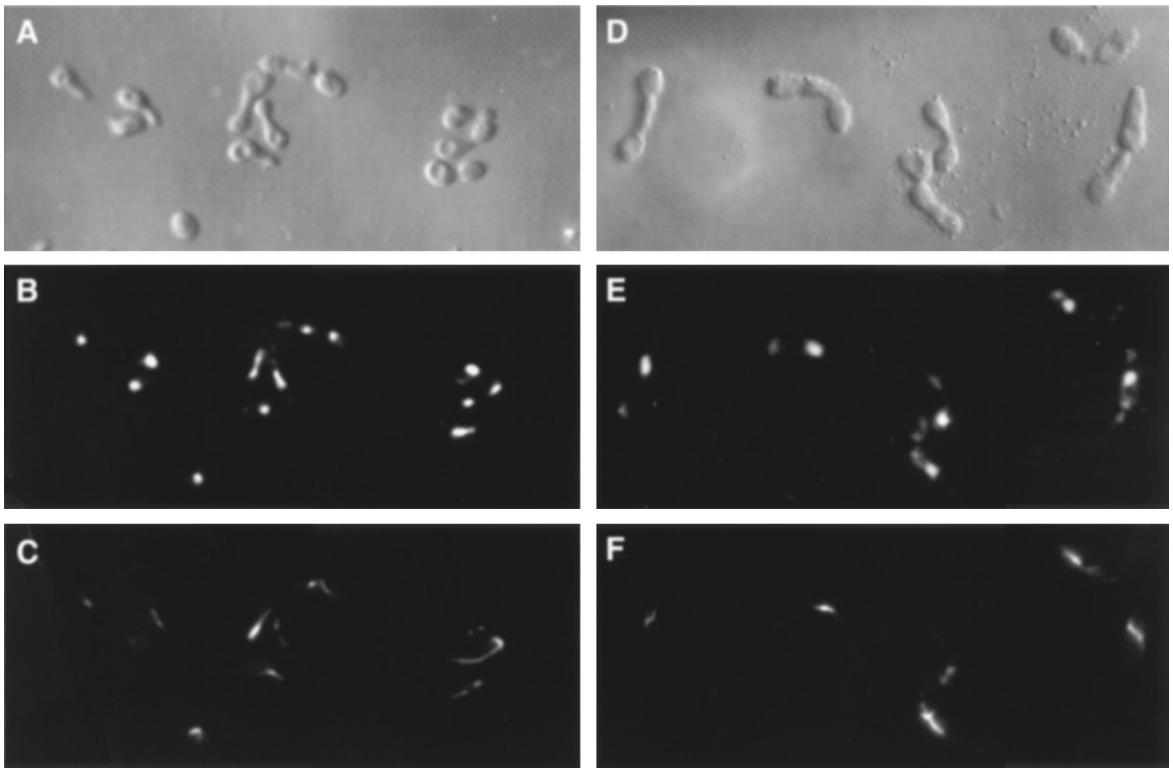

FIG. 1. Cdc$^-$ arrest of the *erc11-2* mutant. Strains 2507-5B (*cln1 cln2 CLN3 GAL-CLN1 ERC11*) (A–C) and 1983-5B (*cln1 cln2 CLN3 GAL-CLN1 erc11-2*) (D–F) were grown to early log phase in YPGal at 38°C, and then glucose was added to repress *GAL-CLN1* expression. Six hours after glucose addition, samples were fixed and cells were examined with Nomarski optics (A and D), by DAPI staining of DNA (B and E), and by indirect immunofluorescent staining of microtubules (C and F).

Fig. 6 and reference 5). These mutations cause an elevated requirement for *CLN* function (Erc$^-$) because *CLN3* is usually sufficient to promote transition through the cell cycle. The screen for *erc* alleles was performed with a *cln1 cln2 CLN3* strain containing *GAL-CLN1* on a *CEN*-based plasmid (5). The Erc$^-$ phenotype (i.e., viability on galactose and inviability on glucose) was determined to be due specifically to the presence of the *GAL-CLN1* construct by both plasmid loss experiments and backcrossing the putative mutants. Only those mutants which required *GAL-CLN1* for their viability on galactose were retained for further analysis (5), ensuring that suppression on galactose was due to the expression of *CLN1.*

Analysis of strains carrying either of the two *erc11* alleles isolated in the screen demonstrated that they are able to grow on glucose-containing media when they are transformed with plasmids containing *CLN1* or *CLN2* under the control of their endogenous promoters (*CEN*-based and 2μ-based vectors; see Fig. 6B). Furthermore, we constructed and analyzed strains containing the *erc11-2* allele and an intact chromosomal copy of either *CLN1* or *CLN2*. It is critical to note that these strains are able to grow on glucose- or galactose-containing media (i.e., *CLN1 cln2 cln3 erc11* and *cln1 CLN2 cln3 erc11* strains are viable, while *cln1 cln2 CLN3 erc11* strains are not). This demonstrates that wild-type levels of *CLN1* or *CLN2*, but not of *CLN3*, are sufficient for rescue of the *erc11-2* allele. It also confirms that the rescue of *cln1 cln2 CLN3 GAL-CLN1 erc11* strains on galactose as opposed to glucose is not simply due to the carbon source. Finally, *CLN3-2* (an activated allele of *CLN3*) and *GAL-CLN3* were unable to suppress *erc11-2*. Taken together, these data demonstrate that the inviability of the *erc11* mutants on glucose is not simply due to a change in growth rate, or to an inability to survive on glucose medium, but rather is due to the specific loss of wild-type levels of the *CLN1* or *CLN2* gene product.

Of 28 complementation groups with the Erc$^-$ phenotype, only those containing *erc11* exhibited first-cycle, cell division cycle (Cdc$^-$) arrest as large-budded cells (55a). *cln1 cln2 erc11* mutant cells with *GAL-CLN1* arrested with this phenotype and with first-cycle kinetics when shifted from galactose to glucose at 38°C (Fig. 1 and 2B) or from glucose at 30°C to glucose at 38°C (data not shown). Furthermore, *erc11-2* strains lacking *GAL-CLN1* also displayed these phenotypes when shifted from galactose at 30°C to galactose at 38°C (a further control for carbon source effects; data not shown). *cln1 cln2 erc11-2* cells arrested with a single nucleus and short spindle under the nonpermissive condition (Fig. 1). The *cln1 cln2 erc11-2* strains gradually lost viability at 38°C, with only 10% viability after 6 h of incubation. We do not know the explanation for the low reversibility of the *erc11* block.

Arrest with the morphology described above is frequently associated with a block in DNA replication (6). To determine if *erc11-2* affects DNA synthesis, we examined the DNA content of mutant *erc11-2* cells by FACS analysis (Fig. 2). *ERC11* strains grown with *CLN1* expressed showed a bimodal distribution of DNA content, with peaks at 1 and 2 N (Fig. 2A). The budding index of the cultures was also consistent with their distribution throughout the cell cycle (Fig. 2B). Upon repression of *CLN1* expression by the addition of glucose, the *cln1 cln2 ERC11* strains showed an increase in the number of unbudded, G_1 cells as they delayed in their transit through Start (1 to 2 h after addition of glucose). For the *ERC11 cln1 cln2 CLN3* strain, the number of budded cells then increased and the population redistributed into approximately equal G_1 and G_2 populations (by 4 to 5 h after addition of glucose). For the

