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Inactivation of the Cyclin-Dependent Kinase Cdc28 Abrogates Cell Cycle Arrest Induced by DNA Damage and Disassembly of Mitotic Spindles in *Saccharomyces cerevisiae*

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Eukaryotic cells may halt cell cycle progression following exposure to certain exogenous agents that damage cellular structures such as DNA or microtubules. This phenomenon has been attributed to functions of cellular control mechanisms termed checkpoints. Studies with the fission yeast *Schizosaccharomyces pombe* and mammalian cells have led to the conclusion that cell cycle arrest in response to inhibition of DNA replication or DNA damage is a result of down-regulation of the cyclin-dependent kinases (CDKs). Based on these studies, it has been proposed that inhibition of the CDK activity may constitute a general mechanism for checkpoint controls. Observations made with the budding yeast *Saccharomyces cerevisiae*, however, appear to disagree with this model. It has been shown that high levels of mitotic CDK activity are present in the budding yeast cells arrested in G_2/mitosis as the result of DNA damage or replication inhibition. In this report, we show that a novel mutant allele of the *CDC28* gene, encoding the budding yeast CDK, allowed cell cycle passage through mitosis and nuclear division in the presence of DNA damage and the microtubule toxin nocodazole at a restrictive temperature. Unlike the checkpoint-defective mutations in CDKs of fission yeast and mammalian cells, the *cdc28* mutation that we identified was recessive and resulted in a loss of the CDK activity, including the Clb2-, Clb5-, and Clb6-associated, but not the Clb3-associated, CDK activities. Examination of several known alleles of *cdc28* revealed that they were also, albeit partially, defective in cell cycle arrest in response to UV-generated DNA damage. These findings suggest that Cdc28 kinase in budding yeast may be required for cell cycle arrest resulting from DNA damage and disassembly of mitotic spindles.

DNA-damaging agents and microtubule toxins are known to block cell cycle progression in a variety of organisms. Cell cycle arrest under these conditions has been conceived to be the manifestation of surveillance mechanisms that monitor the fitness of cells for entry into subsequent cell cycle stages (for reviews, see references 13, 14, and 23 to 25). It is presumed that these *trans*-acting control systems, termed checkpoint controls, couple cell cycle progression with stress-responsive mechanisms, such as DNA repair, to ensure genetic stability (11, 14). One such example is the human tumor suppressor p53. p53 is viewed as a checkpoint protein because of the inducibility of its activity by DNA damage and the requirement of its activity for cell cycle arrest in G_1 after irradiation (11, 14, 19, 20). Cells defective in p53 function fail to arrest after DNA damage and usually exhibit a high degree of genetic instability (11). Inactivation of p53 through mutation or depletion by viral factors appears to be the critical step in cancer development in many types of malignancy (15, 21). The ability of p53 to arrest the cell cycle after DNA damage has been attributed, at least in part, to its transcriptional activation of p21, an inhibitor of cyclin-dependent kinases (CDKs) in mammalian cells (5, 10, 45). Down-regulation of the CDK activity by other means, such as inhibitory phosphorylation, has also been suggested to play a role in the cell cycle response to DNA damage, as mutations that eliminate the inhibitory phosphorylation of rat Cdk4 allow cells to escape from cell cycle arrest in G_1/S after UV-generated DNA damage (40). Similarly, the cell cycle arrest caused by blocking DNA replication in the fission yeast *Schizosaccharomyces pombe* has been ascribed to the absence of the activity of the CDK Cdc2 (4, 7). Mutations that confer constitutive activation of Cdc2 are dominant and permit cell division regardless of inhibition of DNA replication (6, 7, 27).

Given the central function of the ubiquitous CDKs in cell cycle progression in most if not all eukaryotic cells, inhibition of their activities is likely to provide an efficient and convenient means to arrest the cell cycle when such needs arise. It has become an attractive model that checkpoint controls may achieve cell cycle arrest in response to stress treatments by down-regulating CDKs (14, 24, 25, 27). This hypothesis, however, must have certain limitations, since it can not adequately explain the findings for the budding yeast *Saccharomyces cerevisiae*. It has been shown that, unlike Cdk4 of rat and Cdc2 of fission yeast, Cdc28, the CDK of budding yeast, is not subject to inhibition by tyrosine phosphorylation (1, 36). The mutation that confers checkpoint defects in the Cdk4 and Cdc2 CDKs has no effect on Cdc28 with respect to DNA damage-induced cell cycle arrest (1, 36). Furthermore, the mitotic CDK activity in budding yeast remains high in cells arrested in S phase and G_2/M by replication inhibition, DNA damage, or disassembly of mitotic spindles (1, 16, 36, 37), indicating that the inability of these cells to pass the cell cycle arrest is unlikely to be due to inhibition of the Cdc28 activity. These observations suggest that the mechanism of cell cycle arrest following DNA damage in budding yeast may differ from that in fission yeast and mammalian cells.

So far, mutations in the *CDC28* gene that confer a checkpoint defect have not been reported. In order to clarify the role of Cdc28 in checkpoint controls in budding yeast, we attempted to isolate such mutants. In this report, we describe the

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TABLE 1. Yeast strains

Strain[a]	Genotype	Source
YNN413	*MAT***a** *ade2 ura3 leu2 trp1 his3* *MAT*α *ade2 ura3 leu2 trp1 his3*	This study
YNN414	*MAT***a** *ade2 ura3 leu2 trp1 his3*	This study
Ymc323	*MAT***a** *cdc9-8 ade2 ura3 leu2*	This study
Ymc319	*MAT***a** *cdc13-1 ade2 ura3 leu2 trp1 his3*	This study
Ymc336	*MAT***a** *ade2 ura3 leu2 trp1 cdc28Δ::HIS3*	This study
Ymc337	*MAT***a** *cdc9-8 cdc28Δ::HIS3 ura3 leu2 trp1*	This study
Ymc338	*MAT***a** *cdc13-1 cdc28Δ::HIS3 ura3 leu2 trp1*	This study
Ymc374	*MAT***a** *ade2 ura3 leu2 cdc28Δ::HIS3 cdc28-5M* (*TRP1*)	This study
Ymc375	*MAT***a** *cdc9-8 ura3 leu2 cdc28Δ::HIS3 cdc28-5M* (*TRP1*)	This study
Ymc376	*MAT***a** *cdc13-1 ura3 leu2 cdc28Δ::HIS3 cdc28-5M* (*TRP1*)	This study
Ymc309a	*MAT***a** *rad9Δ::TRP1 ade2 ura3 leu2 his3*	This study
US100	*MAT***a** *cdc28-4 ade2 ura3 leu2 trp1 his3*	U. Surana
US43	*MAT*α *cdc28-9*	U. Surana
US44	*MAT***a** *cdc28-13*	U. Surana
US355	*MAT***a** *cdc13-1 rad9Δ ura3 leu2*	U. Surana
Ymc387	*MAT***a** *cdc28-9 ade2 ura3 leu2 trp1 his3*	This study
Ymc388	*MAT***a** *cdc28-13 ade2 ura3 leu2 trp1 his3*	This study

[a] Ymc336, -337, and -338 all contained the *GAL1-CDC28* plasmid (pUS1) for viability.

identification and characterization of a novel allele of *cdc28*, *cdc28-5M*, that results in concomitant losses of the CDK activity and the ability to arrest the cell cycle after DNA damage under nonpermissive conditions. *cdc28-5M* also confers a defect to the checkpoint controls responsible for the integrity of mitotic spindles. Based on these data, we suggest that the Cdc28 kinase plays active roles in cellular responses to stress signals such as DNA damage and disassembly of mitotic spindles.

MATERIALS AND METHODS

Strains, media, and genetic procedures. The strains and the plasmids used in this study are listed in Tables 1 and 2, respectively. Rich (yeast extract-peptone-dextrose [YPD]), minimal, synthetic complete (SC), and dropout media were prepared as described previously (30). The [*rho*^{0}] derivatives of *cdc28-5M* mutants were isolated as described by Fox et al. (8). Restriction enzymes, ligase, and all other DNA-modifying enzymes were from either New England Biolabs or Boehringer. Temperature-sensitive mutants were propagated at 23°C and analyzed at 37°C, unless indicated otherwise. Genetic manipulations were performed according to standard methods described by Rose et al. (30). The lithium acetate method was used for yeast transformation (18). DNA manipulations were performed as described by Sambrook et al. (32). PCRs were performed with *Taq* polymerase (Promega). DNA sequencing was done with Sequenase version 2.0 according to the protocol supplied by the manufacturer.

Construction of the mutant *cdc28* library. A 2.7-kb *Xho*I-*Pst*I fragment of genomic DNA containing *CDC28* with its endogenous promoter was cloned into the *Xho*I-*Pst*I sites of pRS315 (3, 35) to generate pMC186. The low-fidelity PCR method was used to randomly mutagenize the *CDC28* gene. The standard T7 and T3 primers were used for PCR, with the pMC186 plasmid DNA as the template. PCR was carried out in a volume of 50 μl which included 10 ng of plasmid DNA, 5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 0.1% gelatin, and 1 mM dithiothreitol), 1.5 mM $MgCl_2$, 2 μM each primer, 5 μl of 5 mM $MnCl_2$–10 mM Tris-HCl (pH 8.0), and 2.5 U of *Taq* polymerase (Promega). Four reactions were performed in parallel, and in each reaction one of the four nucleotides was used at a concentration five times less than the normal concentration (0.2 mM). Thirty cycles of amplification were used, with the following parameters: 94°C for 1 min, 51°C for 1 min, and 72°C for 3 min. The amplified 2.7-kb fragment was gel purified, digested with *Bam*HI and *Xho*I, and cloned into the *Bam*HI/*Xho*I site of pRS314. Approximately 30,000 *Escherichia coli* transformants were obtained, of which more than 95% were estimated to contain the insert. The plasmid DNA was isolated from the mixture of these *E. coli* transformants.

Integration and gene disruption. The *CDC28* gene was disrupted by the one-step replacement method (31). A 1.2-kb *Eco*RI fragment containing the *CDC28* coding region was cloned into the *Eco*RI site of pBluescript II. The 0.6-kb *Bal*I fragment within the *CDC28* coding region was replaced by a 1.4-kb *Sma*I-*Eco*RV fragment containing the *HIS3* gene. This construct contained 91 nucleotides from the 5′ coding sequence and 188 nucleotides from the 3′ coding sequence of *CDC28*. The *HIS3* gene together with *CDC28* flanking sequences (*cdc28Δ::HIS3*) was excised with *Eco*RI, gel purified, and transformed into a wild-type diploid strain, YNN413. The disruption in His$^+$ transformants was confirmed by Southern analysis. When sporulated and dissected, this diploid *cdc28* strain produced only two viable spores per tetrad.

