Abnormal Kinetochore Structure Activates the Spindle Assembly Checkpoint in Budding Yeast

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Submitted December 16, 1995; Accepted May 29, 1996
Monitoring Editor: David Botstein

Saccharomyces cerevisiae cells containing one or more abnormal kinetochores delay anaphase entry. The delay can be produced by using centromere DNA mutations present in single-copy or kinetochore protein mutations. This observation is strikingly similar to the preanaphase delay or arrest exhibited in animal cells that experience spontaneous or induced failures in bipolar attachment of one or more chromosomes and may reveal the existence of a conserved surveillance pathway that monitors the state of chromosome attachment to the spindle before anaphase. We find that three genes (MAD2, BUB1, and BUB2) that are required for the spindle assembly checkpoint in budding yeast (defined by antimicrotubule drug-induced arrest or delay) are also required in the establishment and/or maintenance of kinetochore-induced delays. This was tested in strains in which the delays were generated by limited function of a mutant kinetochore protein (ctf13-30) or by the presence of a single-copy centromere DNA mutation (CDEII Δ 31). Whereas the MAD2 and BUB1 genes were absolutely required for delay, loss of BUB2 function resulted in a partial delay defect, and we suggest that BUB2 is required for delay maintenance. The inability of *mad2-1* and *bub1* Δ mutants to execute kinetochore-induced delay is correlated with striking increases in chromosome missegregation, indicating that the delay does indeed have a role in chromosome transmission fidelity. Our results also indicated that the yeast RAD9 gene, necessary for DNA damage-induced arrest, had no role in the kinetochore-induced delays. We conclude that abnormal kinetochore structures induce preanaphase delay by activating the same functions that have defined the spindle assembly checkpoint in budding yeast.

INTRODUCTION

The correct temporal order of execution of critical events in the cell cycle is controlled by molecular mechanisms that act at checkpoints (Hartwell and Weinert, 1989; Murray and Kirschner, 1989; Nurse, 1990). Experimental evidence supports the activity of checkpoint controls at the G1/S phase boundary to control proper timing of genome replication and at the G2/M phase boundary to control appropriate timing of cell division processes as conserved functions found in widely divergent organisms (Coleman and Dunphy, 1994; Carr and Hoekstra, 1995). Current models of G2/M checkpoint mechanisms describe controls

responding to two distinct molecular structures monitored by surveillance. One is the chromosomal DNA, because chromosomal damage and incomplete replication serve as inducers of checkpoint-mediated delay or arrest. The other is the mitotic spindle, observed in the effects of agents that interfere with normal microtubule dynamics or stable attachment of chromosomes to the spindle microtubules.

There is strong evidence for a spindle assembly checkpoint in the budding yeast *Saccharomyces cerevisiae*. Tubulin mutants arrest before anaphase of mitosis, with a single large bud and an undivided but duplicated genome (Huffaker *et al.*, 1988; Schatz *et al.*, 1988). Similarly, benzimidazole drugs, which inhibit the formation of microtubules in fungi by binding to unpolymerized tubulin subunits, cause cells to arrest

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before anaphase. (Because prophase and metaphase are not morphologically distinct stages in budding yeast, the precise premitotic or mitotic stage of arrest is not apparent.) Recently, two groups have identified mutant yeast strains that bypass a normally occurring arrest or, at lower concentrations, a delay in the presence of benzimidazole drugs. The *bub* (budding uninhibited by benzimidazole) mutants (Hoyt *et al.*, 1991) were isolated for their inability to recover from brief exposure to high concentrations of drug and the appearance of unscheduled bud formation in the absence of nuclear division. The *mad* (mitotic arrest-defective) mutants (Li and Murray, 1991) were identified by decreased viability in the presence of low concentrations of drug and an inability to slow cell division time under these conditions. In total, there are six genetic loci identified by these mutants (BUB1-3 and MAD1-3).

Recent studies of the molecular genetics of these loci have provided evidence consistent with a surveillance pathway leading to spindle assembly checkpoint activation. Interestingly, the BUB1 gene encodes a kinase activity (Roberts *et al.*, 1994). The BUB3 gene was isolated as a low-copy suppressor of *bub1-1*, and BUB3 protein associates with BUB1 in vitro (Roberts *et al.*, 1994). BUB3 thus may encode either a regulatory subunit or a target of the BUB1 kinase. MAD1 protein exists as a phosphoprotein in vivo and exhibits a higher molecular weight form that depends on the activity of the BUB1 kinase (Hardwick and Murray, 1995). These observations are consistent with the idea that microtubule defects lead to the activation of a kinase (BUB1) or a kinase cascade that includes the MAD1 protein among its targets. These properties are consistent with roles in a signaling pathway.