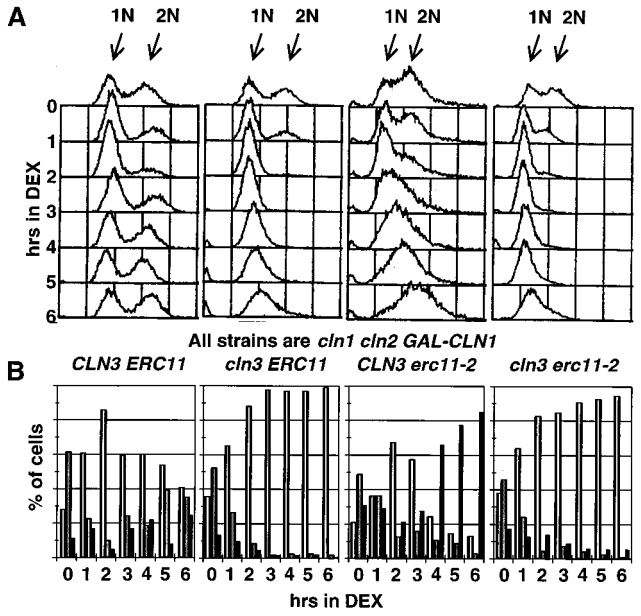

FIG. 2. FACS analysis of the *erc11-2* mutant. Strains 1584-4D (*cln1 cln2 CLN3 GAL-CLN1 ERC11*), 1584-1A (*cln1 cln2 cln3 GAL-CLN1 ERC11*), 1584-4C (*cln1 cln2 CLN3 GAL-CLN1 erc11-2*), and 1584-11A (*cln1 cln2 cln3 GAL-CLN1 erc11-2*) were grown to early log phase in YPGal at 38°C, and then glucose was added at time zero to repress *GAL-CLN1* expression. Samples were fixed, stained with propidium iodide, and counted by using a FACS (A). Aliquots from each time point were analyzed microscopically to determine the budding index (B). Small and medium buds are less than two-thirds the size of the mother, and large buds are at least two-thirds the size of the mother. DEX, dextrose (glucose). □, unbudded cells; ▒, small or medium buds; ■, large buds.

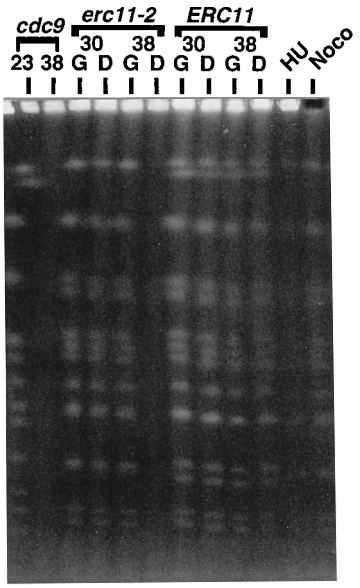

FIG. 3. Chromosomes of *erc11-2* and *ERC11* strains. Strains 2507-5B (*cln1 cln2 CLN3 GAL-CLN1 ERC11*) and 1983-5B (*cln1 cln2 CLN3 GAL-CLN1 erc11-2*) were grown to early log phase in YPGal at 30 and 38°C as indicated. Glucose was added to half of each culture, and incubation was continued for 6 h. Chemical inhibitors (HU, hydroxyurea; Noco, nocodazole) were added to aliquots of 1983-5B in galactose at 30°C. The *cdc9* mutant strain, H9C1A1, was grown at 23°C; the culture was split and incubated at 23 or 38°C for 6 h. Chromosomal DNA was isolated from each sample, separated on a pulsed-field gel, and stained with ethidium bromide. G, galactose; D, dextrose (glucose).

ERC11 cln1 cln2 cln3 strain, cells remained unbudded and accumulated in G_1, as expected for a strain devoid of G_1 cyclin function. The increase in fluorescent staining in these cells may be due to continuing replication of mitochondrial DNA.

When *CLN1* was expressed, the *erc11-2* mutant cells were distributed throughout the cell cycle (Fig. 2), although the profiles of the *erc11-2* strains were somewhat different from that of the wild type. FACS analysis demonstrated that there were more cells in the S and G_2 phases of the cell cycle and the budding index showed a corresponding increase in the number of large-budded cells, suggesting a delay in transit through the S and/or G_2 phases of the cell cycle. The difference between *ERC11* and *erc11-2* mutant cells was more pronounced with the *erc11-2 cln1 cln2 CLN3 GAL-CLN1* mutant strain than the *erc11-2 cln1 cln2 cln3 GAL-CLN1* strain; the presence of *CLN3* may decrease the amount of time spent in the G_1 portion of the cell cycle and lead to accumulation of S/G_2 phase cells.

Upon repression of *GAL-CLN1* expression by the addition of glucose, the *erc11* strains showed an increase in the number of unbudded, G_1 cells, similar to what was observed with wild-type strains. However, in contrast to wild-type cells, the *erc11-2 cln1 cln2 CLN3* cells appeared to pass only slowly through S phase and failed to redistribute into a discrete G_2 peak at 3 to 4 h after repression of *CLN1* transcription. This demonstrates that with repression of *CLN1*, transit through S phase is blocked or significantly delayed in the mutant. The increase in fluorescent staining (possibly due to replication of mitochondrial DNA; see above) makes it difficult to be certain of the exact nuclear DNA content in these cells at later time points. However, comparing the *erc11-2 cln1 cln2 CLN3* and *erc11-2 cln1 cln2 cln3* strains indicates that in the first cycle following shift to restrictive conditions, *erc11-2 cln1 cln2 CLN3* cells synthesized some DNA but may have been unable to complete replication. The percentage of budded cells continued to increase in the *erc11-2 cln1 cln2 CLN3* culture, and by 6 h after the shift, about 85% of the cells were arrested with large buds.

To confirm that *erc11-2* cells arrested in S phase, we used pulsed-field gel electrophoresis since chromosomes isolated from cells arrested in S phase fail to band properly on these gels (26). Most probably this is due to the presence of replication bubbles, forks, or flaps in the DNA which affect its migration ability. As expected, DNA isolated from cells blocked in S phase by a temperature-sensitive DNA ligase allele (*cdc9*) or by hydroxyurea treatment failed to band (Fig. 3, 2nd and 11th lanes), while DNA isolated from cells blocked after DNA replication by the microtubule polymerization inhibitor, nocodazole, demonstrated the characteristic pattern of chromosome bands (12th lane). Under permissive conditions, DNA isolated from an *erc11-2* mutant strain showed a chromosome-banding pattern similar to that of the parental *ERC11* strain (Fig. 3, third through fifth lanes compared with seventh through ninth lanes). In contrast, under nonpermissive conditions, i.e., in the absence of *CLN1* expression at 38°C, the DNA isolated from the *erc11-2* mutant fails to band (Fig. 3, sixth lane). Taken together with the FACS data, this suggests that the *erc11-2* mutant cells arrest in S phase.

Analysis of mutant *erc11-1* strains yielded results similar to those for *erc11-2* strains in the pulsed-field gel electrophoresis assay. Minor alterations in the mobility of specific chromosomes seen for the *erc11-2* mutant under permissive conditions were not observed in the *erc11-1* mutant.

Cloning and characterization of *YKL510*/*ERC11*: homology to *FEN-1* and *RAD2*. We cloned the *ERC11* gene by complementation of the *erc11-2* phenotype. Nineteen plasmids, containing DNA from four different genomic loci, were isolated. Two loci were identified as *CLN1* (three plasmids) and *CLN2*

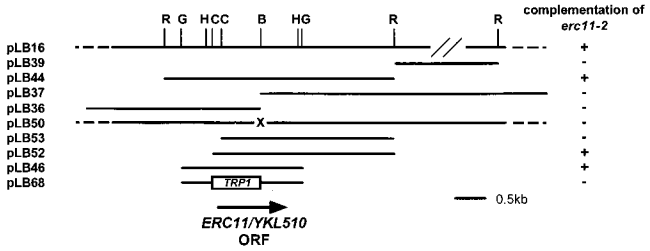

FIG. 4. Localization of *ERC11/YKL510*. The arrow indicates the extent of the *ERC11/YKL510* open reading frame and predicted direction of transcription. Lines indicate the genomic DNA present in plasmids, and X indicates a frameshift mutation. Complementation was scored as the ability of a *CEN*-based plasmid to complement the temperature-sensitive phenotype of 1983-5B (*cln1 cln2 CLN3 GAL-CLN1 erc11-2*) on glucose media at 38°C. Restriction enzyme sites are designated as follows: B, *Bam*HI; G, *Bgl*II; C, *Cla*I; R, *Eco*RI; H, *Hin*dIII.

(four plasmids). These were expected, because *CLN1* and *CLN2*, but not *CLN3* or *CLN3-2* (an activated allele of *CLN3*), were able to suppress the *erc11-2* defect when present on *CEN*-based plasmids (see above). The third locus (11 plasmids) corresponded to *RAD27/ERC11/YKL510*, while the fourth (1 plasmid) corresponded to *SEL1* (see Discussion).