Isolation of the mutants. The double mutants *cdc9 cdc28Δ::HIS3* (Ymc337) and *cdc13 cdc28Δ::HIS3* (Ymc338) were generated by crossing the haploid *cdc28Δ::HIS3* strain (Ymc336) containing *GAL1-CDC28* with *cdc9* and *cdc13* single mutants. The strain Ymc338 was used for transformation with the randomly mutated *cdc28* library DNA. After growing up on SC-Trp galactose plates at 23°C, the transformants were replica plated onto SC-Trp glucose plates and were incubated at 37°C for 6 h before being allowed to grow at 23°C. The replica-plated colonies that grew poorly, if at all, on the plates incubated at 37°C were selected from the master plates and tested again for the rapid-death-at-37°C phenotype. Twenty-seven of 8,000 transformants screened were confirmed to die rapidly at 37°C. They were further tested for cell cycle arrest by flow cytometry (FACScan; Becton Dickinson) analysis. Most of them still showed predominantly a G_2 DNA peak similar to that of the *cdc13* single mutant and were therefore not subjected to further studies. However, there was one rapid-death strain that did not show arrest in G_2. Instead, it was clearly arrested in G_1, as it gave a 1N DNA peak after the shift to 37°C. The plasmid DNA of this strain was extracted, amplified in *E. coli*, and transformed into Ymc337 and Ymc338. After the *GAL1-CDC28* plasmid containing the *URA3* marker was cured on a 5-fluoroorotic acid (5-FOA) plate, this isolated *cdc28* allele was again able to cause the rapid-death-at-37°C phenotype. This allele was named *cdc28-5M*. Mutations in the coding region of *cdc28-5M* were characterized by DNA sequence analysis.

The DNA fragment containing *cdc28-5M* with its endogenous promoter was subcloned into a integration vector, pRS304 (35), containing the *TRP1* marker. It was then linearized at a unique site near the middle of the coding region of *TRP1*, gel purified, and transformed into Ymc336, Ymc337, and Ymc338 cells to generate Ymc374, Ymc375, and Ymc376, respectively (Table 1).

Viability and cell morphology. The overnight cultures of various strains at 23°C in YPD liquid medium were diluted and allowed to resume growth to 10^6 cells/ml (optical density at 600 nm [OD_{600}] = 0.5) at 23°C before being shifted to 37°C. Aliquots of cells were taken at each time point for further analysis. In viability studies, cell samples were sonicated briefly to disrupt clumps, and the number of cells was counted with a hemocytometer. At the same time, the sonicated samples were diluted, plated onto YPD plates, and allowed to form colonies for 3 to 4 days at 23°C. The percent viability was determined by dividing the number of colonies on the YPD plates by the total number of cells plated. In morphology studies, aliquots of cells were sonicated briefly, and at least 200 cells of each sample were examined under the microscope and counted into three categories: unbudded, small-budded, and large-budded cells.

Cell cycle synchronization. The yeast cultures were synchronized by addition of hydroxyurea (HU) to a final concentration of 0.3 M. After incubation at 23°C for 5 h, the majority of the cells were arrested in S phase as budded cells. The cells were then washed by filtration, resuspended in fresh medium without HU prewarmed to 37°C, and incubated at 37°C. Samples were taken from this point. For synchronization with nocodazole, the nocodazole stock solution in dimethyl sulfoxide was added to the fresh culture to a final concentration of 15 μg/ml. After incubation at 23°C for 4 h, the majority of the cells were arrested as large-budded cells.

UV treatment. Overnight cultures in YPD at 23°C were diluted and allowed to grow at 23°C to an OD_{600} of 0.5. To measure the UV sensitivity, 5 ml of each yeast culture was transferred into an empty petri plate and irradiated with UV at various dosages. A fraction of the culture was used for determination of the cell number per milliliter with a hemocytometer, and at the same time, other parts of the culture were appropriately diluted, plated out onto YPD plates, and allowed to form colonies at 28.5°C for 3 days. UV sensitivity was calculated by dividing the number of colonies on plates by the number of cells plated. To measure the cell cycle response to UV radiation, fresh cultures were prepared and irradiated under UV light as described above. The dosage used was 300 J/m^2. The cells were

TABLE 2. Plasmids

Plasmid	Insert
pRS304	*TRP1*, yeast integration vector
pRS315	*LEU2*, yeast centromere vector
pRS314	*TRP1*, yeast centromere vector
pMC186	*CDC28* with its endogenous promoter in pRS315
pMC213	*GAL1-CLB2-myc*, *LEU2*, yeast centromere vector
pMC214	*GAL1-CLB3-myc*, *LEU2*, yeast centromere vector
pMC215	*GAL1-CLB5-myc*, *LEU2*, yeast centromere vector
pMC216	*GAL1-CLB6-myc*, *LEU2*, yeast centromere vector
pUS1	*GAL1-CDC28* in Ycp50

incubated at 23°C, and samples were taken from this point for examination of morphology and DNA profiles. After 150 min at 23°C, the cultures were split into two, with one half remaining incubated at 23°C and the other shifted to 37°C.

Fluorescent staining. Cells were grown in liquid culture under the specified conditions. Aliquots of cells were fixed by the addition of formaldehyde to a final concentration of 3.7%. After 10 min, cells were collected by brief centrifugation and resuspended in KPi buffer (0.1 M KH_2PO_4-K_2HPO_4, pH 6.4) plus 3.7% formaldehyde. Cells were collected 60 min later, washed three times in KPi buffer, resuspended in KPi buffer plus 1.2 M sorbitol, and permeabilized by incubation with 10 μg of Zymolyase 100T (ICN Biomedicals Inc.) per ml for 60 min at 30°C. The cells were then washed once with KPi buffer plus 1.2 M sorbitol and resuspended in the same solution. Ten microliters of the cell suspension was transferred to a polylysine-coated well on a multiwell slide. After 1 min, the cell suspension was removed and the slide was immersed in −20°C methanol for 6 min and in −20°C acetone for 30 s. The slide was air dried, incubated with the primary antibody followed by the secondary antibody, and incubated at 30°C for 60 min. The slides were then washed four times with phosphate-buffered saline (PBS) plus bovine serum albumin. To visualize microtubule structures, the rat monoclonal antibody YOL1/34 (Serotec, Oxford, United Kingdom) was used as the primary antibody and rhodamine-conjugated goat anti-rat immunoglobulin G (Jackson ImmunoResearch) was used as the secondary antibody. Cellular DNA was visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). All stained samples were observed and photographed with a Zeiss Axioplan microscope.

Measurement of DNA content by FACScan analysis. At each time point, cell samples were taken from liquid cultures and fixed with 70% ethanol for 1 h or overnight at 4°C. The cells were then washed once with PBS and resuspended in 0.1 ml of 10 mM Tris-HCl (pH 8.0)–10 mM NaCl–50 μg of propidium iodide per ml–1 mg of RNase A per ml for 4 h at 37°C. Stained cells were subsequently diluted in 1 ml of PBS, and for each sample the DNA content in 10,000 cells was determined with a FACScan flow cytometer.

Plasmid stability assay. Yeast cells containing the centromere plasmid pRS315 (Table 2) were grown overnight in SC-Leu medium at 23°C. The cells were collected by filtration and resuspended in YPD liquid medium to a final OD_{600} of 0.1. The YPD cultures were then shifted to 28.5°C for the *cdc28-4* and *cdc28-5M* mutants and to 34°C for the other *cdc28* mutants. Aliquots of cells were taken at the times when the OD_{600} was 0.1, 0.2, 0.4, 0.8, and 1.6 and then were sonicated briefly, diluted in YPD, plated on YPD plates, and allowed to form colonies at 23°C for 4 days. The colonies on YPD plates were subsequently replica plated on SC-Leu plates and incubated at 23°C for another 2 days. The percentage of cells containing the plasmid was determined by dividing the number of colonies on the SC-Leu plates by the number on the YPD plates.

Immunoprecipitation of Clb proteins and histone H1 protein kinase assay. The plasmids pMC213, -214, -215, and -216, containing *CLB2*, *CLB3*, *CLB5*, and *CLB6*, respectively, were constructed so that each of them was tagged with the c-*myc* epitope at the C terminus and placed under control of the *GAL1* promoter. These constructs were made by our colleagues in this laboratory. The plasmids were transformed into Ymc374 and the isogenic wild-type W303 strain. Overnight cultures of the cells containing various plasmids grown at 23°C in SC-Leu plus raffinose were diluted and allowed to continue to grow at 23°C for 2 h to an OD_{600} of 0.4. HU (Sigma) and galactose were added to final concentrations of 0.3 M and 2%, respectively, to arrest cells in S phase and induce the *CLB* genes. The cultures were incubated at 23°C for 5 h before being shifted to 37°C. At 0 h and 3 h after the shift to 37°C, cells were collected, washed once with stop mix (0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, and 50 mM NaF), and resuspended in 0.2 ml of ice-cold lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 7.2], 1 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml, 0.1 mM sodium orthovanadate, and 15 mM *p*-nitrophenylphosphate). The cell suspensions were then lysed at 4°C by vortexing with glass beads. Crude extracts were obtained after centrifugation at 4°C. The concentration of total protein in each extract was determined. Equal amounts of total protein (800 μg) were taken from each sample and incubated on ice with a rabbit anti-*myc* polyclonal antibody (Santa Cruz Biotechnology, Inc.) for 1 h. Protein A–Sepharose CL-4B (Pharmacia) was then added and incubated at 4°C for 1 h. The beads were precipitated and washed five times with radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2). Each sample was mixed with the loading buffer and loaded onto an SDS–12% polyacrylamide gel. After electrophoresis, the gel was electroblotted to an Immobilon-P membrane (Millipore). The various Clb proteins were detected by probing with a mouse anti-*myc* monoclonal antibody (Oncogene Science) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody and were visualized with the ECL system (Amersham).

For the histone H1 protein kinase assay (39), the monoclonal anti-*myc* antibody was used for immunoprecipitation as described above. After the washing with radioimmunoprecipitation assay buffer, the beads were washed two more times with 25 mM MOPS (morpholinepropanesulfonic acid) (pH 7.2). Six microliters of HBII buffer (60 mM β-glycerophosphate, 25 mM MOPS [pH 7.2], 15 mM *p*-nitrophenylphosphate, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml, and 0.1 mM sodium orthovanadate) was added to each sample. The kinase assay was performed by incubating the beads with 1 mg of histone H1 (Sigma) and 5 mM [γ-^{32}P]ATP (10 mCi/ml) at 23°C for 20 min, followed by addition of SDS loading buffer and SDS-polyacrylamide gel electrophoresis. The gel was then fixed, dried, and exposed to an X-ray film.

