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Differential Requirements for DNA Replication in the Activation of Mitotic Checkpoints in *Saccharomyces cerevisiae*

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Checkpoints prevent inaccurate chromosome segregation by inhibiting cell division when errors in mitotic processes are encountered. We used a temperature-sensitive mutation, *dbf4*, to examine the requirement for DNA replication in establishing mitotic checkpoint arrest. We used γ-irradiation to induce DNA damage and hydroxyurea to limit deoxyribonucleotides in cells deprived of *DBF4* function to investigate the requirement for DNA replication in DNA-responsive checkpoints. In the absence of DNA replication, mitosis was not inhibited by these treatments, which normally activate the DNA damage and DNA replication checkpoints. Our results support a model that indicates that the assembly of replication structures is critical for cells to respond to defects in DNA metabolism. We show that activating the spindle checkpoint with nocodazole does not require prior progression through S phase but does require a stable kinetochore.

The fidelity of chromosome segregation is achieved by checkpoint controls that prevent cell cycle progression when damage is encountered or key processes are not completed. The feedback regulation is under the strict genetic control of checkpoint genes, and some are believed to play a pivotal role in preventing tumor progression. Checkpoint genes are defined empirically by mutations which relieve critical cell cycle dependency relationships. Two checkpoints respond to errors in DNA metabolism and prevent nuclear division when replication is inhibited or DNA is damaged (12, 37). A distinct checkpoint monitors the mitotic spindle. In metazoans, the spindle checkpoint ensures that chromosome segregation is inhibited if even a single chromosome is not attached to the spindle or is oriented toward only one pole (mono-oriented) (12, 49, 67). Similarly, yeast cells delay cell division in response to a variety of mitotic defects which likely alter proper orientation or successful attachment of chromosomes on the spindle. These perturbations include mutations in centromeric DNA, dicentric-chromosomes, multiple minichromosomes, altered kinetochore activity, and perturbations of spindle structure (12, 49, 67).

An interesting class of mutants identified in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* can enter anaphase in the absence of DNA replication (3, 11, 19, 26, 44, 50, 51, 57, 61). These mutants pose conceptual problems for the DNA synthesis and the spindle checkpoints because chromosome segregation is occurring even though DNA replication is not completed, and all (unreplicated) chromosomes must be mono-oriented. "Reductional anaphase" results from mutations in genes that encode components which are physically associated with the origin recognition complex or are part of the DNA replication or DNA repair complex (5, 10, 29, 35, 57).

The cyclical activity of the $p34^{cdc2/CDC28}$ cyclin-dependent kinase (CDK) during the cell cycle corresponds with its periodic association with cyclin subunits. An evolutionarily conserved multisubunit complex, the anaphase-promoting complex (APC; also called the cyclosome), is required to degrade B-type cyclins and additional proteins which regulate anaphase events (23, 27, 28, 43, 56, 62, 69, 70). Kinase inactivation is not required for cells to traverse the metaphase-to-anaphase transition but, rather, may be necessary for cells to exit mitosis (20, 58). The biochemical target(s) of the checkpoints is unknown, but recent evidence suggests that the checkpoints may prevent cell cycle progression by impinging upon the cell cycle-regulatory machinery (12, 27, 49, 67). In fission yeast, phosphorylation of essential inhibitory residues on $p34^{cdc2/CDC28}$ has been implicated as an effector of the DNA-responsive checkpoints (13, 30, 46). In higher eukaryotes, the requirement to phosphorylate these residues for the DNA replication and DNA damage checkpoints appears to be species specific (30). Budding yeast cells arrested by the DNA-responsive or spindle checkpoints have elevated levels of CDK activity (2, 54, 59). Inhibitory CDK phosphorylations are not critical for arrest of cells in response to DNA damage, suggesting an alternate target for the checkpoints in budding yeast (2, 54). Recently, an effector of the APC, *PDS1*, has been implicated in both the DNA-responsive and spindle checkpoint pathways (6, 68). The checkpoints could effectively prevent the anaphase events of cyclin destruction and sister chromatid separation at multiple levels by inactivating the APC directly or by modulating substrates targeted for destruction (7, 27).

An elegant model has been proposed to explain how cells evade the DNA replication checkpoint. This model posits that the signal generated to arrest cell division in response to incomplete DNA synthesis comes from proteins assembled at replication forks (31). Cells that do not initiate DNA replication have not assembled the protein complex required to detect or signal incomplete replication. This model explains how cells enter anaphase in the absence of DNA replication but cannot explain how cells in which all chromosomes are mono-oriented appear to elude the spindle checkpoint. Perhaps none of the mitotic checkpoints function in cells which have not initiated DNA replication.