To identify the *ERC11* plasmid, a genomic fragment from the locus was meiotically mapped by targeted integration of *URA3* followed by tetrad analysis of diploids heterozygous for the *URA3*-marked locus and for *erc11-2.* No recombinant *URA3*$^+$ *erc11-2* spores were generated in 23 tetrads, demonstrating tight linkage between *ERC11* and the plasmid DNA.

To confirm that the cloned DNA contained the wild-type *ERC11* gene, we targeted duplicative integration of a plasmid containing *URA3* and DNA from the putative *ERC11* locus into an *erc11-2* mutant strain, 1983-5B. Sixteen of 16 transformants were complemented for the Erc$^-$ defect, as evidenced by their ability to grow at 38°C in the absence of *CLN1* expression. Subsequently, cells which had removed the duplication by homologous recombination were selected on 5-fluoro-orotic acid. Of the 36 colonies tested, 31 were Erc$^+$, demonstrating that the plasmid contained wild-type *ERC11* DNA capable of repairing the *erc11-2* mutation.

We mapped the *ERC11* gene to the right arm of chromosome XI, and we identified a *Bam*HI restriction site within the region required for complementation of the *erc11-2* mutation (Fig. 4). Following bidirectional sequence analysis from this site, we determined that *ERC11* had been sequenced as part of the chromosome XI sequencing project and had been named *YKL510* (GenBank accession no. S93804 [28]). *YKL510* was later renamed *RAD27* on the basis of its homology to *RAD2* and the phenotypic analysis of a null allele (43) after our work had been submitted for publication. In this paper, we refer to the gene as *RAD27/ERC11* to be consistent with our figures and the mutations that we have characterized as *erc11* alleles; however, we recognize that the *RAD27* nomenclature has precedence over the *ERC11* designation.

RAD27/ERC11 is highly homologous to the mouse flap endonuclease 1 (*FEN-1*) gene (60% identical amino acids over the entire predicted open reading frame) (22, 23), as well as to *Schizosaccharomyces pombe rad2*$^+$ (56% identical) and a human *rad2*$^+$ homolog (58% identical) (37). The products of these genes have been implicated biochemically and genetically to have roles in DNA synthesis and repair. They are members of a larger family which includes *Saccharomyces cerevisae RAD2*, *Schizosaccharomyces pombe rad13*$^+$, and human XP-G, which also have roles in DNA repair (7, 20, 48).

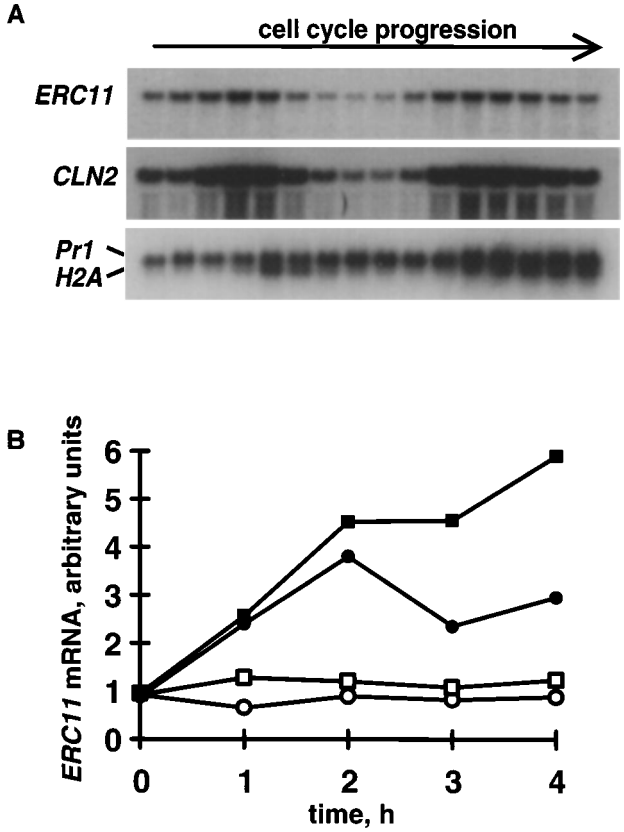

FIG. 5. Transcriptional regulation of *ERC11* mRNA. (A) *cdc15-2* (K2944) cells were grown at 25°C and then arrested in late M phase by shifting the culture to 36°C for 3 h. Synchronous division was induced by shifting cells back to 25°C, and samples for Northern analysis were taken every 12 min. (B) *cln1 cln2 CLN3 GAL-CLN1* (2507-5B *MAT*α or 2101-13C *MAT***a** *bar*$^-$) cells were grown in glucose at 30°C. To arrest cells with pheromone before the addition of MMS, α-factor was added to 2101-13C cells. MMS was added to a final concentration of 0.05%, and samples were taken at 1-h intervals. Quantification of *ERC11* induction was performed by using a Molecular Dynamics phosphoimager, and mRNA levels were normalized by using *TCM1* as a loading control. ○, glucose; ●, glucose plus MMS; □, glucose plus pheromone; ■, glucose plus pheromone plus MMS.

The FEN-1 protein has been demonstrated to have DNA endonuclease and 5′→3′ exonuclease activity. FEN-1 has also most likely been purified as a protein, sometimes called MF-1, required for replication in vitro (18, 27, 56, 58). This factor has been demonstrated to be required for completion of lagging-strand DNA synthesis. In addition to its activities on DNA, it has 5′→3′ RNA exonuclease activity on RNA-DNA hybrids and is likely required for the removal of ribonucleotides at the 5′ end of Okazaki fragments.

***RAD27/ERC11* transcription is cell cycle regulated and induced by DNA damage.** The transcription of many genes involved in DNA synthesis is cell cycle regulated, peaking in late G_1 and being dependent upon promoter elements called *Mlu*I cell cycle boxes (MCB elements) (reviewed in reference 35). The upstream region of *RAD27/ERC11* contains three *Mlu*I sites, as well as three elements with a single base pair change. The *RAD27/ERC11* transcript was cell cycle regulated, peaking in late G_1 similarly to *CLN2* (Fig. 5A). The cell cycle regulation of transcription was evident by two different methods of cell synchronization. In the experiment shown in Fig. 5A, cells were synchronized by arrest in M phase by incubation of *cdc15-2* mutant cells at the nonpermissive temperature and

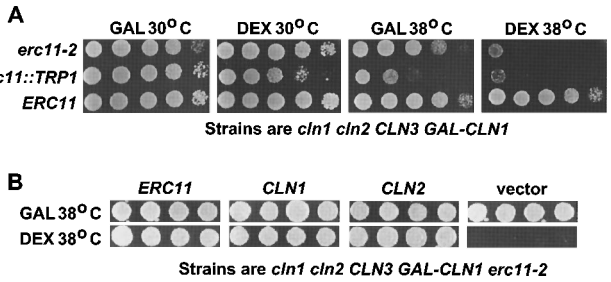

FIG. 6. (A) Plating efficiency of *erc11* mutant strains. Five-microliter volumes of 10-fold serial dilutions were made from fresh stationary-phase cultures, plated, and incubated for 2 days at the indicated temperatures. (B) Suppression of *erc11-2* by high-copy plasmids. Strain 1983-5B (*cln1 cln2 CLN3 GAL-CLN1 erc11-2*) was transformed with 2μm-based plasmids with the indicated genomic DNA inserts. Single colonies from four transformants of each genotype were inoculated onto plates by using a 48-prong inoculator and incubated for 2 days. DEX, dextrose (glucose); GAL, galactose.

released (39). We have also analyzed the transcription of *RAD27/ERC11* after synchronization by arrest of *cln1 cln2 cln3 GAL-CLN3* cells in raffinose followed by refeeding with galactose (36) and found similar results (data not shown).