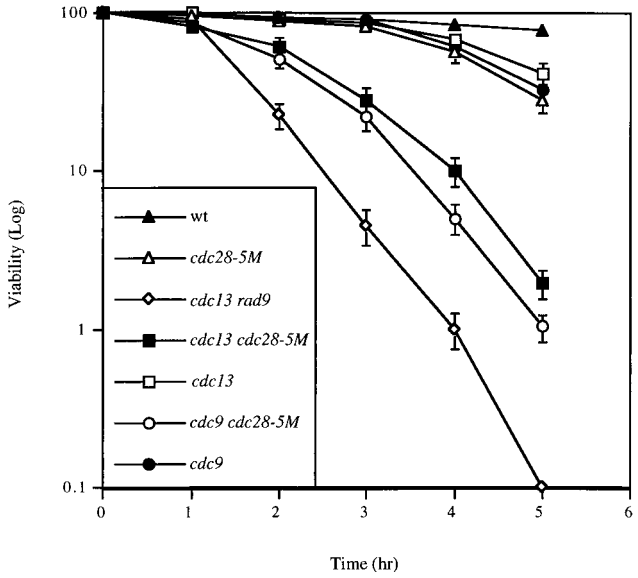

FIG. 1. Rapid-death phenotype of the *cdc13 cdc28-5M* and *cdc9 cdc28-5M* mutants at the restrictive temperature. Log-phase cultures in liquid YPD at 23°C were shifted to the restrictive temperature 37°C for the times indicated. Cell viability was determined as described in Materials and Methods. The strains were YNN414 (wild type [wt]), Ymc374 (*cdc28-5M*), US355 (*cdc13 rad9*Δ), Ymc376 (*cdc13 cdc28-5M*), Ymc319 (*cdc13*), Ymc375 (*cdc9 cdc28-5M*), and Ymc323 (*cdc9*). Results are presented as means ± standard deviations.

RESULTS

Isolation of a *cdc28* mutant by rapid death in *cdc13* cells. To determine whether Cdc28 plays a role in checkpoint controls in budding yeast, we carried out a genetic screen for *cdc28* mutants that would exhibit defects in cell cycle arrest in response to DNA damage. The screening scheme involved the *cdc13* mutant. *cdc13* cells are thought to arrest in G_2 after a shift to a restrictive temperature (37°C), due to the presence of damaged DNA in the telomere region (9). We have chosen to designate the arrest point of *cdc13* cells as G_2/M because budding yeast lacks morphological markers for determination of cell cycle stages between postreplication and onset of anaphase. The cell cycle arrest in *cdc13* cells has been attributed to checkpoint control, since it is dependent on the function of a group of designated checkpoint proteins such as Rad9 (13, 41–44). The *cdc13 rad9*Δ mutant fails to arrest in G_2/M at 37°C and loses viability rapidly, because, presumably, entry into mitosis in the presence of DNA damage is lethal to the cells (44). The rapid-death phenotype of *cdc13* cells in the background of checkpoint gene mutations has been used to isolate additional checkpoint mutants that are defective in cell cycle arrest after DNA damage (44). We adopted this strategy to search for *cdc28* mutants with similar checkpoint defects. The *CDC28* gene was mutagenized in vitro by low-fidelity PCR. The mutagenized DNA, inserted into a single-copy plasmid, was used to transform a *cdc13* strain (Ymc338) (Table 1) whose *CDC28* locus had been deleted and whose viability had been maintained by the presence of a galactose-inducible *CDC28* gene (*GAL1-CDC28*) on a plasmid. The transformants were grown at 23°C and screened for colonies that died rapidly on glucose plates at 37°C (see Materials and Methods). We obtained 27 transformants (of 8,000) which showed a rapid-death pheno-

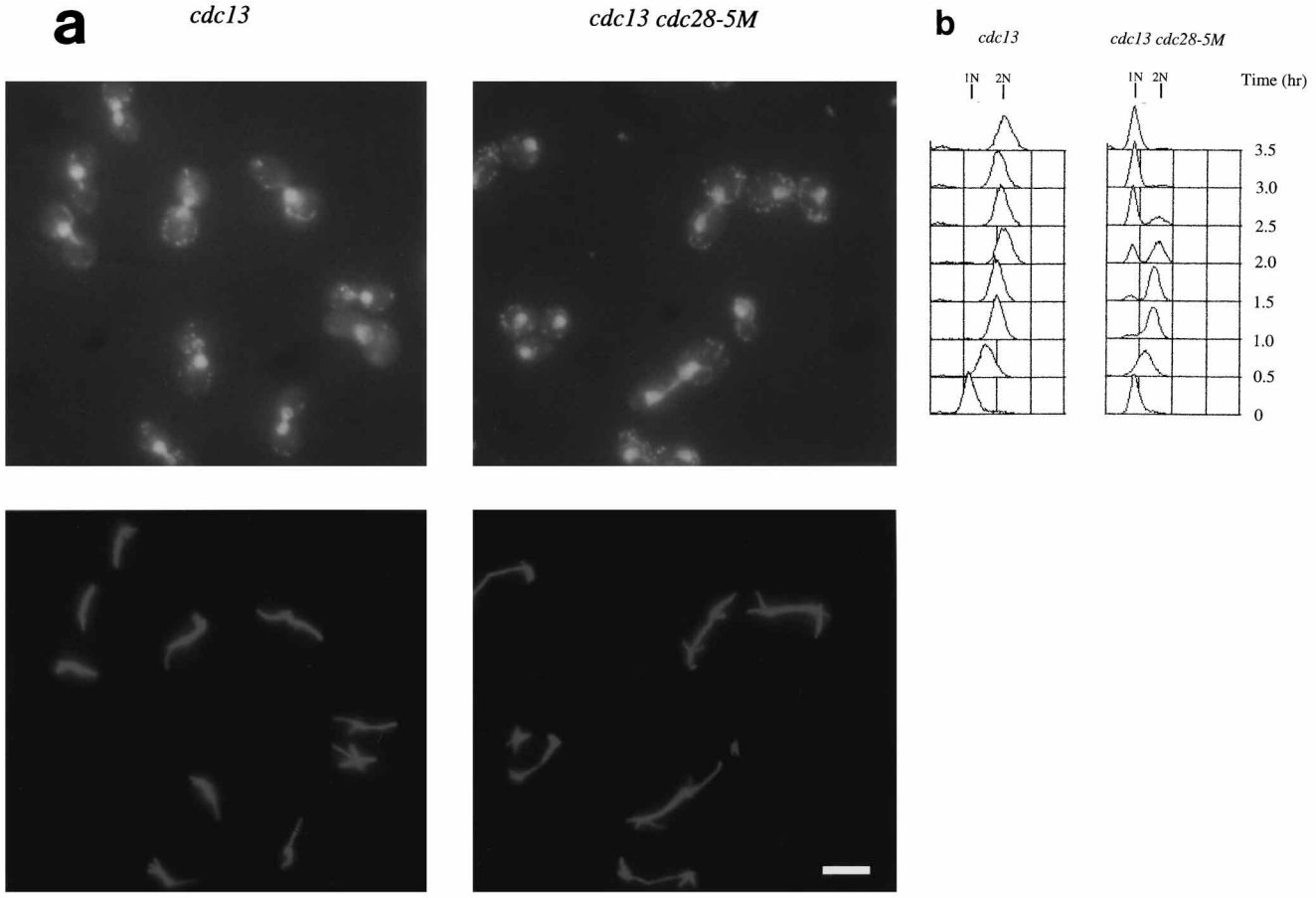

FIG. 2. Nuclear division of *cdc13 cdc28-5M* cells at 37°C after release from the HU block. The [*rho*^{0}] derivative of Ymc376 (*cdc13 cdc28-5M*) containing either pMC186 or pRS315 was synchronized in S phase by treatment with HU (0.3 M) at 23°C for 5 h. After being washed, the cells were resuspended in fresh medium without HU and incubated at 37°C. Samples were taken every 30 min for examination. (a) Fluorescent photographs of samples taken at 1.5 h after release from HU arrest at 37°C. The cells were stained with DAPI to visualize the nucleus (upper panels) and with the antitubulin antibody to visualize mitotic spindles (lower panels). Left panels, *cdc13 CDC28* (Ymc376::pMC186) cells; right panels, *cdc13 cdc28-5M* (Ymc376::pRS315) cells. Bar, 8 μm. (b) Flow cytometry analysis of the *cdc13 CDC28* and *cdc13 cdc28-5M* cells from time zero to 3.5 h after the shift to 37°C.

type at 37°C. By visual examination of cell morphology under a microscope and flow cytometry (FACScan) analysis of DNA content, most of the rapid-death strains were deemed unpromising since they exhibited a dumbbell shape and a 2N DNA content at 37°C similar to that of *cdc13* cells. However, there was one strain that displayed mainly an unbudded morphology and a 1N DNA peak after the shift to 37°C (data not shown) (see below). Upon retrieval of the plasmid containing mutated *cdc28* and its introduction into a *cdc28* deletion strain (Ymc336) by plasmid shuffling, it was found that this *cdc28* allele was temperature sensitive for cell growth and produced a G_1 arrest phenotype at 37°C (data not shown). It was named *cdc28-5M*.

To facilitate analysis of mutant phenotypes, we integrated the *cdc28-5M* allele into the *TRP1* locus of the *cdc28*Δ strain Ymc336 to generate Ymc374 (Table 1). A *cdc13 cdc28-5M* double mutant (Ymc376) (Table 1) was derived from a cross between this strain and *cdc13*. The cell viability of this double mutant at 37°C was compared with those of the single mutants as well as a *cdc13* mutant containing a known checkpoint mutation, *rad9*Δ (US355) (Table 1). *cdc13 cdc28-5M* cells lost viability considerably more rapidly than either of the single mutants at 37°C (Fig. 1). The loss of viability of *cdc13 cdc28-5M* cells at 37°C, however, was not as dramatic as that of *cdc13 rad9*Δ cells (Fig. 1).