We have used a variety of treatments and two different assays to determine if mitotic checkpoints could be induced in cells that did not initiate S phase. We used antitubulin immunofluorescence as a cytological marker and CDK activity as a biochemical signature for cells arrested by checkpoint controls. We show that cells have differential abilities to induce the DNA-responsive and spindle assembly checkpoints. Double-strand DNA breaks induced in G_1 arrest cells in G_2/M only if cells have initiated DNA synthesis. We infer that double-strand breaks induced in G_1 can persist into S phase, where the DNA

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TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Source
L1282D	*MAT***a** *ade5 trp1 ura3 dbf4-1*	L. Johnston
1612-6-4	*MAT***a** *ade5 his7 ura3 ctf13-30 dbf4-1*	This study
1626-7-3	*MAT***a** *ade3 his3 trp1 ura3 leu2 swi5::LEU2 dbf4-1*	This study
1294R (s30)	*MAT***a** *ade2 his3 leu2 lys2 ura3 trp1 ctf13-30*	P. Hieter
5943-4	*MATα his7 lys5 ura3 can1 cyh2 GAL*$^+$	Burke lab

replication checkpoint responds to the damage. Our results support the proposed model that the ability to detect errors in DNA metabolism requires the initiation of DNA replication in order to assemble the sensor or signaling structure. Activation of the spindle checkpoint with nocodazole can arrest cells in the absence of S phase. The response to nocodazole in the absence of DNA replication requires the essential kinetochore protein Ctf13p. We suggest that cells monitor kinetochore-microtubule occupancy as part of the spindle assembly checkpoint in *S. cerevisiae.*

MATERIALS AND METHODS

Yeast strains, growth, and media. The genotypes and sources of relevant yeast strains are shown in Table 1. Strains were constructed by standard genetic techniques with defined rich or synthetic minimal media (yeast extract-peptone-dextrose and synthetic complete) (17, 52). Cells were synchronized by growth to stationary phase by using a modification of a previously published procedure (58). In the standard experiment, synchronized cultures were obtained by growth in rich liquid medium (YM-1) supplemented with 2% raffinose (Sigma) for 2 days, until approximately 90% of the cells were unbudded. Cells were then inoculated into prewarmed YM-1 medium containing 2% glucose (Sigma). Cultures were incubated at 38°C (the restrictive temperature for *dbf4-1* and *ctf13-30* strains) and were shaken with vigorous aeration. Cells for irradiation experiments were grown to stationary phase as described above, concentrated by centrifugation, placed on ice, and exposed to 16 kilorads from a ^{137}Cs source. The effectiveness of irradiation was assayed by plating the haploid cells onto yeast extract-peptone-dextrose plates and measuring the viability, with direct observation of colony formation by microscopy as described previously (63). In every case, the plating efficiency was less than 10%. Hydroxyurea (HU) (Sigma) was used at a concentration of 100 mM. Nocodazole (Sigma) was used at 13.5 or 20 μg/ml in a final concentration of 1% dimethyl sulfoxide (DMSO). Benomyl (Dupont) was used at 30 μg/ml in a final concentration of 1% DMSO. We found variability in the effectiveness of nocodazole, especially at high temperatures as described previously (16, 24). A combination of nocodazole (20 μg/ml) and benomyl (30 μg/ml) (NOC-BEN) was used in a final concentration of 1% DMSO (16). For extended incubations in NOC-BEN, additional drug mixture was added every 2.5 h to ensure complete microtubule depolymerization. Effectiveness of antimicrotubule drug treatment was confirmed by indirect immunofluorescence as described below.

Indirect immunofluorescence and photomicroscopy. Antitubulin indirect immunofluorescence and DNA staining with 4′,6-diamidino-2-phenylindole (DAPI) were as described previously (1). Rat antitubulin monoclonal antibody (YOL1/34) and secondary (fluorescein isothiocyanate-conjugated) goat anti-rat antibody (Seralabs) were diluted 50-fold in phosphate-buffered saline containing 1% bovine serum albumin. Photomicroscopy and image processing were performed as described previously (63).

Histone H1 kinase assays. Cells were synchronized as described above. Approximately 1×10^8 to 2×10^8 cells were harvested every 30 min, and total CDK activity from protein extracts was measured by ability to phosphorylate histone H1 (Boehringer Mannheim) as described previously (59). Protein concentrations were determined by the Bradford method with bovine serum albumin as a standard (Bio-Rad). Equal amounts of total cell extract (2 μg) were used in kinase reactions. Equal loading of the gel was confirmed by Coomassie staining the gels prior to phosphorimager analysis. Phosphorylated histone H1 was detected by phosphorimager analysis (Molecular Dynamics). Relative histone H1 kinase levels were determined by measuring the pixel intensities corresponding to phosphorylated histone H1 by ImageQuaNT software (Molecular Dynamics). Lane-specific background was subtracted for each time point. The lane with the peak pixel intensity was chosen as the relative standard for intensity comparison. Each lane represents a consecutive time point in a 7-h time course. The first time point was when unbudded cells were diluted into fresh YM-1 medium containing 2% glucose at 38°C. There was some variability between experiments in the time required for cells to enter the cell cycle from stationary phase. Each experiment was done at least twice, and in every case we obtained reproducible profiles whose primary difference was the time at which kinase levels peaked. We attribute this variation to differences between cultures in the kinetics of reentering the cell cycle from stationary phase.