In addition to its being cell cycle regulated, *RAD27/ERC11* transcription was induced by a DNA-alkylating agent, MMS. *cln1 cln2 CLN3 GAL-CLN1* strains were treated with MMS. Steady-state levels of *RAD27/ERC11* transcript increased within 1 h of MMS treatment, and levels showed a 3- to 10-fold increase at their peak in both glucose and galactose (Fig. 5B and data not shown). This is similar to the induction we observed for *RAD2* (data not shown), a gene previously described to be induced by DNA damage (45). For two reasons, the increase in transcript levels is not likely to be due to an accumulation of cells at a point of the cell cycle when the level of *RAD27/ERC11* transcription is high. First, the percentage of unbudded cells does not change significantly during MMS treatment, suggesting that cells are not accumulating at any specific stage of the cell cycle (data not shown). Second, cells prearrested in the G_1 phase of the cell cycle by treatment with α-factor show a similar increase in *RAD27/ERC11* transcription upon treatment with MMS (Fig. 5B). From these experiments, we conclude that *RAD27/ERC11* transcription is regulated during cell cycle progression and independently inducible by DNA damage.

An *erc11* null allele causes a severe growth defect which is partially suppressed by *GAL-CLN1*. The suppression of *erc11-2* by *CLN1* could be due to an increase in *RAD27/ERC11* activity or to activation of another pathway that can substitute for *RAD27/ERC11* function. To differentiate between these possibilities, we constructed a deletion-insertion allele of the *RAD27/ERC11* open reading frame as shown in Fig. 4. Transplacement of *erc11*::*TRP1* into a *cln1 cln2 CLN3 GAL-CLN1* diploid strain, followed by sporulation and dissection, allowed recovery of *erc11*::*TRP1* haploid cells.

Mutant *erc11*::*TRP1* strains displayed a more severe growth defect than the original *erc11-2* mutants did and were not suppressed to the same extent by overexpression of *CLN1* (Fig. 6A). The *erc11-2* point mutant shows approximately the same plating efficiency on galactose as it does on glucose at 30°C and a similar plating efficiency on galactose at 38°C. In contrast, compared with the level on galactose at 30°C, the disruption allele has about a 10-fold decrease in plating efficiency on glucose at 30°C and approximately a 100-fold decrease on galactose at 38°C. Both alleles cause about a 10,000-fold decrease in plating efficiency on glucose at 38°C.

The growth of strains containing the *erc11*::*TRP1* allele, as well as the suppression by *GAL-CLN1*, was somewhat variable among different isogenic isolates. Perhaps the growth defect of the *erc11* disruptant strains, coupled with the increased mutation rate caused by *erc11* mutations (see below), results in the accumulation of extragenic suppressor mutations. We compared four *erc11*::*TRP1 GAL-CLN1* strains and four *erc11*::*TRP1* strains. Strains with *GAL-CLN1* demonstrated about a 3- to 11-fold increase in plating efficiency on galactose compared with the efficiency on glucose at 30°C and a 10- to 900-fold increase in plating efficiency on galactose compared with that on glucose at 38°C. In contrast, *erc11*::*TRP1* strains lacking *GAL-CLN1* showed only a 1- to 2-fold increase in plating efficiency on galactose compared with glucose at 30°C and a 1- to 10-fold increase in plating efficiency on galactose compared with glucose at 38°C. These data demonstrate that suppression of the *erc11*::*TRP1* growth defect by galactose medium is largely due to the presence of the *GAL1*::*CLN1* gene (although a minor role for galactose as opposed to glucose but independent of *GAL1*::*CLN1* cannot be excluded).

The arrest morphology of *erc11*::*TRP1* cells is similar to the phenotype of cells with the point mutation (data not shown), although the Cdc$^-$ phenotype is slightly less uniform (generally 70 to 80% large-budded cells in *erc11*::*TRP1* arrested strains compared with 85 to 94% in *erc11-2* arrested strains). Furthermore, like the *erc11-2* mutation, the null allele also appears to block completion of S phase, as assayed by pulsed-field gel electrophoresis (data not shown).

The more extreme phenotype associated with the *erc11* null allele demonstrates that the point mutants isolated in the Erc$^-$ screen do have some residual activity, which may be increased by *CLN1*. However, since *GAL-CLN1* partially suppresses the phenotype associated with the disruption allele, the mechanism of suppression may not be due solely to an increase in the activity of the Erc11 mutant protein.

Analysis of cell-cycle-regulated transcription and Cdc28-associated kinase activity in *erc11-2* strains. The activity of the G_1 cyclins has been demonstrated to be important for the transcription of MCB-regulated genes; the Cdc28-Cln kinase may increase the activity of the MCB-binding protein, MBF (3). The presence of MCB elements in the *RAD27/ERC11* promoter, coupled with the incomplete rescue of the *erc11* null allele by *GAL-CLN1*, suggested that suppression of *erc11-2* by *GAL-CLN1* might be the result of increasing the levels of *RAD27/ERC11* transcription. Therefore, we analyzed *RAD27/ERC11* mRNA levels in the presence and absence of *GAL-CLN1* expression (Fig. 7A). In *cln1 cln2 GAL-CLN1* strains that contained *CLN3*, the levels of *RAD27/ERC11* transcript decreased transiently when *CLN1* expression was repressed, most likely because of the accumulation of cells in the G_1 phase of the cell cycle. Then the levels of *RAD27/ERC11* mRNA increased and were similar to the steady-state levels in the presence of *GAL-CLN1* expression. This was true for both wild-type and *erc11-2* strains. However, when strains were devoid of *CLN* function upon repression of *CLN1*, *RAD27/ERC11* transcript levels decreased substantially when *CLN1* was repressed. Taken together, these data demonstrate that *CLN3* activity is sufficient for the transcriptional activation of *RAD27/ERC11* and make it unlikely that *GAL-CLN1* suppresses *erc11-2* by transcriptional activation of *RAD27/ERC11.*

Although *GAL-CLN1* does not appear to suppress *erc11-2* by a direct effect on the transcription of *RAD27/ERC11*, *GAL-CLN1* might suppress *erc11-2* through an effect on the transcription of other genes. One class of notable candidates are the B-type cyclins, *CLB5* and *CLB6*. *CLB5* and *CLB6* appear to be MCB-regulated genes, and their activity is important for