***cdc28-5M* confers a checkpoint defect to *cdc13* and *cdc9* mutants.** The failure of the Ymc338::*cdc28-5M* cells to arrest in G_2/M at 37°C may be alternatively explained by the possibility that of the two mutated proteins, Cdc28 lost function first after the temperature shift, so that the cells did not have a chance to escape from the G_1 block imposed by the *cdc28-5M* mutation before they could be arrested in G_2/M by the *cdc13* mutation. To establish unequivocally that the *cdc28-5M* mutation conferred a mitotic checkpoint defect, we investigated the cell cycle progression from the S phase stage to the telophase stage in the *cdc13 cdc28-5M* cells under nonpermissive conditions. To avoid potential interference from different strain backgrounds, we examined only the *cdc13 cdc28-5M* mutant (Ymc376) carrying either plasmid pRS315 or pMC186 (pRS315-*CDC28*) (Table 2). In addition, a [*rho*^{0}] derivative of Ymc376 was used to avoid the interference of mitochondrial DNA with DAPI staining and FACScan. These strains were first synchronized in S phase by 5 h of treatment with the DNA synthesis inhibitor HU at 23°C and then transferred to fresh medium at 37°C. Samples were drawn at intervals to examine the nuclear morphology and the FACScan pattern. Cells that exhibited clear signs of late anaphase and telophase, i.e., two well-separated nuclei with one localized in the mother cell and one localized in the daughter cell and joined by elongated nuclear spindles, were counted. The *cdc13 CDC28* (Ymc376::pMC186) cells

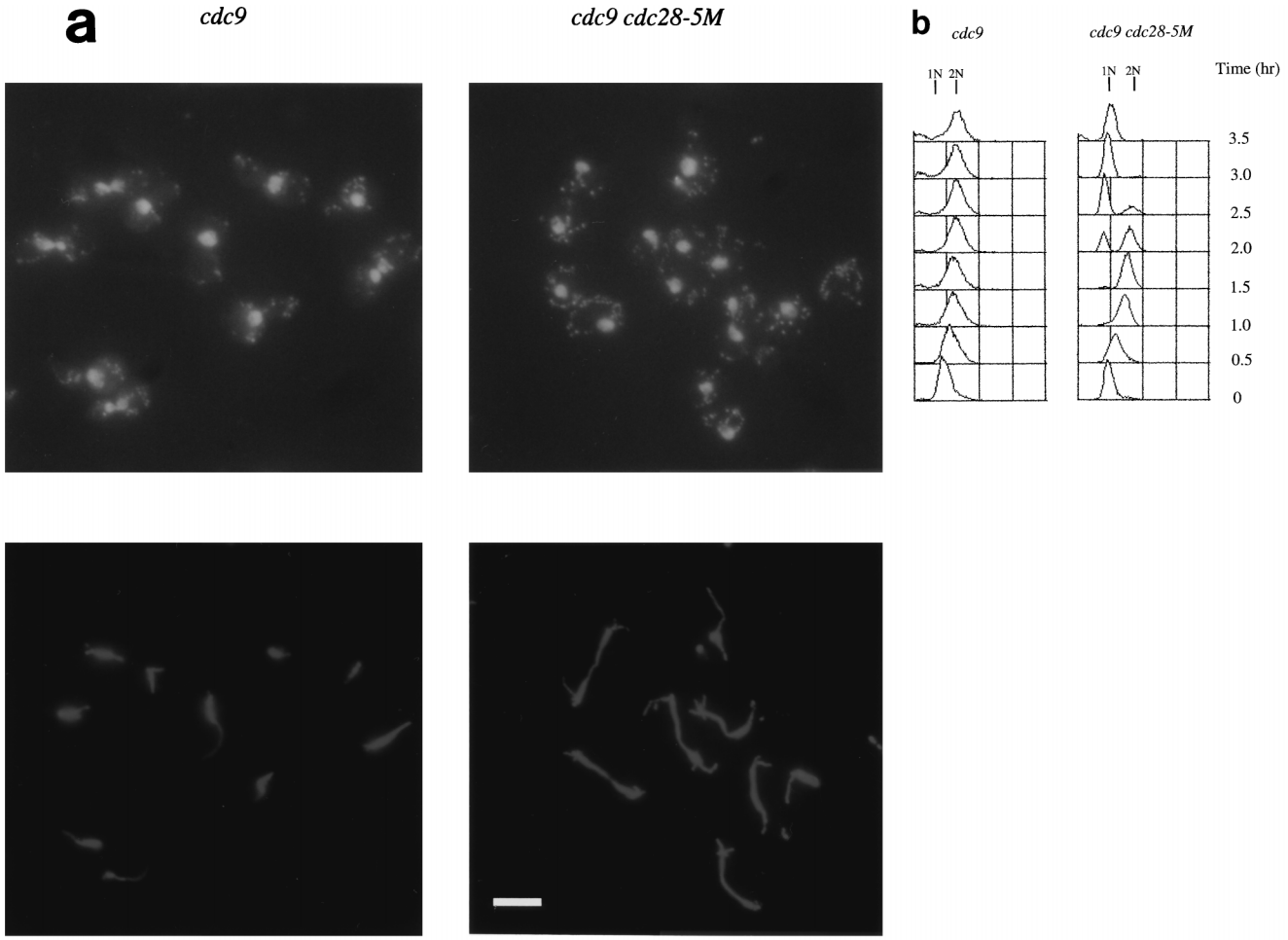

FIG. 3. Nuclear division of *cdc9 cdc28-5M* cells at 37°C after release from the HU block. The [*rho*^{0}] derivative of Ymc375 (*cdc9 cdc28-5M*) containing either pMC186 or pRS315 was analyzed at 37°C by the same procedure as described in the legend to Fig. 2. (a) Fluorescent photographs of samples taken at 1.5 h after release from the HU block at 37°C. The cells were stained with DAPI (upper panels) and with the antitubulin antibody (lower panels). Left panels, *cdc9 CDC28* (Ymc375::pMC186) cells; right panels, *cdc9 cdc28-5M* (Ymc375::pRS315) cells. Bar, 8 μm. (b) Flow cytometry analysis of the *cdc9 CDC28* and *cdc9 cdc28-5M* cells from time zero to 3.5 h after the shift to 37°C.

were uniformly arrested as large-budded cells with an undivided nucleus and short spindles after release to 37°C for 1.5 h (Fig. 2a), which is typical of the *cdc13* single mutant at the restrictive temperature. This phenotype was unchanged with prolonged incubation at 37°C (up to 4 h; see Fig. 4). The *cdc13 cdc28-5M* (Ymc376::pRS315) strain, on the other hand, gave rise to many cells that were clearly in late anaphase or telophase under the same conditions (Fig. 2a). Results for later time points indicated that these cells underwent cell division and accumulated as unbudded G_1 cells (see below). FACScan analysis also demonstrated that the Ymc376::pMC186 cells remained arrested with a 2N DNA content after 3.5 h of incubation at 37°C (Fig. 2b), whereas the Ymc376::pRS315 cells proceeded through nuclear division and accumulated as G_1 cells with a 1N DNA content (Fig. 2b). These results showed that the *cdc28-5M* mutation indeed conferred a mitotic checkpoint defect to *cdc13* cells. Similar experiments using α factor-synchronized cells also led to the same conclusion (data not shown).

We further tested whether the effect of the *cdc28-5M* mutation on cell cycle arrest in *cdc13* cells was applicable to other DNA damage-arrested mutants by performing similar studies with Ymc375, a *cdc9 cdc28-5M* double mutant (Table 1). The *cdc9* mutant, like the *cdc13* mutant, arrests in G_2/M at 37°C in a checkpoint-dependent manner (13, 43). The *cdc9 cdc28-5M* mutant also died more rapidly than either the *cdc9* or *cdc28-5M* single mutant at 37°C, as shown in Fig. 1. Indeed, with the same regimen employed with the Ymc376 cells as described above, the HU-synchronized culture of *cdc9 cdc28-5M* (Ymc375::pRS315) cells similarly showed a checkpoint defect. While the *cdc9 CDC28* (Ymc375::pMC186) cells were arrested as large-budded cells with a single nucleus and short nuclear spindles after 1.5 h of release to 37°C (Fig. 3a), many of the Ymc375::pRS315 cells were found to be in late anaphase and telophase, displaying elongated mitotic spindles connecting two nuclei well separated into the mother and the daughter cells under the same conditions (Fig. 3a). This checkpoint defect was also confirmed by FACScan analysis where the incubation at 37°C lasted 3.5 h (Fig. 3b). The DNA content patterns of the *cdc9 cdc28-5M* cells were nearly identical to those of the *cdc13 cdc28-5M* cells (compare Fig. 3b and 2b), indicating that the *cdc28-5M* mutation conferred similar checkpoint defects in both *cdc9* and *cdc13* cells. Figure 4 shows the percentage of the cells in each strain undergoing nuclear division at 37°C after release from the HU block along the time course for the experiments presented above. Whereas the numbers of *cdc9*

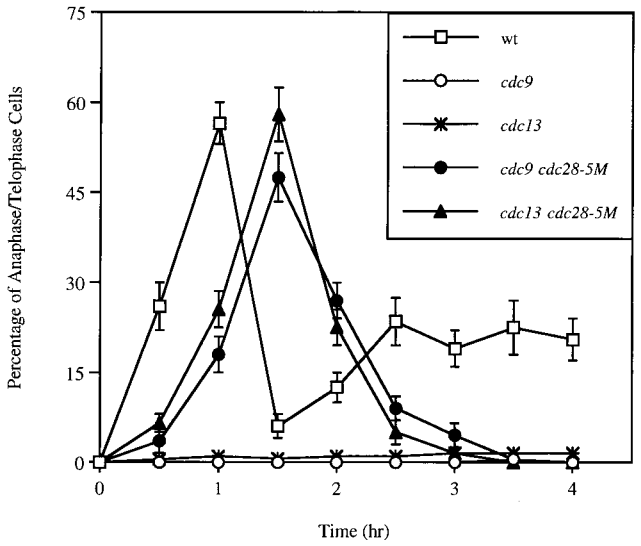

FIG. 4. Quantitative measurement of cells undergoing nuclear division at 37°C after release from the HU block. The percentage of cells that displayed the late anaphase and telophase nuclear morphology at each time point from the experiments presented in Fig. 2 and 3 was counted and plotted. The late anaphase and telophase cells were those that contained two nuclei, with one localized in the mother cell and one localized in the daughter cell and connected by long mitotic spindles, as shown in the right panels of Fig. 2a and 3a. For each time point, a total of at least 200 cells were counted. The strains were YNN414 (wild type [wt]), Ymc375::pMC186 (*cdc9 CDC28*), Ymc376::pMC186 (*cdc13 CDC28*), Ymc375::pRS315 (*cdc9 cdc28-5M*), and Ymc376::pRS315 (*cdc13 cdc28-5M*). Results are presented as means ± standard deviations.