RESULTS

***dbf4* mutants segregate unreplicated chromatids.** We used *dbf4* mutants to examine the requirement for cells to proceed through S phase in order for mitotic checkpoints to be functional. The mutant has been extensively characterized and executes a reductional anaphase (61). Interaction between Dbf4p and the Cdc7p kinase is required to initiate DNA replication (8). Temperature-sensitive mutations in *CDC7* or *DBF4* render the cell incapable of initiating DNA synthesis, and cells segregate the unreplicated chromatids (61). The ability of *dbf4* mutants to undergo a reductional anaphase is dependent upon strain background (61). We were concerned about potential strain background differences. We confirmed that, similar to the strains characterized by Toyn et al. (61), all of our *dbf4* mutants underwent a reductional anaphase. We found that temperatures below 38°C were not fully restrictive for *dbf4* mutants. However, we could reproduce all of the previously described *dbf4* phenotypes by growing cells at 38°C (61). We analyzed spindle morphology by antitubulin immunofluorescence and showed that the elongated spindles in cells from strain 1626-7-3 (*dbf4*) were distinct from wild-type anaphase spindles (Fig. 1A). The microtubule staining was less intense at the center of the elongated spindle, giving rise to a spindle which appeared fractured compared to wild-type anaphase length spindles (Fig. 1A). Under these conditions, *dbf4* mutants did not replicate DNA as measured by flow cytometry (data not shown) (61). We determined CDK activity as a measurement of the ability to phosphorylate histone H1 in clarified cell extracts from synchronized cell populations. Histone H1 kinase levels rose and fell in cells from strain L1282D (Fig. 1B), indicating that destruction of the CDK activity was not inhibited in cells which had not initiated DNA replication. L1282D is a *dbf4* strain characterized by Toyn et al. (61). We confirmed that neither the DNA-responsive checkpoint nor

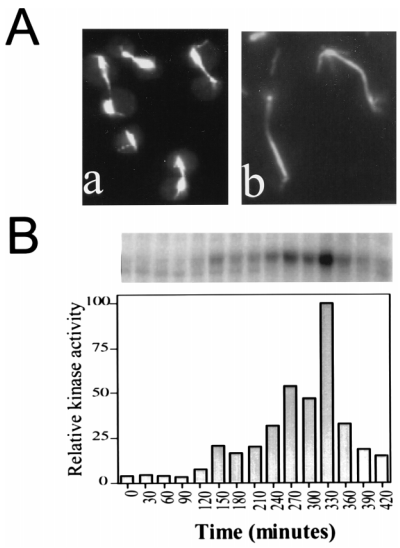

FIG. 1. Spindle morphology and CDK activity of *dbf4* mutants. (A) Cells were grown for 4 h at 38°C before being harvested for antitubulin immunofluorescence. (a) Synchronized cells from strain 1626-7-3 (*dbf4*). (b) Asynchronous cells from wild-type strain 5943-4. (B) Relative histone H1 kinase activity in synchronous cells from strain L1282D (*dbf4*) grown at 38°C. Cells were harvested every 30 min over 7 h. The first time point was when cells were diluted into fresh medium.

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TABLE 2. Cell morphology and spindle lengths in response to activated checkpoints

Mutation(s)	Treatment (4 h)	% of total cells		
		U[a]	MND[b]	LND[c]
dbf4	38°C	20.4	8.3	74.3
	HU at 38°C	28.4	24.4	47.2
	γ-irradiation at 23°C	2.9	91.3	5.8
	γ-irradiation at 38°C	12.2	19.5	68.3
ctf13	38°C	25.6	70.6	3.8
ctf13 dbf4	38°C	24.2	10.5	65.3

[a] U, unbudded with a single nucleus.
[b] MND, medial nuclear division phenotype (budded with an undivided nucleus and a spindle shorter than 1.5 μm).
[c] LND, late nuclear division phenotype (budded with a divided nucleus and a spindle longer than 1.5 μm).

the spindle assembly checkpoint was active, because CDK activity was not stabilized. We used this mutant and induced lesions to determine whether mitotic checkpoints could operate in the absence of S phase.

Double-strand DNA breaks induced in G_1 do not restrain mitosis in *dbf4* mutants. The arrest in response to DNA damage induced in G_1, during S, and in G_2 is under the control of the DNA damage checkpoint (12, 37). Haploid cells treated with a lethal dose of γ-irradiation arrest irreversibly as large-budded cells at G_2/M. To determine whether the DNA damage checkpoint was operational in the absence of DNA replication, we treated strain 1626-7-3 (*dbf4*) with 16 kilorads of γ-irradiation. Cells were grown to stationary phase and incubated on ice during radiation exposure. When cells were diluted into fresh medium and allowed to complete DNA replication by growth at the permissive temperature, 90% of the cells were inviable and arrested with a short spindle (Table 2; Fig. 2A). Cells that were unable to initiate DNA synthesis by growth at the restrictive temperature also resulted in 90% inviability. However, spindles elongated (Table 2; Fig. 2A) and appeared similar to those of the *dbf4* mutant (Fig. 1A). These data suggest that the DNA damage checkpoint cannot be induced in the absence of S phase. We measured CDK activity in strain L1282D (*dbf4*) treated with γ-irradiation as an independent measure of checkpoint activity. Histone H1 kinase levels peaked after 4 h at the restrictive temperature, decreased, and then increased again (Fig. 2B). The number of cells with large buds accumulated concomitantly with the increase in kinase activity. However, when kinase levels decreased, we did not observe a decrease in the number of budded cells (Fig. 2B and C). We also examined the spindle morphology at each time point and found that most cells had elongated spindles when kinase levels were lowest (data not shown). We presume that cells proceeded to the next cell cycle in the presence of double-strand DNA breaks in the absence of DNA replication. Therefore, CDK activity was not stabilized by γ-irradiation as is typical for wild-type cells, suggesting that the DNA damage checkpoint is not competent to respond to this type of lesion when cells fail to initiate DNA replication.