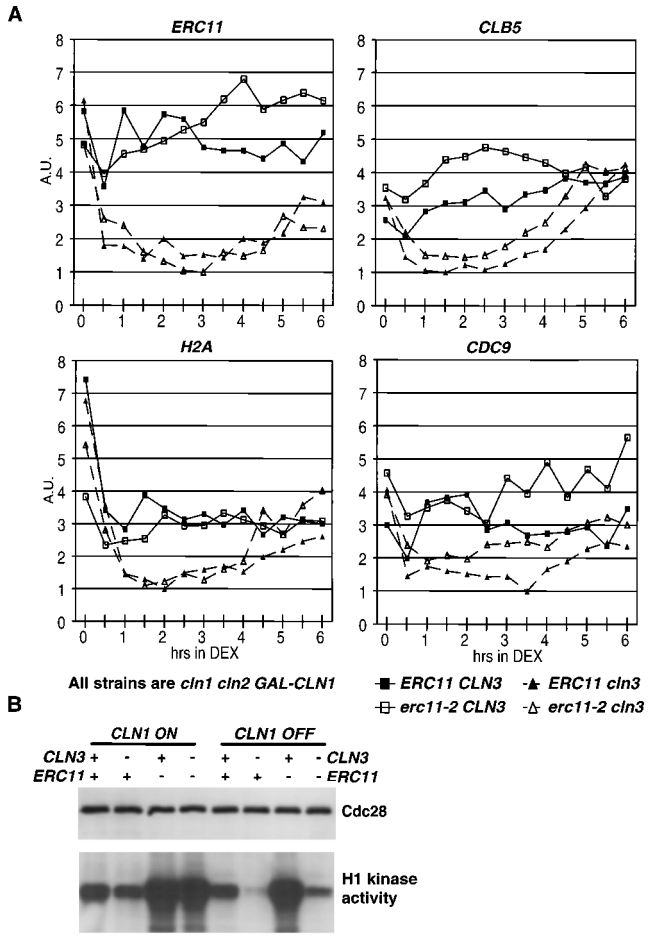

FIG. 7. Strains 1584-4D (*cln1 cln2 CLN3 GAL-CLN1 ERC11 CDC28*::*HA*), 1584-1A (*cln1 cln2 cln3 GAL-CLN1 ERC11 CDC28*::*HA*), 1584-4C (*cln1 cln2 CLN3 GAL-CLN1 erc11-2 CDC28*::*HA*), and 1584-11A (*cln1 cln2 cln3 GAL-CLN1 erc11-2 CDC28*::*HA*) were grown to early log phase in YPGal at 38°C, and then glucose was added at time zero, to repress *GAL-CLN1* expression. (A) Transcriptional regulation in *erc11-2* mutant cells. Samples were taken at 30-min intervals. Probes were as indicated, quantification was performed with a Molecular Dynamics phosphoimager, and mRNA levels were normalized by using *TCM1* as a loading control. DEX, dextrose (glucose). (B) Cdc28-associated kinase activity in *erc11-2* mutant strains. Early-log-phase cultures in YPGal at 38°C were split, glucose was added to half, and incubation continued for 4 h. Cdc28::HA was immunoprecipitated under native conditions from cell extracts made from the indicated strains. A fraction of the immunoprecipitation was Western blotted to analyze Cdc28 levels. The remainder was used for a kinase assay with histone H1 as a substrate to assay Cdc28-associated kinase activity.

transit through S phase (17, 51). To address whether *GAL-CLN1* suppresses *erc11-2* through activation of MCB-regulated genes, in particular, *CLB5* and *CLB6*, we have analyzed the ability of *CLB5* to suppress *erc11-2*, as well as the transcript levels of *CLB5* and the activity of the Cdc28 kinase.

When we compared the suppression of *erc11-2* by *GAL-CLB5* with that by *GAL-CLN1*, we found that *erc11-2* strains with *GAL-CLB5* had a 10-fold decrease in plating efficiency and much smaller colonies than *erc11-2 GAL-CLN1* strains (data not shown). *erc11-2* strains with both *GAL-CLN1* and *GAL-CLB5* resembled *GAL-CLN1* strains, demonstrating that *GAL-CLB5* is not toxic to *erc11-2* mutant cells. Given the weak suppression of *erc11-2* by *GAL-CLB5* (this plasmid construct results in strong overexpression of *CLB5* [data not shown]), it is unlikely that the suppression of *erc11-2* by *GAL-CLN1* is entirely mediated through *CLB5*.

We analyzed both the transcript levels of *CLB5* and the activity of the Cdc28 kinase in *cln1 cln2 CLN3* strains that contained *ERC11* or *erc11-2*, in the presence and absence of *GAL-CLN1* expression. Similar to the situation with *ERC11* mRNA, levels of the *CLB5* transcript transiently decreased upon repression of *GAL-CLN1* in *cln1 cln2 CLN3* strains and then increased to levels comparable to those present before *CLN1* repression (Fig. 7A). The *ERC11* and *erc11-2* strains behaved comparably to one another. In *cln1 cln2 cln3 GAL-CLN1* strains, the level of *CLB5* mRNA decreased more dramatically and remained lower than in the *cln1 cln2 CLN3 GAL-CLN1* strains for about 4 h after *CLN1* repression. For unknown reasons, *CLB5* transcript levels increased at later times after *CLN1* repression. This experiment demonstrates that in *cln1 cln2 CLN3* cells, *CLN3* is capable of activating *CLB5* transcription to a level similar to the level found when *GAL-CLN1* expression is present, in both *ERC11* and *erc11-2* strains. Therefore, the inefficient replication of *cln1 cln2 CLN3 erc11-2* strains is not due to a deficit in *CLB5* expression. The transcription levels of *CDC9*, another MCB-regulated gene, were also comparable in the *ERC11* and *erc11-2* strains in the presence and absence of *GAL-CLN1* expression (Fig. 7A).

When we examined the levels of the *H2A* transcript, which is not MCB regulated (41), we observed a slight reduction in response to turning off *GAL-CLN1*. This difference was more pronounced in the *ERC11* strain than in the *erc11-2* strain. We do not yet know the significance of this difference; *CLN1* may have a direct effect on the levels of histone transcription, or this may be the result of a difference in the cell cycle distributions between these strains.

Finally, we also examined the transcription of an SCB-regulated transcript, *PCL1* (*HCS26*) (40, 57). The regulation of the levels of the *PCL1* transcript was similar to that of *CLB5* and was comparable in the *ERC11* and *erc11-2* strains, in both the presence and absence of *GAL-CLN1* expression (data not shown).

Overall, these measurements of transcript levels indicate that in either *erc11-2* or *ERC11* strains, the presence of *CLN3* is entirely sufficient for activating transcription of both SCB- and MCB-regulated genes. *CLN3* is required for this activation, at least for several hours following inactivation of *GAL-CLN1* (Fig. 7A). Therefore, it is unlikely that rescue of *erc11-2* inviability by *CLN1* is due to differential activation of transcription by *CLN1* as opposed to *CLN3*.

Although transcriptional levels of *CLB5* are similar in *ERC11* and *erc11-2* strains in both the presence and absence of *CLN1* expression, posttranscriptional effects on the activity of the Cdc28-cyclin kinase could account for the cell cycle arrest of *erc11* strains. For example, the inactivation of $p40^{SIC1}$, the Cdc28-Clb kinase-specific inhibitor, requires Cdc28-Cln kinase function. We analyzed the levels of Cdc28-associated kinase activity in *ERC11* and *erc11-2* strains (Fig. 7B). Because the Cdc28-Clb kinase activity is much greater than the Cdc28-Cln kinase activity (19), most of the kinase activity detected in this assay can be attributed to the Cdc28-Clb kinase.

In the *erc11-2* strains grown in the presence of *CLN1* expression, there was a high level of Cdc28-associated kinase activity compared with the level in *ERC11* control strains. This is probably due at least in part to the increase in the number of cells in the S and G_2 phases of the cell cycle (Fig. 2), when the level of Cdc28-Clb kinase activity is high. After repression of *CLN1*, the levels of kinase activity in both the *ERC11 CLN3* and *erc11-2 CLN3* strains were similar to the levels present before inactivation of *CLN1*. In the *cln3* strains, which contained no G_1 cyclin activity upon repression of *CLN1*, the levels of Cdc28-associated kinase activity decreased when

TABLE 1. Spontaneous mutation rates in *erc11* mutant cells

Genotype	Rate of Can^R events[a] (10^{-8})	
	GAL	DEX
ERC11	0.68	2.7
erc11-2	21	40
erc11::*TRP1*	99	12
ERC11	1.1	2.4
erc11::*TRP1*	62	64

[a] Cells were plated on YPGal and SCGal lacking Arg and containing canavanine to determine viable and Can^R cells. Rates were calculated by the Lea-Coulson method of the median (33) from the analysis of 9 independent cultures for each carbon source for the first experiment and 15 independent cultures for each carbon source for the second experiment. GAL, galactose; DEX, dextrose (glucose).

CLN1 was repressed. Although the Cdc28-associated kinase activity assayed is not due directly to Cdc28-Cln kinase, G_1 cyclin activity is required for both *CLB* transcription and inactivation of $p40^{SIC1}$. Taken together with the transcriptional activation of *CLB5*, these data demonstrate that *CLN3* activity in *erc11-2* mutant cells is sufficient for the activation of Cdc28-associated kinase, making it unlikely that the block to cell cycle progression is due to a general failure to activate *CLB*-dependent kinase activity in the *erc11* mutant. We do not know the contribution of specific Clb proteins to the bulk Cdc28-associated kinase we have measured. However, the results of Schwob et al. (50) indicate that the six *CLB* genes are redundant for activation of DNA replication.

***ERC11* is important for repair of DNA damage.** Many genes involved in DNA replication also have a role in DNA repair. Furthermore, like defects in DNA synthesis, defects in DNA repair might also lead to cell cycle arrest, due to DNA damage checkpoints. Therefore, we analyzed *erc11-2* and *erc11*::*TRP1* strains for phenotypes associated with the repair of DNA damage. Although *erc11* mutants are not UV sensitive (see below), they do display phenotypes associated with a defect in DNA repair. Surprisingly, *CLN1* expression does not suppress any of these *erc11* defects.