CDC28 and *cdc13 CDC28* cells capable of dividing at 37°C are negligible, the *cdc9 cdc28-5M* and *cdc13 cdc28-5M* cells show a high level of nuclear division comparable to that of the wild-type control (Fig. 4), suggesting that the mitotic checkpoint function in these mutants is probably completely disabled. The slight delay in onset of nuclear division in the double mutants compared to that in the wild-type control may be due to a slower recovery of the mutants from HU arrest.

We conclude from these results that the *cdc28-5M* mutation allowed nuclear division to occur in the *cdc9* and *cdc13* mutants at the restrictive temperature and thereby conferred a mitotic checkpoint defect to these cells. The fact that transformation of Ymc375 and Ymc376 cells with the wild-type *CDC28* gene on a single-copy plasmid restored the G_2/M arrest phenotype indicated that the checkpoint defect of *cdc28-5M* was recessive.

***cdc28-5M* confers a checkpoint defect to UV-irradiated cells.** Exposure of wild-type yeast cells to X-ray or UV light radiation leads to G_2/M arrest which is dependent on the same checkpoint functions required for the arrest of *cdc9* and *cdc13* cells at 37°C (13, 14, 24, 25, 41, 44). It is therefore anticipated that *cdc28-5M* may also affect the cell cycle arrest induced by irradiation. To confirm this, we examined the response of the *cdc28-5M* mutant to UV radiation. The *cdc28-5M* mutant showed an increased sensitivity to UV radiation at 28.5°C compared to those of wild-type cells and cells carrying several other alleles of the *cdc28* mutation, as shown in Fig. 5. However, it survived better than the *rad9*Δ mutant (Fig. 5). We again used the pMC186 and pRS315 plasmids to avoid potential interference of strain background in the following analysis. The *cdc28-5M* (Ymc374) cells carrying either of the plasmids were grown exponentially at 23°C and irradiated under UV light, and samples were taken at intervals to examine the cell morphology and the FACScan pattern. As shown in Table 3 and Fig. 6, both the *CDC28* and *cdc28-5M* strains responded to UV radiation with a cell cycle arrest. After 150 min of incubation at 23°C following irradiation, nearly 80% of the cells in both strains were seen as large-budded cells with 2N DNA content (Table 3; Fig. 6, lower panels). Fluorescent staining for nuclear morphology confirmed that these cells were arrested in G_2/M (data not shown). This indicated that the mitotic checkpoint function in the *cdc28-5M* mutant was nearly as normal as that of *CDC28* cells at the permissive temperature. However, the UV-induced G_2/M arrest of *cdc28-5M* cells could not be sustained after a temperature shift to 37°C, as the majority of the cells underwent cell division and accumulated as G_1 cells with a 1N DNA content after the temperature shift to 37°C for 80 min (Table 3; Fig. 6, upper panels). In contrast, the *CDC28* cells remained arrested in G_2/M after the same treatment (Table 3; Fig. 6, upper panels), as did the *cdc28-5M* cells incubated continuously at 23°C for same length of time (Table 3; Fig. 6, lower panels). It is clear, therefore, that the *cdc28-5M* mutation conferred a checkpoint defect to UV-irradiated cells and that the defect was temperature dependent.

***cdc28-5M* can not override the cell cycle arrest imposed by HU.** The *cdc28-5M* mutant could be synchronized in S phase by treatment with HU at 23°C, as described above (Fig. 2b and 3b). Five hours of treatment with HU at 23°C resulted in 88 and 80% budded cells in the *CDC28* and *cdc28-5M* strains, respectively. A subsequent temperature shift to 37°C in the presence of HU for up to another 3 h did not noticeably change the budding profile in either of the strains (data not shown). No discernible signs of nuclear division could be observed in the *cdc28-5M* mutant at 37°C in the presence of HU as judged by fluorescent staining for nuclear morphology (data not shown). In addition, the *cdc28-5M* mutant did not lose viability any more rapidly than the *CDC28* cells in the presence of HU at either 23 or 37°C (data not shown). It was therefore

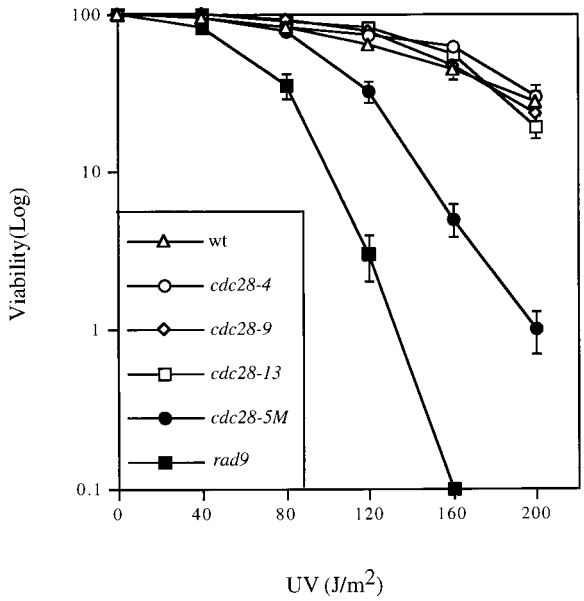

FIG. 5. UV sensitivity of the *cdc28-5M* mutant and several other *cdc28* mutants at 28.5°C. Log-phase cultures were irradiated with UV at the dosages indicated. After the irradiation, cells were plated out on YPD plates and incubated at 28.5°C for 3 days. UV sensitivity was calculated by dividing the number of viable colonies on the YPD plates by the number of cells plated. The strains were Ymc387 (*cdc28-9*), Ymc388 (*cdc28-13*), YNN414 (wild type [wt]), Ymc374 (*cdc28-5M*), US100 (*cdc28-4*), and Ymc309a (*rad9*Δ). Results are presented as means ± standard deviations.

TABLE 3. Budding profile of the *cdc28-5M* mutant after UV irradiation

Time (min) at:		Strain[a]	% of cells (mean ± SD) with:		
23°C	37°C		No bud	Small bud	Large bud
0		wt	48.5 ± 3.5	22 ± 1	29.5 ± 2.5
		cdc28-5M mutant	43 ± 5	27 ± 2	30 ± 3
150		wt	13.5 ± 1.5	7.5 ± 1.5	79 ± 3
		cdc28-5M mutant	18 ± 2	4.5 ± 1.5	77.5 ± 3.5
190		wt	12.5 ± 1.5	5 ± 1	82.5 ± 2.5
		cdc28-5M mutant	19.5 ± 2.5	5.5 ± 0.5	75 ± 3
230		wt	14 ± 1	6 ± 1	80 ± 2
		cdc28-5M mutant	17.5 ± 2.5	8 ± 1	74.5 ± 3.5
150	40	wt	11 ± 1	7.5 ± 1.5	81.5 ± 2.5
		cdc28-5M mutant	49 ± 5	14 ± 1	37 ± 4
150	80	wt	15.5 ± 1.5	8.5 ± 0.5	76 ± 2
		cdc28-5M mutant	90.5 ± 1.5	2 ± 0	7.5 ± 1.5

[a] wt, wild type.

concluded that the *cdc28-5M* mutation did not affect the checkpoint functions designated to control the HU-arrested cell cycle stage (24, 25, 44).

The *cdc28-5M* mutation results in inactivation of CDKs. The *cdc28-5M* allele was so named because it contained five mutations in the *CDC28* coding region. DNA sequence analysis revealed that there were amino acid changes of L to H, L to S, S to C, E to K, and N to D at residues 11, 183, 216, 217, and 232, respectively, in the *cdc28-5M* allele. We attempted to determine which of these mutations was responsible for the checkpoint defect by introducing each of them individually into the *CDC28* gene via in vitro mutagenesis. It was found that none of the five mutations alone could confer the checkpoint defect as measured by response to UV irradiation, nor could they produce a temperature-sensitive phenotype individually (data not shown). Based on these results, we conclude that the phenotype of *cdc28-5M* is a combined effect of multiple mutations. We then tested if any of the Clb-associated kinase activities was affected in the *cdc28-5M* mutant. This experiment, however, would be futile if the mutant kept at the restrictive temperature was not prevented from being arrested in G_1, where even wild-type cells do not exhibit high levels of Clb-associated Cdc28 kinase activities (17). Fortunately, the *cdc28-5M* mutant was still capable of normal S phase arrest upon HU treatment (see above), where Clb-associated kinases are known to be highly active in wild-type cells (1, 36, 37). It was therefore possible to compare various Clb-associated kinase activities in the mutant by using HU-arrested cultures. The *CLB2*, *CLB3*, *CLB5*, and *CLB6* genes were each tagged with the *myc* epitope at the 3′ ends of their coding regions and placed under the control of the *GAL1* promoter. Wild-type and *cdc28-5M* cells containing the *GAL1-CLB* constructs were grown at 23°C in either galactose or raffinose and synchronized in S phase by treatment with HU for 5 h. They were then shifted to 37°C for another 3 h in the presence of the drug before collection and analysis. Samples taken from wild-type cells grown in galactose showed similar levels of activity for all four Clb-associated kinases tested, and no kinase activity was detected in the raffinose-grown cells (Fig. 7a, top panel). There was a slight decline in the kinase activity after 3 h of incubation in HU at 37°C in all cases (Fig. 7a, top panel, and b). Samples taken from the *cdc28-5M* cells, however, showed a remarkable difference from the wild-type cells in kinase activity. With the exception of the Clb3-associated kinase, the activities of all Clb-associated kinases were greatly reduced in the galactose-grown *cdc28-5M* cells at both the permissive and nonpermissive temperatures. At 23°C, the Clb2-associated kinase activity was only 49% of that of the wild type, and it further decreased to 18% after 3 h at 37°C (Fig. 7b). Similarly, the Clb5-associated kinase activity decreased from 31 to 11% of the wild-type level after 3 h of incubation in HU at 37°C (Fig. 7b). More strikingly, the Clb6-associated kinase activity in the HU-arrested *cdc28-5M* mutant was completely undetectable even at the permissive temperature (Fig. 7a and b). The absence of this activity was not due to the failure of expression of the *myc*-tagged *CLB6* gene in the mutant, since the tagged Clb6 protein was present at a level similar to that in the wild-type control in the immunoprecipitates of the mutant cells, as were all other tagged Clb proteins (Fig. 7c). From these results, we conclude that the Cdc28 kinase activity in the *cdc28-5M* mutant is grossly affected by the mutation and that the Clb5- and Clb6-associated kinases were especially crippled.