HU treatment does not restrain mitosis in *dbf4* mutants. Wild-type cells in which DNA replication is perturbed by treatment with the ribonucleotide reductase inhibitor, HU, arrest with elevated levels of CDK activity and short mitotic spindles (45, 54). If depleting cells of deoxyribonucleotides activates the DNA replication (S/M) checkpoint in the absence of S phase, then treatment with HU should restrain mitosis in *dbf4* mutants. Synchronized cells from strain L1282D (*dbf4*) were incubated in medium containing 100 mM HU at the restrictive temperature. CDK activity cycled, and spindle elongation was not delayed (Fig. 2B; Table 2). The percentage of large-budded cells increased as kinase activity accumulated, and it remained high, although kinase levels decreased and rose again (Fig. 2B). We observed a small increase in the number of unbudded cells during the later time points when kinase levels decreased, suggesting that some cells had completed cytokinesis (Fig. 2C). These data suggest that activation of the S/M checkpoint with HU requires the initiation of DNA replication.

The spindle checkpoint can be induced in the absence of DNA replication. Treatment of yeast cells with the microtubule-depolymerizing drugs nocodazole and benomyl arrests cells prior to anaphase with unseparated chromatids and high levels of CDK activity (15, 21, 32, 55). CDK activity is often used as a molecular signature for the spindle assembly checkpoint (21, 32, 64, 66). The spindle assembly checkpoint has been defined by mutations in two classes of genes, *MAD* (mitotic arrest deficient) and *BUB* (budding uninhibited by benzimidazoles), which are required for the arrest in response to treatment with an antimicrotubule drug (21, 32). We wanted to determine if the spindle assembly checkpoint could be induced

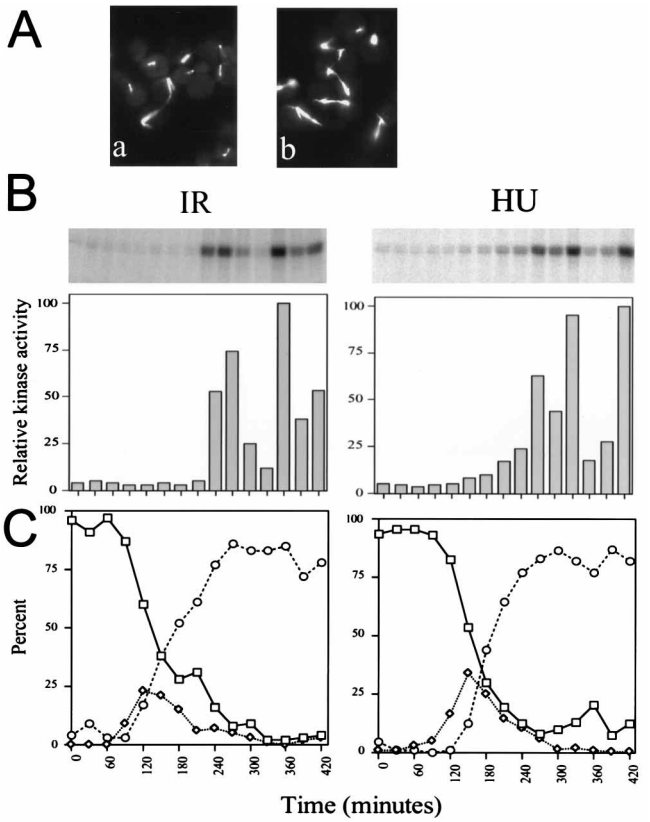

FIG. 2. CDK activity and budding morphology in *dbf4* mutants in response to activation of the DNA replication and DNA damage checkpoints. (A) Antitubulin immunofluorescence of *dbf4* mutant strain 1626-7-3 treated with γ-irradiation. Synchronized cells were grown for 4 h at 23°C (a) or 38°C (b) before being harvested for antitubulin immunofluorescence. (B) Relative histone H1 kinase activity in synchronous cells of strain L1282D (*dbf4-1*) treated with γ-irradiation (IR) or HU (100 mM). The first time point was when cells were diluted into fresh medium. Cells were harvested every 30 min. HU was added to the unbudded cells at 0 h. (C) Budding morphology of cells harvested for kinase activity (B). Squares, unbudded cells; diamonds, small-budded cells; circles, large-budded cells.