We analyzed the spontaneous mutation rate in wild-type and mutant cells by assaying the frequency of canavanine-resistant cells (Table 1). Cells containing either the *erc11-2* or *erc11*::*TRP1* allele mutated to Can^R at a higher frequency than did wild-type cells.

Mutant *erc11* strains are about 10-fold more sensitive than wild-type cells to the DNA-damaging agent MMS, as indicated by a halo assay (Fig. 8A).

Mutations in *erc11* cause a striking increase in the recombination rate. Both *erc11-2* and *erc11*::*TRP1* cause an approximately 100-fold increase in the loss of heterozygosity at the *MAT* locus, as determined by the increase in mating proficiency of diploid cells. This increase is due almost exclusively to recombination, as most events resulting in mating competency were associated with the retention of a marker on the opposite side of the centromere from the *MAT* locus, as would be expected for recombination (but not for chromosome loss) (Fig. 8B).

We also assayed for DNA damage more directly by testing for the presence of nicks. We analyzed the amount of radioactive nucleotide incorporated by Klenow enzyme into DNA isolated from *erc11* mutant and wild-type strains as well as the size of the radiolabelled DNA fragments. Analysis of the radiolabelled products by denaturing alkaline agarose gel electrophoresis demonstrated that DNA isolated from both *erc11-2* and *erc11* null mutant strains had incorporated more

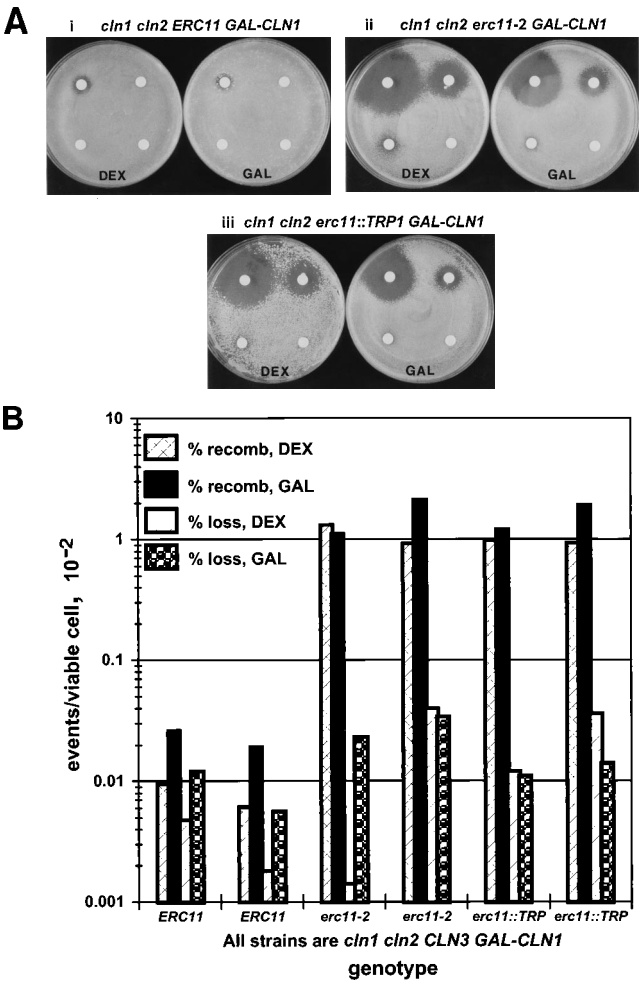

FIG. 8. (A) Sensitivity of strains to the DNA-damaging agent MMS. Exponential-phase cultures were grown in YPGal, and an aliquot (0.2 ml) was spread on YPD or YPGal plates. Filter paper discs containing 20 μl of MMS at 5% (upper left spot), 1.5% (upper right), 0.5% (lower left), and 0.15% (lower right) were placed on the surface. Plates were incubated at 30°C for 2 days. Panels: i, 2507-5B; ii, 1983-5B; iii, 2562-5A. (B) Recombination and chromosome loss levels in *erc11* mutant strains. A quantitative mating assay was used to score for the loss of heterozygosity at the *MAT* locus in diploids. Chromosome loss events are those which result in loss of both *MAT* and a linked marker; recombination events result in loss of heterozygosity at *MAT* and retention of a linked marker on the opposite side of the centromere. Data shown are from the *MAT***a** cross; similar results were found with crosses to a *MAT*α tester. DEX, dextrose (glucose); GAL, galactose; recomb, recombination.

label and was on average, smaller than DNA isolated from wild-type strains (data not shown). This effect was largely independent of *CLN1* expression, consistent with the observations above suggesting that *erc11* causes defects in DNA synthesis and/or repair that are not suppressed by *CLN1*.

***ERC11* and *RAD2* have distinct roles in vivo.** *RAD27*/*ERC11* and *RAD2* are members of the same gene family by sequence. To test for genetic interactions and functional similarities between them, we transplaced *rad2* null mutations into *cln1 cln2 CLN3 GAL-CLN1* strains. These null mutations did not result in an Erc$^-$ phenotype. We crossed *erc11*::*TRP1* and *rad2*::*URA3* haploids and examined the meiotic progeny. Spores containing both mutations were recovered at the expected frequency for unlinked genes. The *erc11*::*TRP1* and *erc11*::*TRP1 rad2*::*URA3* mutants displayed similar plating efficiencies on glucose and galactose at 30 and 38°C. *erc11*::*TRP1* mutants are

not UV sensitive, and the *rad2*::*URA3* and *erc11*::*TRP1 rad2*::*URA3* mutants displayed similar sensitivities to UV irradiation (data not shown). These results suggest that although Rad2 and Erc11/Rad27 share significant homology, they may have little functional overlap in vivo.

***CLN1*, *erc11*, and cell cycle checkpoints.** Many mutations that block DNA replication cause a Cdc^- cell cycle arrest due to checkpoint control: the failure to complete replication activates a regulatory process blocking cell cycle progression (24, 59). The *RAD9* and *MEC1* genes encode components of this checkpoint pathway (59, 62). To test if the Cdc^- arrest of *erc11 cln1 cln2* strains was due to a checkpoint control assaying the completion of DNA replication, we wanted to construct *erc11 rad9* and *erc11 mec1* strains. *GAL-CLN1 rad9 erc11-2* strains were inviable on both galactose and glucose at 38°C (data not shown). The *cln1 cln2 rad9 erc11-2* mutants failed to arrest with a Cdc^- phenotype in either the presence or absence of *CLN1* expression, demonstrating that the arrest morphology of *cln1 cln2 erc11-2* mutant cells is dependent upon *RAD9*. Because the *cln1 cln2 rad9 erc11-2* mutants now fail to show Cdc^- arrest, we conclude that Cdc^- arrest in the absence of Cln1 is caused by a checkpoint mechanism assaying the completion of DNA replication. The inviability of *erc11-2 rad9* mutants suggests that *GAL-CLN1* is dependent upon a *RAD9* checkpoint for suppression of the *erc11-2* defect. The requirement for *RAD9* is also consistent with the high level of Cdc28-associated kinase activity in the *erc11-2* strains; the *RAD9*-dependent G_2/M checkpoint arrest or pause is associated with high levels of Cdc28-associated kinase activity (55).

We were unable to analyze the effect of the *mec1-1* mutation on *erc11* mutants, because *mec1-1 ERC11 GAL-CLN1* cells demonstrated a severe growth defect on galactose in comparison with *mec1-1* cells without *GAL-CLN1* (Fig. 9A). On galactose, *mec1-1 ERC11 GAL-CLN1* cells continued cell division, which led to an increase in cell number; however, inviable cells accumulated in the population. After 12 h of growth in galactose, approximately 90% of the cells in the culture were unable to form colonies when plated.

To analyze this effect of *GAL-CLN1*, we monitored chromosome loss and recombination events in diploids by assaying for the formation of mating cells (Fig. 9B). *mec1-1* caused about a 10-fold increase in loss and recombination, even in the absence of *GAL1*::*CLN1* expression, from the levels obtained with *MEC1* strains. A 12-h pulse of galactose to activate *GAL1*::*CLN1* transcription resulted in a 100-fold increase in loss and recombination compared with levels for *MEC1* controls. Therefore, the inviability caused by *GAL-CLN1* expression may be due to effects on DNA synthesis or chromosome segregation.