Checkpoint defects of other Start-deficient *cdc28* mutants. Since the G_1 arrest phenotype of *cdc28-5M* was similar to those of several known alleles of *cdc28* defective in Start functions, such as *cdc28-4*, *-9*, and *-13* (28, 29), we determined whether these mutants would also exhibit mitotic checkpoint defects by

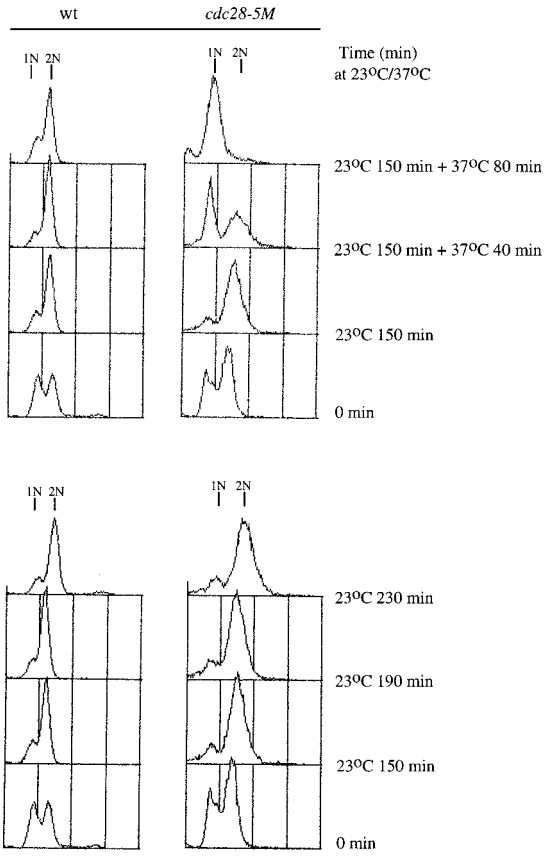

FIG. 6. The *cdc28-5M* mutation abolished the G_2/M arrest induced by UV radiation at 37°C. Log-phase cultures were irradiated with UV at a dosage of 300 J/m^2 and incubated at 23°C for 150 min. Each culture was then split into two halves to be incubated at 23 and 37°C, respectively. Samples were taken every 40 min for analysis of the DNA content as well as the budding profile (Table 3). The strains were Ymc374::pMC186 (wild type [wt]) and Ymc374::pRS315 (*cdc28-5M*).

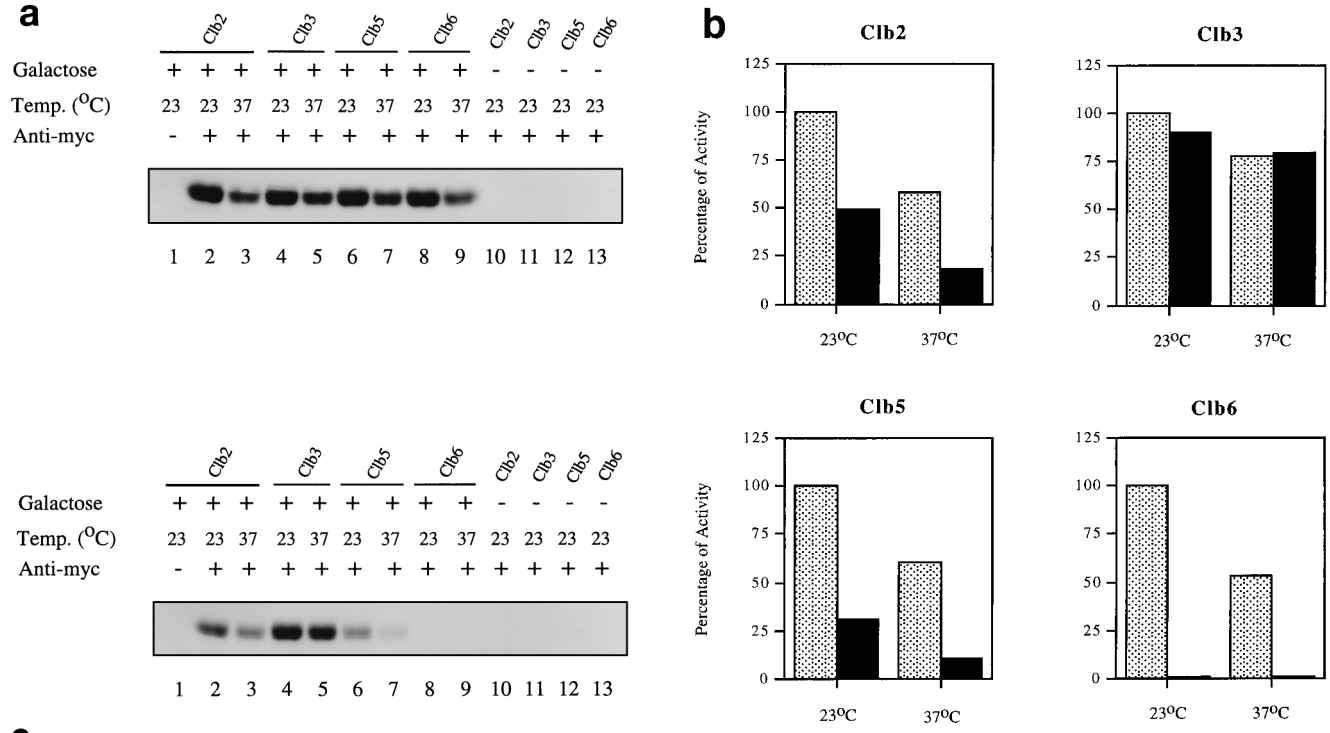

FIG. 7. Several Clb-associated kinase activities were impaired in the *cdc28-5M* mutant. Ymc374 (*cdc28-5M*) and YNN414 (wild-type) cells containing *GAL1-CLB2*, *GAL1-CLB3*, *GAL1-CLB5*, *GAL1-CLB6*, or the empty vector plasmids were grown in a selective medium containing raffinose at 23°C. HU and galactose were added to log-phase cultures to final concentrations of 0.3 M and 2%, respectively, and the cells were incubated at 23°C for 5 h to arrest in S phase while expression of the *CLB* genes was induced. The cultures were then shifted to 37°C in the presence of HU. Aliquots of cells were withdrawn, and protein extracts were prepared as described in Materials and Methods. (a) Clb2-, Clb3-, Clb5-, and Clb6-associated histone H1 kinase activities at 23 and 37°C in wild-type cells (top) and in the *cdc28-5M* mutant (bottom). Lanes 1, 2, 4, 6, 8, 10, 11, 12, and 13 contain samples taken before the temperature shift, and lanes 3, 5, 7, and 9 contain samples taken 3 h after the temperature shift to 37°C. Lane 1, no antibody; lanes 10 to 13, samples from the raffinose cultures. (b) Quantitative presentation of the data in panel a. The signals were quantified with a phosphorimager, and the values were normalized to that for the wild type. Stippled bars, wild type; solid bars, *cdc28-5M* mutant. (c) Levels of various Clb proteins immunoprecipitated from cultures incubated for 3 h after a temperature shift to 37°C in the presence of HU as described above. Lanes 1, 3, 5, 7, 9, and 11, wild-type cells; lanes 2, 4, 6, 8, 10, and 12, *cdc28-5M* mutant. Lanes 1 and 2, extracts from cells containing *GAL1-CLB6* with no anti-*myc* antibody added; lanes 11 and 12, extracts from cells containing the vector without a *CLB* gene insert. Numbers on the left are molecular weights in thousands. IgG, immunoglobulin G.

analyzing their cell cycle responses to UV radiation. As shown in Table 4 and Fig. 8, both *cdc28-9* and *-13* cells responded to UV radiation with an apparently normal G_2/M arrest at 23°C. The *cdc28-4* cells, however, remained asynchronous after same treatment (Table 4; Fig. 8, lower panels). Upon a temperature shift to 37°C, some, but not all, of the *cdc28-9* and *-13* cells divided and emerged as G_1 cells (Table 4; Fig. 8, upper panels) (note that this experiment was done together with that with the results shown in Fig. 6, with an extra time point shown). There was a slight increase of G_1 cells in the *cdc28-4* strain at 37°C as well. The asynchronous nature of the *cdc28-4* culture at both temperatures and of the other two mutants at 37°C was not due to their continued division, since the cell number of these mutants did not double during the course of the experiment, and neither was it likely to be attributable to cell death, as these mutants were considerably more resistant to UV killing than the *cdc28-5M* cells (Fig. 5). It is therefore possible that under these conditions, some of the cells were arrested in G_2/M whereas others were in G_1. Nevertheless, the fact that some G_2/M-arrested cells of the *cdc28-9* and *-13* mutants could not sustain the arrest at 37°C indicated that the mitotic checkpoint controls in these mutants were at least partially defective.

Instability of minichromosomes in the *cdc28-5M* mutant. Mutants defective in checkpoint functions usually exhibit a higher rate of chromosome instability than wild-type cells. The rate of chromosome loss, for example, is enhanced by the *rad9* mutation (42). Maintaining chromosome stability is precisely the suggested role for checkpoint proteins (11, 14, 24). In view of the checkpoint defects displayed by *cdc28-5M* and other alleles of *cdc28* mutants, it was possible that these mutants might likewise cause a higher-than-normal rate of chromosome instability. This possibility was tested by measuring the inheritance of a centromeric plasmid, pRS315, in these mutants. The wild-type strain and various *cdc28* mutants, as well as the *rad9*Δ mutant, were cultured at either 28.5°C (semipermissive for *cdc28-5M* and *-4* cells) or 34°C (semi-permissive for *cdc28-9* and *-13* cells) in the absence of selective pressure for the plasmid. Samples were drawn at intervals, and the percentage of cells containing the plasmid was assessed. As shown in