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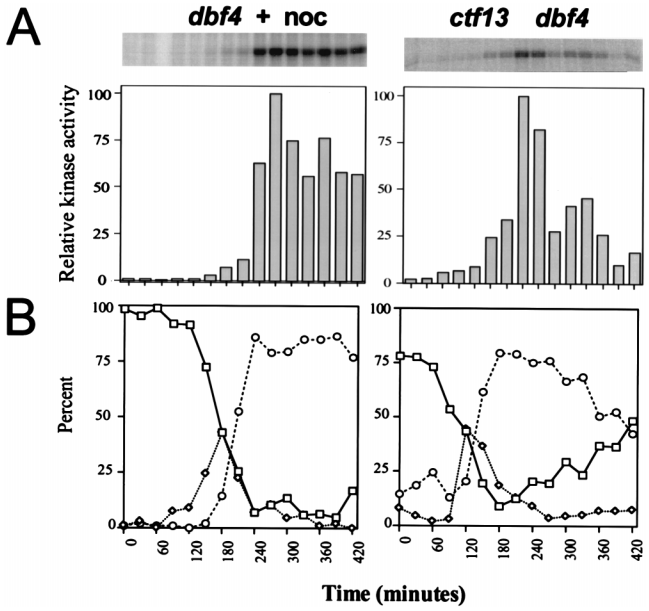

FIG. 3. CDK activity and budding morphology in response to activation of the spindle checkpoint. Synchronized cells were grown at 38°C, and cells were harvested every 30 min over 7 h. The first time point was when cells were diluted into fresh medium. (A) Relative histone H1 kinase activity for L1282D (*dbf4*) treated with 13.5 μg of nocodazole per ml and 1612-6-4 (*ctf13-30 dbf4-1*). The nocodazole was added to the unbudded cells at 0 h. (B) Budding morphology at time points corresponding to kinase activity in panel A. Squares, unbudded cells; diamonds, small-budded cells; circles, large-budded cells.

by nocodazole in the absence of S phase. Synchronized cells (strain L1282D *dbf4*) were incubated in medium containing 13.5 μg of nocodazole per ml. We did not assay mitotic restraint by antitubulin staining in nocodazole-treated cells, since microtubules were depolymerized. However, previous reports documented a variable effect of nocodazole treatment on microtubules at elevated temperatures (16, 24). We were concerned about this variability, so we examined microtubule structures by antitubulin staining and confirmed that the microtubules were depolymerized (data not shown). Treating *dbf4* mutants with nocodazole resulted in continuous high levels of CDK activity and an accumulation of cells with large buds, which corresponded to the increase in kinase activity (Fig. 3). We conclude that in contrast to γ-irradiation and HU, nocodazole induces a checkpoint in the absence of DNA replication.

Mutations in the *CTF13* gene, a component of the centromere binding complex CBF3, also activate the spindle checkpoint (41, 63). Temperature-sensitive *ctf13* mutants show defects in chromosome segregation and arrest at G_2/M with a short spindle (Table 2) (9). We used strain 1612-6-4 (*ctf13 dbf4*) to test whether the spindle checkpoint could be induced by impaired kinetochore function in the absence of S phase. We first determined that cells defective for *DBF4* function underwent a reductional anaphase in this strain by transforming a wild-type copy of *CTF13* on a CEN-based plasmid and analyzing the spindle morphology. We examined the spindle morphology of cells from this strain (*ctf13 dbf4 pCTF13 TRP1*) grown in minimal medium lacking tryptophan at the restrictive temperature for 4 h. These cells underwent a reductional anaphase similar to cells of strain L1282D (*dbf4*) (61) (data not shown). To determine if inactivating Ctf13p induced an arrest in the absence of DNA replication, we grew synchronized cells from strain 1612-6-4 (*ctf13 dbf4*) at the restrictive temperature to simultaneously inactivate Ctf13p and Dbf4p. Surprisingly, cells from strain 1612-6-4 were not mitotically restrained (Table 2), and CDK activity was not maintained (Fig. 3A). The accumulation of cells with large buds correlated with the rise in kinase activity (Fig. 3B). As kinase levels decreased, we observed an increase in the number of unbudded cells, suggesting that some cells had completed cytokinesis and had progressed to the next cell cycle. Therefore, there is a requirement for S phase for *ctf13* mutant kinetochores to induce the spindle checkpoint.