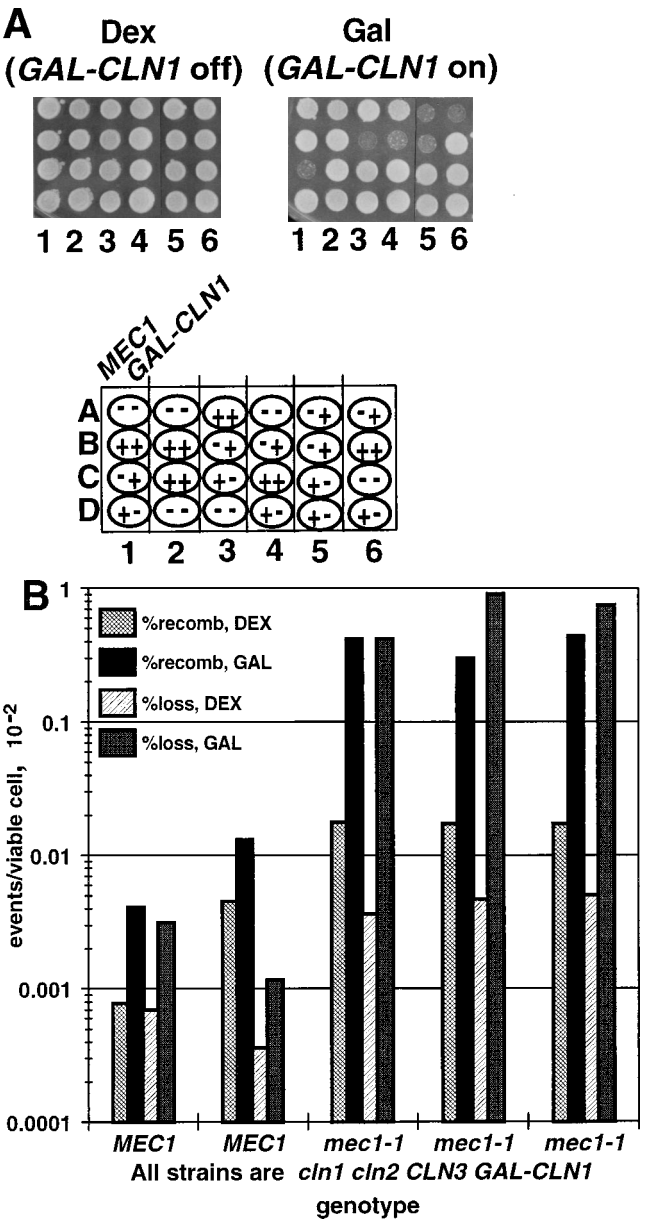

FIG. 9. (A) Inviability of *mec1-1 GAL-CLN1* strains. Spores from a diploid strain heterozygous for *mec1-1* and *GAL-CLN1* were inoculated onto glucose and galactose media and grown for 2 days. The *mec1-1* genotype was assigned to spores on the basis of complementation testing with known *mec1-1* mutant strains for hydroxyurea sensitivity. The *GAL-CLN1* genotype was assigned on the basis of Leu^+ prototrophy; the integrated construct is a *LEU2*::*GAL1*::*CLN1* cassette. Dex, dextrose (glucose); Gal, galactose. (B) Recombination and chromosome loss levels in *MEC1* and *mec1-1* mutant strains. A quantitative mating assay was used to score for the loss of heterozygosity at the *MAT* locus in diploids after 12 h of growth in galactose or glucose. Chromosome loss events are those which result in loss of both *MAT* and a linked marker; recombination events result in loss of heterozygosity at *MAT* and retention of a linked marker on the opposite side of the centromere. Data shown are from the *MAT***a** cross; similar results were found with crosses to a *MAT*α tester. DEX, dextrose (glucose); GAL, galactose; recomb, recombination.

DISCUSSION

We describe the isolation and characterization of mutations in *RAD27/ERC11/YKL510*. Mutations in *erc11* result in cell cycle arrest of *cln1 cln2 CLN3* strains, a phenotype that is suppressed by expression of *CLN1* or *CLN2*. Characterization of the *erc11* arrest demonstrates that cells are blocked in the completion of DNA synthesis. First, cells arrest with a large bud, a short spindle, and a single nucleus. This phenotype is identical to that associated with other mutations that affect DNA replication. Second, FACS analysis demonstrates that cells are crippled in their capability to synthesize DNA, as their progression is greatly delayed compared with that of wild-type cells, and they may not actually complete synthesis. Finally, DNA isolated from *erc11* mutants is unable to band on CHEF gels, as has been described for mutations and conditions with which cell growth is arrested in S phase.

Mutations in *erc11* also cause defects in DNA repair, including an increased spontaneous mutation rate, increased sensitivity to a DNA-damaging agent, and increased recombination.

Although *CLN1* expression suppresses the block in replication caused by *erc11-2*, it fails to suppress any of the defects associated with DNA damage.

The phenotypes associated with *erc11* alleles, as well as the cell-cycle-regulated and damage-inducible transcription of *RAD27*/*ERC11*, are in vivo evidence for functions for the protein in both DNA replication and repair. A role in these functions was suggested by the in vitro biochemical activity described for Rad27/Erc11/Ykl510, and its mammalian homolog, FEN-1, as a DNA flap endonuclease (22, 23). It was postulated that FEN-1 activity might be required during DNA repair to cleave a damaged strand of DNA after unwinding and during lagging-strand DNA synthesis for cleavage and ligation of displaced Okazaki fragments. After this manuscript was submitted, Reagan et al. published an article characterizing the phenotypes associated with disruption of Rad27/Erc11/Ykl510 in *CLN1 CLN2 CLN3* cells (43). They found that disruption of *RAD27*/*ERC11* caused temperature-sensitive cell inviability with defects associated with a block in the completion of DNA replication. Their analysis and conclusions are consistent with the data presented here demonstrating that *RAD27*/*ERC11* has critical roles in both DNA replication and repair.

Isolation of *erc11*/*ykl510* in a screen for mutations that require increased G_1 cyclin dosage. The isolation of mutant alleles of *RAD27*/*ERC11* synthetically lethal with *cln1 cln2* defines a link between Start and DNA replication and/or repair. One interpretation of the isolation of *rad27*/*erc11* in this context is that either the fidelity or the rate of DNA replication is broadly compromised in *cln1 cln2* cells and, therefore, that a number of mutations in the DNA replication or repair pathway could cause synthetic lethality with *cln1 cln2* (an Erc^- phenotype). For a number of reasons, we do not favor this hypothesis. First, we have been unable to observe any differences between *cln1 cln2 CLN3* cells and *cln1 cln2 CLN3 GAL-CLN1* cells in sensitivity to MMS, hydroxyurea, or UV irradiation (55a, 57a). Second, the numbers of cells in S phase, as assayed by FACS, are not strikingly different for the two genotypes, suggesting that this is not a kinetic slow point in *cln1 cln2 CLN3* cells. Third, deletion of *rad2*, a gene known to be required for some types of DNA repair, does not cause the Erc^- phenotype. Fourth, many genes required for DNA replication have been identified; mutations in most cause Cdc^- arrest. *RAD27*/*ERC11* is the only complementation group identified in our screen thus far which results in the budded-cell arrest phenotype characteristic of mutants defective in DNA synthesis. Taking these data together, it appears that if *cln1 cln2* cells do make defective DNA, the defects are subtle, specific, and not synergistic with those associated with other DNA-damaging agents; if synthesis is indeed defective, Erc11 and only a few other proteins may be specifically required to repair the defects generated in *cln1 cln2* cells.