TABLE 4. Budding profiles of several other *cdc28* mutants after UV irradiation

Time (min) at:		Mutant	% of cells (mean ± SD) with:		
23°C	37°C		No bud	Small bud	Large bud
0		*cdc28-9*	36.5 ± 3.5	24 ± 2	39.5 ± 5.5
		cdc28-13	31.5 ± 2.5	26.5 ± 1.5	42 ± 4
		cdc28-4	49 ± 3	20 ± 1	31 ± 2
150		*cdc28-9*	12.5 ± 1.5	9 ± 1	78.5 ± 2.5
		cdc28-13	16 ± 2	7.5 ± 1.5	76.5 ± 3.5
		cdc28-4	33.5 ± 1.5	14.5 ± 0.5	52 ± 2
190		*cdc28-9*	11 ± 2	8 ± 1	81 ± 3
		cdc28-13	15 ± 1	9.5 ± 0.5	75.5 ± 1.5
		cdc28-4	38.5 ± 1.5	11 ± 1	50.5 ± 2.5
230		*cdc28-9*	13.5 ± 0.5	9.5 ± 1.5	77 ± 2
		cdc28-13	18 ± 3	9 ± 1	73 ± 4
		cdc28-4	43 ± 2	12 ± 1	45 ± 3
270		*cdc28-9*	16 ± 1	9.5 ± 0.5	74.5 ± 1.5
		cdc28-13	19.5 ± 1.5	8.5 ± 1.5	72 ± 3
		cdc28-4	41.5 ± 2.5	10 ± 1	48.5 ± 3.5
150	40	*cdc28-9*	29.5 ± 3.5	8.5 ± 1.5	62 ± 5
		cdc28-13	33 ± 2	11 ± 1	37 ± 3
		cdc28-4	46 ± 4	6.5 ± 1.5	47.5 ± 2.5
150	80	*cdc28-9*	41.5 ± 2.5	6 ± 1	52 ± 3.5
		cdc28-13	48.5 ± 3.5	5.5 ± 0.5	46 ± 4
		cdc28-4	58 ± 3	7 ± 1	35 ± 2
150	120	*cdc28-9*	65.5 ± 2.5	2.5 ± 0.5	32 ± 2
		cdc28-13	59 ± 4	4 ± 1	37 ± 3
		cdc28-4	61.5 ± 3.5	5 ± 1	33.5 ± 2.5

Fig. 9, the wild-type strain maintained the plasmid quite well at both temperatures, whereas the *cdc28-4*, *-9*, and *-13* cells lost the plasmid slightly more rapidly than the wild type. In contrast, the *cdc28-5M* cells lost the plasmid very rapidly, faster than any of the strains tested, including the *rad9*Δ mutant (Fig. 9). This result confirmed that the *cdc28-5M* mutant was genetically unstable, and even more so than the known *rad9*Δ checkpoint mutant, consistent with its phenotype of the checkpoint defect.

***cdc28-5M* also abolishes nocodazole-induced cell cycle arrest.** Microtubule toxins such as nocodazole promote disassembly of mitotic spindles and block the cell cycle in ways which, superficially, have certain similarities with the effects caused by DNA damage in budding yeast. Cells arrested upon treatment with these drugs are large budded with an undivided nucleus (16). This cell cycle arrest, however, has been thought to involve a unique pathway of checkpoint controls (14, 16, 25). Nevertheless, the observation that the nocodazole-arrested wild-type cells maintain a high level of Cdc28 kinase activity whereas the checkpoint mutants incapable of such arrest do not (16) prompted us to examine whether this arrest could be affected by the *cdc28-5M* mutation. After 4 h of incubation at 23°C in the presence of nocodazole, both wild-type and *cdc28-5M* mutant cells were predominantly arrested as large-budded cells with a 2N DNA content (Table 5 and Fig. 10). Upon a temperature shift to 37°C, however, the *cdc28-5M* cells failed to sustain the arrest even though the drug was still present. By 2 h at the restrictive temperature, the majority of the *cdc28-5M* cells had undergone cell division and stood as unbudded cells with a 1N DNA content, while the wild type remained arrested as large-budded cells with a 2N DNA content after the same treatment (Table 5; Fig. 10). This result was essentially the same as that obtained from the UV test shown in Fig. 5 and suggested that the spindle checkpoint functions were also disabled in the *cdc28-5M* mutant.

DISCUSSION

The pathways of checkpoint controls are conceived to include multiple steps, such as detection of damage signals, transmission of the signals to the cell cycle machinery, and inhibition of cell cycle progression (22–25). Although unproven, it is generally accepted that the targets to which the DNA damage signal is transmitted are likely the CDKs (14, 22–25) and hence that the cell cycle arrest following DNA damage is a result of down-regulation of CDKs by checkpoint-elicited negative regulators. This model is so far supported by studies of several systems, with, however, the notable exception of the budding yeast *S. cerevisiae*. The budding yeast CDK, Cdc28, does not seem to play roles in the checkpoint controls equivalent to those of the CDKs in other systems, as it is highly active in checkpoint-arrested cells (1, 16, 36, 37). Therefore, the DNA damage-caused cell cycle arrest in budding yeast is unlikely to be a result of down-regulation of Cdc28. Due to a lack of studies on *cdc28* mutants that exhibit checkpoint deficiencies,

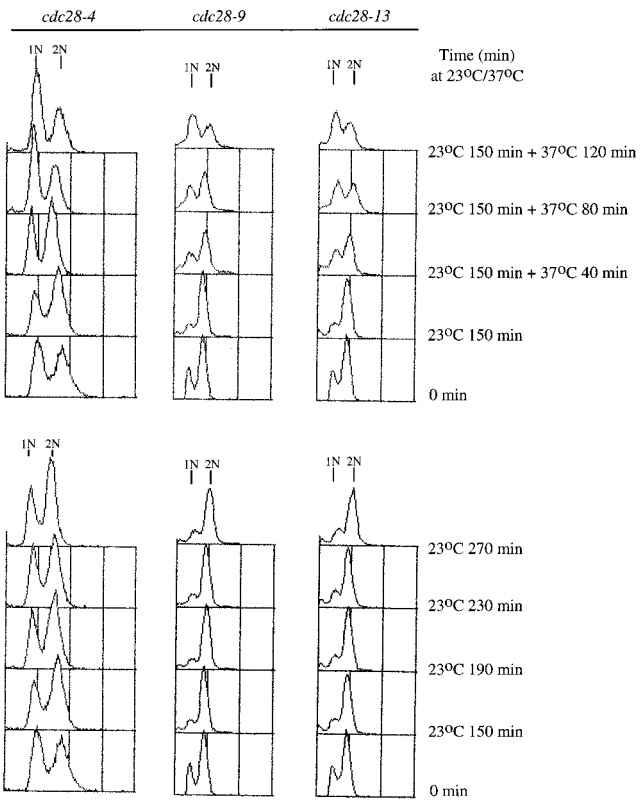

FIG. 8. Flow cytometry analysis of other *cdc28* mutants after UV irradiation. Log-phase cultures of the *cdc28-4*, *-9*, and *-13* mutants were irradiated with UV and analyzed by the same procedures described in the legend to Fig. 6. Samples were taken every 40 min for analysis of the DNA and budding profiles (Table 4). This experiment was done together with that with the *cdc28-5M* and wild-type control cells shown in Fig. 6. The strains were US100 (*cdc28-4*), US43 (*cdc28-9*), and US44 (*cdc28-13*).

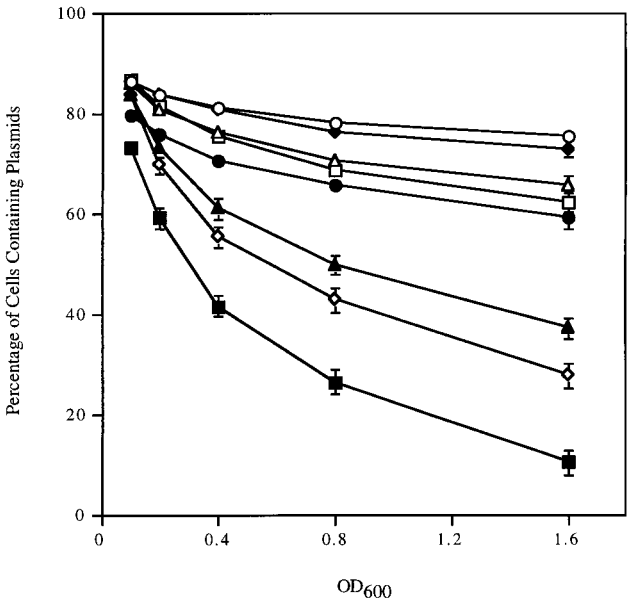

FIG. 9. Plasmid stability in various *cdc28* mutants at semipermissive temperatures. Cells from each strain containing pRS315 were grown in liquid SC-Leu medium at 23°C, collected by filtration, and resuspended in liquid YPD to a final OD_{600} of 0.1. These cultures were then shifted to either 28.5 or 34°C. Aliquots of cells were taken at the times when the OD_{600} was 0.1, 0.2, 0.4, 0.8, and 1.6 to be plated onto YPD plates to form colonies at 23°C for 4 days. The colonies were then replica plated on SC-Leu plates and incubated at 23°C for 2 days. The percentage of cells retaining the plasmid was calculated by dividing the number of colonies on the SC-Leu plates by the number on the YPD plates. Open circles, YNN414 (wild type) at 28.5°C; closed diamonds, YNN414 at 34°C; closed circles, US100 (*cdc28-4*) at 28.5°C; open triangles, US43 (*cdc28-9*) at 34°C; open squares, US44 (*cdc28-13*) at 34°C; closed squares, Ymc374 (*cdc28-5M*) at 28.5°C; closed triangles, Ymc309a (*rad9*Δ) at 28.5°C; open diamonds, Ymc309a at 34°C. Results are presented as means ± standard deviations.

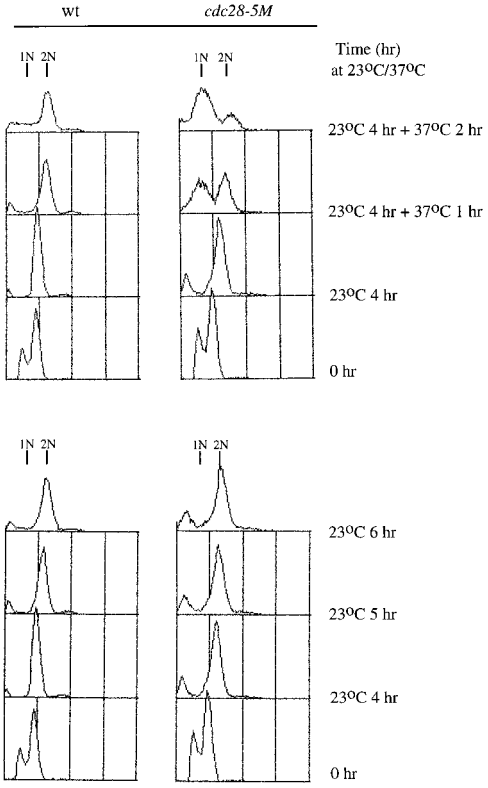

FIG. 10. The *cdc28-5M* mutation abolished the G_2/M arrest induced by nocodazole. Log-phase cultures of *cdc28-5M* mutant and wild-type cells were grown in liquid YPD at 23°C. Nocodazole was added to a final concentration of 15 μg/ml, and the cells were incubated at 23°C for 4 h. The cultures were then split into two halves to be incubated at 23 and 37°C, respectively. Samples were taken every hour for analysis of the DNA and budding profiles (Table 5). The strains were YNN414 (wild type [wt]) and Ymc374 (*cdc28-5M*).

the role of CDK in checkpoint control in budding yeast has remained a mystery.