The spindle checkpoint requires *CTF13* function. The arrest in response to nocodazole and impaired kinetochore function in a *ctf13* mutant requires the function of the same set of spindle checkpoint genes (21, 32, 41, 63). We were surprised that these two methods of inducing the spindle checkpoint showed different requirements for S phase. We considered two models to explain this differential requirement. One model is that nocodazole and *ctf13* produce different lesions that each induce independent mitotic checkpoints. Ctf13p is a kinetochore component, and the mutant has altered microtubule binding activity in vitro (9, 53). If there are two independent checkpoints, signaling in response to the impaired binding of kinetochores to microtubules (*ctf13*) may require S phase but signaling in response to depolymerized microtubules (nocodazole) may not. Perhaps the gross changes in spindle architecture in cells treated with microtubule-depolymerizing drugs induce a distinct mitotic checkpoint. The differential requirement for S phase may reflect the complex action of nocodazole treatment. The second possibility is that *ctf13* and nocodazole-treated cells arrest for the same reason. The nocodazole effect in the absence of DNA replication may be mediated exclusively through the kinetochore. Sorger et al. (53) have proposed that an assembled kinetochore complex may be essential for the spindle checkpoint to sense defects in the attachment of chromosomes to the spindle (22, 53). These researchers have shown that *ctf13* mutants retain some residual centromere-DNA binding activity and postulate that this may be sufficient to assemble a few kinetochores which are capable of signaling to arrest mitosis. Perhaps the aberrant kinetochore that assembles in a *ctf13* mutant in the absence of DNA replication is incapable of activating the spindle checkpoint.

If nocodazole's effect on the cell cycle was due to a complex action of the drug or to a distinct spindle checkpoint, then nocodazole should arrest a *ctf13* mutant in the absence of DNA replication. However, if the arrest in response to nocodazole in the absence of DNA replication requires Ctf13p, nocodazole should be unable to mitotically restrain a *ctf13 dbf4* mutant. We measured CDK activity in synchronized cells from strain 1612-6-4 (*ctf13 dbf4*) treated with 13.5 μg of nocodazole per ml. CDK activity was not maintained at mitotic levels (Fig. 4A). The percentage of large-budded cells peaked when kinase levels were highest (Fig. 4B). There was an increase in the number of unbudded cells during late time points, suggesting that some of these cells executed cytokinesis. We examined microtubules by antitubulin immunofluorescence and noticed faint microtubule staining reappearing at the restrictive temperature (data not shown). We found that NOC-BEN (16) was effective for complete depolymerization of microtubules in strain 1612-6-4 at elevated temperatures. Strain 1612-6-4 entered the cell cycle with better synchrony if cells were allowed to initiate budding prior to the addition of NOC-BEN, which was added directly to the culture at 38°C when more than 50% of the cells were budded. The first time point was 30 min prior to drug addition. We found that additional treatment with the drug mixture at later time points resulted in sustained microtubule depolymerization. CDK activity was not maintained by

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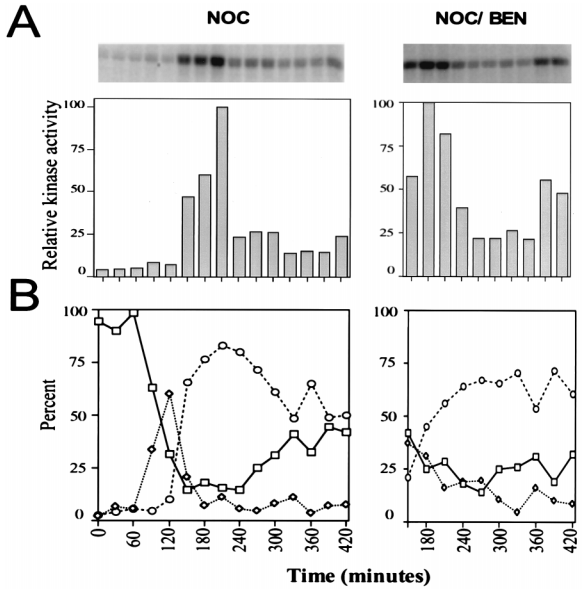

FIG. 4. CDK activity and budding morphology in cells from strain 1612-6-4 (*ctf13-30 dbf4-1*) treated with 13.5 μg of nocodazole per ml or with NOC-BEN. The nocodazole was added to the unbudded cells at 0 h. The NOC-BEN mixture was added when the majority of cells had started to bud (t = 180 min). The first time point was 30 min prior to drug addition. Additional NOC-BEN was added to the culture at 5.5 h to ensure the complete depolymerization of microtubules throughout the experiment. Depolymerization of microtubules was monitored by antitubulin immunofluorescence at 1-h intervals after the addition of NOC-BEN. (A) Relative histone H1 kinase activity. (B) Budding morphology corresponding to kinase activity in panel A. Squares, unbudded cells; diamonds, small-budded cells; circles, large-budded cells.

the NOC-BEN treatment (Fig. 4A). The majority of cells were large budded throughout the experiment, although there was a small increase in the number of unbudded cells at later time points when kinase levels again increased (Fig. 4B). These data suggest that nocodazole requires Ctf13p to induce the spindle checkpoint in the absence of S phase.

DISCUSSION

We used haploid *dbf4* mutants to determine whether mitotic checkpoints function in mutants which enter anaphase with unreplicated DNA and mono-oriented chromosomes. We monitored spindle morphology and CDK activity to determine if we could induce mitotic checkpoints in the absence of DNA replication. Treatments that normally activate the DNA-responsive checkpoints do not restrain mitosis in the absence of DNA synthesis. γ-Irradiation in G_1 and HU treatment were unable to prevent mitosis in *dbf4* mutants. Activation of the spindle checkpoint with nocodazole treatment could stabilize CDK activity in the absence of DNA replication, but this arrest required Ctf13p function.