One possible mechanism of suppression of *erc11* by *CLN1*: activation of Erc11 and/or another pathway. Mutant *erc11* alleles result in lethality in *cln1 cln2 CLN3* cells. The phenotype of these cells demonstrates that in the absence of *CLN1* and *CLN2*, cells are dependent upon *RAD27*/*ERC11* for progression through S phase. One interpretation is that *CLN1* and *CLN2* can activate another pathway for DNA replication that is at least partially *RAD27*/*ERC11* independent, while *CLN3* is unable to activate this pathway. This does not necessarily imply that *RAD27*/*ERC11* is a substrate of the Cdc28-Cln kinase, only that in the absence of *CLN1* and *CLN2*, cells are dependent upon a pathway within which *RAD27*/*ERC11* is an essential component. The ability of *CLN1* and *CLN2* to activate a DNA synthesis pathway that *CLN3* is unable to activate is consistent with a variety of observations that *CLN1* and *CLN2* promote cell cycle progression in a way qualitatively different from that of *CLN3* (see the introduction).

Previously identified roles for the G_1 cyclins in control of DNA synthesis include the activation of transcription of genes required for S phase, including the *CLB* genes, as well as the activation of Cdc28-Clb kinases, probably by inhibition of $p40^{SIC1}$ (50, 57). *CLN3* appears to be at least as potent an activator of transcription as *CLN1* (57; also see above), making it unlikely that *CLN1* and *CLN2* suppress *erc11* by activating the general transcription of genes required for DNA synthesis. In particular, we have tested *cln1 cln2* strains (both *erc11-2* and *ERC11*) for induction of *RAD27*/*ERC11* itself, as well as the MCB-controlled genes *CDC9* and *CLB5* and the SCB-controlled gene *PCL1*, and have observed no defect in their induction in the absence of *CLN1* expression. At most, a modest defect in histone *H2A* transcription as a result of turning off *CLN1* expression was observed. Therefore, we consider it unlikely that the defect in *cln1 cln2 erc11-2* strains that is suppressed by *CLN1* or *CLN2* expression is due to defects in transcriptional induction of other genes. However, it remains possible that *CLN1* and *CLN2* do suppress *erc11* via the activation of the transcription of genes not yet identified.

In addition to being proficient in activating transcription of SCB- and MCB-regulated genes, *cln1 cln2 erc11-2* strains contained high levels of Cdc28p-associated histone H1 kinase activity, suggesting that the Sic1p inhibitor of Cdc28-Clb kinase activity was not functioning. These results suggest a direct role for *CLN1* and *CLN2* in regulation of DNA replication independent of activating Clb protein expression and kinase activity (50).

We have identified two possible components of the hypothetical *CLN1*- or *CLN2*-dependent pathway, *SEL1* (suppressor of *erc11*, encoding a potential helicase) and *CDC9* (encoding DNA ligase). Increased levels of these proteins can suppress the *erc11-2* DNA synthesis defect (57b). Furthermore, deletion of *SEL1* and *ERC11* is synthetically lethal, even in the presence of *CLN1*, as would be predicted for the disruption of a component in each of two functionally redundant pathways. Finally, like *CLN1* and *CLN2*, *SEL1* and *CDC9* can suppress the temperature-sensitive lethality of *erc11-2* but are unable to suppress the MMS-sensitive *erc11-2* phenotype (associated with defects in DNA repair).

The observation that *CLN1* and *CLN2* are unable to complement the requirement for *RAD27*/*ERC11* in DNA repair could cast doubt on the idea that *CLN1* and *CLN2* may activate a parallel pathway that can at least partially bypass the requirement for *RAD27*/*ERC11* function in replicative DNA synthesis. Ad hoc explanations to accommodate this fact could include the following: *CLN1* and *CLN2* can interact with Erc11 at only a subset of the temporal or spatial domains at which Erc11 functions; *RAD27*/*ERC11* has two different activities in vivo, loss of only one of which (required for DNA synthesis) can be suppressed by an alternative pathway activated by *CLN1* and *CLN2*; and a function activated by *CLN1* and *CLN2* is present at the time appropriate for its having a role in DNA synthesis but is not expressed at a time appropriate for its having a role in DNA damage repair. However, we lack direct evidence in favor of any of these explanations. An alternative explanation is presented below.

Possible mechanism of suppression of *erc11* by *CLN1*: bypass of a checkpoint arrest. Although *CLN1* and *CLN2* suppress the inviability and cell cycle arrest caused by *erc11-1* and *erc11-2* in *cln1 cln2 CLN3* cells, they are apparently unable to suppress any other *erc11* phenotypes. One interpretation of this surprising result is that *cln1 cln2 CLN3* cells arrest because of the defects caused by the *erc11* alleles and that *CLN1* allows

the cells to progress through the cell cycle by ignoring these defects, not by repairing them. It may be that the *erc11* damage results in a response which inhibits Cdc28-Cln3 kinase but fails to inhibit Cdc28-Cln1 kinase. This differential inhibition may be the result of quantitative or qualitative differences between the Cdc28-Cln kinases.

If *CLN1* and *CLN2* function causes the bypass of a checkpoint-mediated arrest, the levels of DNA damage or disrepair generated by reducing *erc11* function must not be lethal. Otherwise, bypass of the checkpoint would result in cell death. Apparently the levels of damage caused by *erc11* are sublethal only when *RAD9* is functional. The requirement for *RAD9* is consistent with the presence of DNA damage and demonstrates that some checkpoint response is, in fact, required for suppression. Perhaps *CLN1* causes a bypass of one checkpoint in *erc11* mutant cells, allowing replicative DNA synthesis, but that then a pause at another checkpoint (*RAD9* dependent) is required to allow successful completion of DNA synthesis or some repair before mitosis.

It is also interesting in this context that *CLN1* overexpression is lethal in cells defective for the *MEC1*-dependent DNA damage checkpoint (Fig. 9). Perhaps *mec1-1* strains are dependent for viability on the same checkpoint bypassed by *CLN1* in the *erc11-2* background. This idea could lead to the prediction that *mec1-1* might be synthetically lethal with *rad9*; however, this appears not to be the case (unpublished results). Alternatively, *CLN1* overexpression could somehow activate defective or error-prone DNA replication, requiring a pause at a *MEC1*-dependent checkpoint for repair.

Previous work has indicated that some checkpoints do function through the inhibition of cyclin-dependent kinases and cyclins. In *Schizosaccharomyces pombe*, inhibitory phosphorylation of cdc2p, the Cdc28p homolog, is required for hydroxyurea-induced cell cycle arrest; in the absence of phosphorylation, cells experience mitotic catastrophe (15, 34). Furthermore, in mammalian cells, DNA damage in G_1 causes cell cycle arrest and decreased CDK activity. This effect is p53 dependent (29, 32) and is due, at least in part, to inhibition of the activity of cyclin E-cdk2 complexes by the p53-inducible $p21^{WAF/CIP1}$ protein and reduction of the levels of cyclins A and D1 (13, 14).

It has recently been demonstrated that in *Saccharomyces cerevisiae*, *RAD9*, previously shown to be required for arrest in G_2/M, is also required for arrest in the G_1/S phase after UV irradiation (52, 53). Perhaps *RAD9* affects both Cdc28-Cln and Cdc28-Clb kinase activity. Although *RAD9*-mediated arrest is characterized by high kinase levels at the G_2/M checkpoint (55), the size of complexes containing Cdc28-cyclin from checkpoint-arrested cells is different from that of complexes from nonarrested cells. This demonstrates that there is some effect on the Cdc28-cyclin complex. Another possible connection between the Cdc28 kinase and checkpoints is that a loss-of-function mutation in *CKS1*, a Cdc28-cyclin binding protein and the budding yeast homolog of $p13^{suc1}$, can suppress a mutation in the checkpoint gene *RAD53* (*MEC2*/*SAD1*) (2). Rad53p may inhibit Cdc28 kinase activity or alter kinase specificity by affecting *CKS1* function. Our observations that *GAL-CLN1* dramatically affects the viability of *mec1-1* strains may reveal an additional link between checkpoints and cyclin-dependent kinases. High, constitutive levels of *CLN1* may either cause insensitivity to DNA damage or result in DNA synthesis that is partially defective.

Suppression of *erc11* by activation of a partially *erc11*-independent pathway and suppression by bypass of a checkpoint arrest are not mutually exclusive hypotheses. *CLN1* and *CLN2* may have multiple effects on the regulation of DNA synthesis.

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