The data presented in this report unequivocally establish a requirement for Cdc28 in checkpoint controls governing cell cycle events defined by DNA damage-induced arrest and the arrest following loss of microtubule function. Our conclusion that *cdc28-5M*, a novel mutation we isolated based on its effect on the viability of *cdc13* cells at a restrictive temperature, conferred a DNA damage checkpoint defect is strongly supported on the basis of three criteria. First, the *cdc13* and *cdc9* mutants, which normally arrest in G_2/M at the restrictive temperature due to checkpoint functions, no longer showed the arrest when *cdc28-5M* was introduced into them. Detailed analysis of nuclear morphology and DNA profiles with synchronized cultures has left no doubt of the ability of *cdc9 cdc28-5M* and *cdc13 cdc28-5M* cells to carry out nuclear division under nonpermissive conditions. In addition, the fact that transformation of these double mutants with a single copy of wild-type *CDC28* restored the G_2/M arrest demonstrates that the *cdc28-5M* mutation is solely responsible for the checkpoint defect. Abrogation of the cell cycle arrest in *cdc13* or *cdc9* mutants has been a classical criterion for assignment of the checkpoint functions, advocated initially by Hartwell and Weinert and utilized later by those authors and their coworkers to isolate and characterize various checkpoint genes such as the *RAD9*, *RAD17*, *MEC1*, *MEC2*, and *MEC3* genes (13, 41, 43, 44). On this ground, *cdc28-5M* deserves to be regarded as a new member of the checkpoint mutant family. Second, the failure of the *cdc28-5M* cells to arrest in G_2/M at the restrictive temperature following UV irradiation further proves that the *cdc28-5M* mutant was defective in DNA damage checkpoint controls. Asynchronous wild-type yeast cultures respond to UV

TABLE 5. Budding profile of the *cdc28-5M* mutant in the presence of nocodazole

Time (h) at:		Strain[a]	% of cells (mean ± SD) with:		
23°C	37°C		No bud	Small bud	Large bud
0		wt	35 ± 6	28 ± 2	37 ± 4
		cdc28-5M mutant	38.5 ± 3.5	21 ± 2	40.5 ± 5.5
4		wt	6.5 ± 1.5	5.5 ± 1.5	88 ± 3
		cdc28-5M mutant	17 ± 2	7 ± 1	76 ± 3
5		wt	4.5 ± 1.5	3.5 ± 0.5	91 ± 2
		cdc28-5M mutant	18 ± 3	3.5 ± 0.5	78.5 ± 2.5
6		wt	8.5 ± 2.5	5 ± 1	86.5 ± 3.5
		cdc28-5M mutant	21 ± 2	4 ± 0	75 ± 2
4	1	wt	13.5 ± 2.5	4 ± 1	82.5 ± 3.5
		cdc28-5M mutant	41 ± 5	5.5 ± 1.5	53.5 ± 6.5
4	2	wt	15 ± 2	7 ± 1	78 ± 3
		cdc28-5M mutant	71.5 ± 3.5	3.5 ± 0.5	25 ± 3

[a] wt, wild type.

radiation with a more-or-less uniform G_2/M arrest which not only is morphologically similar to the arrest of *cdc13* or *cdc9* cells at the restrictive temperature but also is checkpoint dependent (41, 42, 44). The *cdc28-5M* mutant showed a normal G_2/M arrest at 23°C after UV irradiation, but it could not sustain the arrest after a shift to 37°C, indicating that the checkpoint defect was temperature sensitive. The fact that Cdc28 was required for maintaining the UV-induced arrest suggests that the involvement of Cdc28 in checkpoint controls is specific. The third criterion for *cdc28-5M* to be a checkpoint mutant is that it was genetically unstable at a semipermissive temperature, as it lost minichromosomes five times more frequently than wild-type cells. This type of trait, exhibited also by the *rad9* mutant (42), has been interpreted to be a consequence of checkpoint defects (11, 13, 14). It is worth noting that the genetic instability of the *cdc28-5M* mutant was even higher than that of the *rad9*Δ mutant, at least as judged by the minichromosome stability. Together, these results have firmly established that the *cdc28-5M* mutant was defective in the DNA damage-dependent checkpoint controls in budding yeast. Examination of other known alleles of *cdc28* further supports a role of Cdc28 in checkpoint controls, as they were also found to be at least partially defective in DNA damage-induced cell cycle arrest. It is obvious, however, that these mutations affected checkpoint functions to a lesser degree than *cdc28-5M*, since under comparable conditions they did not disrupt the G_2/M arrest completely as *cdc28-5M* did, were more resistant to UV killing than *cdc28-5M*, and allowed minichromosomes to be inherited with a fidelity close to that of the wild type.

One important difference between *cdc28-5M* and other known checkpoint-deficient CDK mutations such as *cdc2-3w* of *S. pombe* (6, 7) and $Cdk4^{F17}$ of rat (40) is that *cdc28-5M* was recessive and caused a loss of histone H1 kinase activity. The B-type cyclins are normally present at high levels in S phase and M phase (1, 17, 26, 34, 36, 37) and in HU-arrested cells (1, 36, 37). The fact that the *cdc28-5M* mutant was capable of S phase arrest in the presence of HU allowed us to compare the activities of various B cyclin-associated Cdc28 kinases. It was found that several Clb-associated Cdc28 kinase activities were crippled in the HU-arrested mutant. The Clb5- and Clb6-associated kinases were apparently most severely affected. The Clb6-associated kinase activity was even undetectable at the permissive temperature. Since the *cdc28-5M* mutant possessed a nearly normal checkpoint function at the permissive temperature, it is unlikely that its checkpoint defect is solely attributable to loss of Clb6-associated kinase activity. Consistent with this, the *clb6*Δ mutant (in an otherwise wild-type background) did not display a visible DNA damage checkpoint defect, nor did the *clb5*Δ mutant or the *clb5*Δ *clb6*Δ double mutant (data not shown). These results, however, do not suffice to rule out the possibility that the Clb5 and Clb6 cyclins may be principally important for checkpoint functions, as it is known that other B-type cyclins are able to substitute for Clb5 and Clb6 in their absence (34). Unless other B cyclin-associated kinases are also defective, the loss of *CLB5* and *CLB6* functions may not lead to severe defects. We speculate, therefore, that the Clb5- and Clb6-associated kinases may be important for checkpoint functions but that the severe checkpoint defect of *cdc28-5M* results from the overall impairment of several Clb-associated kinases. This speculation is in line with the observation that no one of the five mutations in *cdc28-5M* could produce temperature sensitivity or checkpoint defects.

Our finding that the loss-of-function mutation in *CDC28* resulted in a checkpoint defect is completely consistent with earlier reports that cells arrested by checkpoint controls still display high Cdc28 kinase activity (1, 36, 37). It is an interesting phenomenon that yeast cells arrested in G_2/M for various reasons, such as DNA damage, loss of microtubule functions, and certain *cdc* mutations, even including the mutation in the *CDC28* gene itself (*cdc28-1N* [38]), generally exhibit high Cdc28 mitotic kinase activity. The cell cycle arrest in the presence of microtubule toxins has been ascribed to checkpoint mechanisms distinct from the DNA damage-responsive ones. However, the data we have presented suggest that this arrest also depends on the activity of Cdc28, as the *cdc28-5M* mutant escaped from nocodazole-induced arrest upon a temperature shift to 37°C. The fact that Cdc28 was required for maintaining the arrest again suggests a specific role for Cdc28 in this type of checkpoint control. The third checkpoint control pathway, which governs the progression of S phase, remained fully functional in the *cdc28-5M* mutant, as it could still be arrested by HU as effectively as the wild type at the restrictive temperature. Since an S phase Cdc28 kinase, the Clb3-associated kinase, was not impaired by the *cdc28-5M* mutation (Fig. 7), the phenotype of the mutant may not warrant any conclusion on whether Cdc28 is required for the HU-induced cell cycle arrest.

The data presented in this report suggest that in budding yeast, cell cycle arrest in response to DNA damage and loss of microtubule functions is not accomplished by downregulation of the CDK activity. On the contrary, the CDK kinase is needed at least for the maintenance of these types of arrest. How Cdc28 participates in cell cycle arrest induced by damage is still open for speculation. Considering that Cdc28 (in complexes with different cyclins) is required for G_2/M arrest as well as the cell cycle events preceding mitosis, such as DNA replication, budding, and spindle pole body duplication and separation (26), it is conceivable that the stage of the arrest by mitotic checkpoint controls may also be a rate-limiting step in the normal cell cycle and may be under suppression by Cdc28 activities even in the absence of damage. This mitotic suppression, imaginably important for coordinating parallel cell cycle events as mentioned above, may be lifted only when these events are completed correctly. In this regard, it is noteworthy that the Clb5-Clb6 pair of cyclins may play an important role in cell cycle arrest after damage, as their activities are most severely impaired in the *cdc28-5M* mutant. The Clb5 and Clb6 cyclins are known to be required for initiation of DNA replication (33, 34). It is reasonable to speculate that they may not only initiate S phase but also inhibit nuclear division until the cell is ready to divide. A similar function has been postulated for the Cdc6 protein, which, like Clb5 and Clb6, is required for the initiation of DNA replication (2, 12). Interestingly, Cdc6 was also found to act as an inhibitor of nuclear division (2). The mutant defective in Cdc6 function exhibited a high rate of chromosome loss when grown at a semipermissive temperature (12), supporting the involvement of the Cdc6 protein in checkpoint controls.

In addition to *CDC28*, there are seven more genes (*RAD9*, *RAD17*, *RAD24*, *MEC1*, *MEC3*, *RAD53*, and *POL2*) that have been identified to be required for cell cycle arrest after DNA damage (23, 44). A pressing task at present is to find out whether these checkpoint genes are in any way linked to the cell cycle machinery, particularly the Cdc28 kinase. The data shown in this report have brought them one step closer. There are, however, still many questions to be answered before we have a coherent understanding of the checkpoint controls.

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