One possible explanation for the aberrant division in *dbf4* mutants is that Dbf4p is an integral component of the S/M checkpoint. However, Toyn et al. (61) showed that if *dbf4* cells are allowed to initiate DNA replication at the permissive temperature in the presence of HU, followed by treatment with HU at the restrictive temperature, then cells do not undergo an aberrant division. These results demonstrate that cells which have initiated DNA replication can activate the S/M checkpoint in the absence of Dbf4p. A model to explain how cells which undergo a reductional anaphase appear to elude the S/M checkpoint proposes that the assembly of replication structures is required in order to detect or to signal in response to errors in DNA replication (31). In support of this model, previous data show that treatment with HU to activate the S/M checkpoint does not prevent the segregation of unreplicated chromatids in cells which fail to initiate DNA replication (3, 11, 50, 57). In each case, mitotic restraint was assayed cytologically and therefore may not provide an accurate assessment of the function of the DNA replication checkpoint. A formal possibility is that the checkpoints were activated but cells could not respond in the absence of a sister chromatid. We have extended these studies by using CDK activity as a biochemical marker and a molecular signature for checkpoint activation to examine the requirement for cells to initiate DNA replication for the function of mitotic checkpoints. We infer that the DNA replication checkpoint is not fully activated by treatment with HU, because CDK activity was not stabilized in the absence of DNA replication. Therefore, the DNA replication checkpoint does not respond to changes in the deoxyribonucleotide pools induced by HU treatment in the absence of DNA replication.

Induced DNA damage in haploid cells in G_1 did not mitotically restrain *dbf4* mutants. CDK activity provided us with a biochemical marker to address the nature of the G_2/M arrest in response to DNA damage in the absence of S phase. Haploid cells irradiated in G_1 arrest irreversibly at G_2/M because they lack a sister chromatid to provide the homology necessary to repair the lesion (18). Our data show that double-strand DNA breaks induced with ionizing radiation in G_1 arrest cells in G_2/M only if the cells have previously initiated DNA synthesis. However, activation of the DNA damage checkpoint with a different lesion is capable of arresting cell division in *dbf4* mutants. Toyn et al. (61) showed that DNA damage induced by treatment with the UV mimetic 4-nitroquinoline oxide (NQO) prevents aberrant chromatin division in a *dbf4* mutant. Cell cycle arrest in response to DNA damage requires processing of the DNA damage lesion to intermediates, which are then competent to signal an arrest (36). Multiple types of DNA damage lesions are able to activate the DNA damage checkpoint (37). The differential effects of NQO and γ-irradiation may be resolved if the ability to detect or process the dimer lesions induced by NQO treatment does not require initiation of DNA replication.

Our results suggest that the activation of the DNA damage checkpoint that arrests cells in G_2/M is dependent on the assembly of replication complexes. We propose that double-strand DNA breaks induced by ionizing radiation in haploid G_1 cells persist into S phase, where they are recognized by the DNA damage checkpoint. Cells arrested after DNA replication by treatment with benomyl are capable of activating the DNA damage checkpoint in response to X-irradiation or UV DNA damage (39, 42, 65). One model to explain the requirement for cells to initiate DNA replication for the DNA-responsive checkpoints to function is that assembly of complexes implicated in detecting or processing damage takes place at origins of replication or in association with replication forks. Perhaps the aspect of replication structures required for the DNA damage checkpoint is still assembled in benomyl-treated cells. In the absence of DNA replication, these complexes are precluded from assembling or associating with newly replicated DNA and hence, cells cannot respond to DNA damage. The checkpoint arrests in response to limited deoxyribonucleotide pools and γ-irradiation are genetically separable but overlap components in the signaling pathway (37). Our results demonstrate that the ability to signal in response to both these perturbations in DNA metabolism requires cells to initiate DNA replication. We suggest that assembly of replication complexes

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is required for cells to process damage in the form of double-strand DNA breaks and stalled replication forks.

Micromanipulation and cytological observations of higher eukaryotic cells have provided insight into the lesion which is monitored by the spindle checkpoint. Cytological observations made with living mitotic cells suggest that a single unattached kinetochore is sufficient to activate the spindle checkpoint (48). Disrupting kinetochore structure by microinjection of antibodies to centromere protein C (CENP-C) prolongs metaphase in mammalian cells (60). Elegant micromanipulation studies in insect spermatocytes suggest that lack of tension at an unattached kinetochore is the event which is sensed and arrests cell division (33). The 3F3 antibody recognizes a phosphoepitope present on the kinetochores of unattached chromosomes (14). Tension can alter the phosphorylation state of proteins recognized by the 3F3 antibody (40). This has led to the model that cells monitor bipolar attachment of chromosomes to the mitotic spindle by preventing the onset of anaphase when kinetochores do not experience opposing tension. It has been proposed that tension-sensitive chromosomal proteins between sister chromatids provide the mechanism to detect alterations in the balance of forces (38). Kinetochores which are not experiencing tension activate the spindle checkpoint, and an inhibitory signal is sent to prevent cell division. However, this mechanism may not activate the spindle checkpoint universally. Tension has been proposed to play the opposite role in *Drosophila* oogenesis (25). Laser ablation studies with mammalian mitotic cells suggest that it is the lack of kinetochore-microtubule binding which initiates signaling, although the possibility of a role for tension has not been excluded (47). Although there is a clear association between kinetochore function and the spindle checkpoint in budding yeast, none of the experiments addressed the role of tension in activating the spindle checkpoint (12, 67).

Unreplicated chromatids are mono-oriented and therefore are not under tension. If lack of tension activated the spindle checkpoint in yeast, then *dbf4* mutants which do not initiate DNA replication should be arrested in mitosis. Piatti et al. (44) proposed a model to explain how *cdc6* mutants enter anaphase in the absence of DNA synthesis. These researchers assumed that cells can be physically restrained at metaphase by mitotic checkpoints only when there are cohesive proteins that bind sister chromatids. The strongest evidence is that reductional anaphase in *cdc6* mutants cannot be prevented by a mutation in *CDC23*, which encodes a component of the APC, suggesting that unreplicated chromatids are incapable of preventing spindle elongation (23). Even if the spindle checkpoint was activated due to the lack of tension in cells with unreplicated chromatids, spindle elongation would be impossible to constrain. If the yeast spindle checkpoint monitors tension at the kinetochore of mono-oriented chromatids, we predict that the activated checkpoint will result in stable levels of CDK activity. We and others have demonstrated that CDK activities are unstable in *dbf4* mutants (Fig. 1B) (61). One possibility is that the spindle checkpoint is not activated by a lack of tension and that yeast cells possess a different mechanism to monitor bipolar orientation of chromosomes prior to anaphase. Alternatively, tension may play a role but yeast cells may not be able to detect the lack of tension on an unreplicated chromatid. Unreplicated chromatids may not be able to restrain mitosis because the structure of the tension-sensing complex is not assembled in the absence of a sister chromatid.

Unlike the DNA-responsive checkpoints, the spindle checkpoint could be induced in the absence of S phase. Our results are consistent with previous data indicating that nocodazole can suppress the reductional anaphase and inviability of *dbf4* mutants (61). These data suggest that the spindle checkpoint is functional in the absence of DNA replication but that the lesions are not present to induce the checkpoint in *dbf4* mutants. In a *dbf4* mutant, all kinetochores may be attached to microtubules, and this may be sufficient to allow progression through the cell cycle. If tension plays a role in the spindle checkpoint, it does not enforce a cell cycle arrest in the absence of DNA replication. However, perturbation of kinetochore-microtubule attachment is sufficient to inhibit cell cycle progression in the absence of DNA replication. Therefore, yeast cells may possess multiple sensing mechanisms to ensure proper alignment of chromosomes on the spindle. Perhaps only the kinetochore attachment-sensing checkpoint is operational in the absence of a sister chromatid.

We propose that a component of the yeast spindle checkpoint monitors the attachment of kinetochores to microtubules. Therefore, we were initially surprised that *ctf13* was unable to activate the spindle checkpoint in the absence of DNA synthesis. The same checkpoint genes are required to arrest cells in response to nocodazole treatment and impaired kinetochore function in *ctf13* mutants (41, 63). We demonstrated that the ability to stabilize CDK activity by nocodazole treatment required Ctf13p in cells which had not initiated DNA replication. We conclude that in the absence of DNA replication, the nocodazole checkpoint functions through the kinetochore. These data suggest that the checkpoint which arrests cells impaired for kinetochore function and the checkpoint which is activated by nocodazole are not independent.

Proper assembly of some aspect of kinetochore structure may be required to detect or signal errors in chromosome attachment (22, 53, 67). We suggest that in *dbf4* mutants, the spindle checkpoint is not activated, because the kinetochores of unreplicated chromatids are competent to bind to microtubules. However, perturbing microtubule attachments in a *ctf13* mutant could not stabilize CDK activity in the absence of DNA replication. One possibility is that the kinetochore which is assembled in a *ctf13* mutant in the absence of S phase is unstable. In the absence of a functional kinetochore complex, cells may be incapable of detecting or signaling errors in attachment, even when all chromosomes are not attached to the spindle. Recently, the *Xenopus* and human homologs of the *MAD2* checkpoint were shown to localize to the kinetochore in cells where the spindle checkpoint has been activated by treatment with microtubule-depolymerizing drugs (4, 34). The ability to assemble a kinetochore competent to signal may reflect the proper localization of checkpoint components. Ctf13p does not appear to be essential for the spindle checkpoint to arrest nocodazole-treated cells which have completed DNA replication (59a). We suggest that the Ctf13p requirement to elicit a nocodazole-mediated arrest in the absence of DNA replication may simply reflect a kinetochore stability or assembly defect rather than implicating Ctf13p in the generation of the checkpoint signal.

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