In many distantly related eukaryotes, abnormal kinetochore configuration, like microtubule disruption, can induce preanaphase arrest or delay. Kinetochore-induced arrests or delays have been reported in several systems after experimental interference, including injection of anticentromere antibodies into human (Bernat *et al.*, 1990; Tomkiel *et al.*, 1994; Campbell and Gorbisky, 1995) and mouse (Simerly *et al.*, 1990) cultured cells, breakage of spindle fibers with a UV microbeam (Zirkle, 1970), and breakage of spindle fibers with a glass needle (Nicklas, 1967). An anaphase delay associated with the spontaneous occurrence of single, unattached kinetochores has also been observed in cultured cells that are otherwise unperturbed (Rieder *et al.*, 1994). Recent micromanipulation experiments have demonstrated that tension of the kinetochore fibers is important for progression into anaphase (Li and Nicklas, 1995; Nicklas *et al.*, 1995).

Investigation of the structure and function of the budding yeast kinetochore (centromere DNA and associated proteins) has generated materials that facilitate the manipulation of these structures in vivo with

the use of molecular genetic approaches (reviewed in Hegemann and Fleig, 1993). We have previously observed a preanaphase mitotic delay induced by mutant centromere DNAs at single copy in budding yeast (Spencer and Hieter, 1992). The cell cycle alteration is observed as advanced bud growth in cells with a G2 DNA content and a single nucleus at the mother/bud neck. This cellular morphology is similar to the arrest morphology of wild-type cells in benzimidazole drugs or the arrest morphology of G2/M *cdc* mutants. In all of these instances, the increase in bud volume is indicative of the passage of additional time before entry into anaphase. We and others have reported a morphologically similar delay in temperature-sensitive kinetochore protein mutants (Doheny *et al.*, 1993; Goh and Kilmartin, 1993; Strunnikov *et al.*, 1995) under nonpermissive or semipermissive growth conditions.

The similarity between the mitotic delay in yeast because of the presence of one defective kinetochore and a cell cycle delay in animal cells with a single, unattached chromosome is striking and provides strong evidence for conservation of a kinetochore-induced mitotic delay. We and others have proposed that this phenomenon indicates the action of "kinetochore surveillance" at a conserved checkpoint acting after bulk DNA synthesis and before anaphase (McIntosh, 1991; Spencer and Hieter, 1992; Rieder *et al.*, 1994; Nicklas *et al.*, 1995). In this paper, we find that yeast genes defective in the spindle assembly checkpoint, as defined by microtubule disruption, are required for kinetochore-induced delays. These data provide additional support for the hypothesis that kinetochore surveillance exists in *Saccharomyces* and that this surveillance pathway is an important component of the benzimidazole-induced arrest or delay at the spindle assembly checkpoint.

MATERIALS AND METHODS

Yeast Strains

Media. All yeast media are as described in Rose *et al.* (1990), with the exception of YPD-rich. This medium is YPD containing an additional 60 $\mu\text{g/ml}$ uracil, 90 $\mu\text{g/ml}$ adenine, and 90 $\mu\text{g/ml}$ L-tryptophan supplementation. Addition of these extra supplements to YPD prevents these from becoming limiting for growth and prevents the appearance of interstrain variability caused by auxotrophic markers in growth assays.

For the analysis of red-sectoring phenotypes, each strain was plated at a density of ~200 colonies per plate on minimal (SD) medium including (in $\mu\text{g/ml}$): 20 uracil, 40 L-lysine, 6 adenine sulfate, 20 L-histidine, 30 L-tryptophan, and 220 L-leucine. The limiting adenine supplementation was used to facilitate red pigment development in *ade2-101* cells.

Strain Backgrounds. Experimental and control strain sets within any single experiment were congenic (with one exception described below). Strain sets were constructed by DNA-mediated transformation, inbreeding, or single chromosome transfer by *Kar1⁻* matings. The *mad2-1*, *bub1 Δ* , and *bub2 Δ* experiments were all conducted in an S288c background currently in use in our laboratory (established by M. Carlson). The *bub2-1*-containing strains and their controls

were congenic in a closely related but distinct S288c-derived background (kindly provided by A. Hoyt). The *rad9Δ::HIS3* strains used for the single-delaying kinetochore assay were conducted in a congenic strain set in a W303 laboratory strain background (kindly provided by T. Weinert). We note that the published increase in chromosome loss frequency engendered by *rad9Δ::HIS3* (Weinert and Hartwell, 1993) is not visible with our test chromosome (Figure 5), as would be expected for an induced loss rate of 2×10^{-4} loss events per cell division. All *His⁺* spores from crosses carried out in the W303 background were radiation sensitive, as were the original parental strains.

The *rad9Δ::HIS3* *ctf13-30* strains were analyzed in products from an S288c $\times$ W303 hybrid cross. As there was no effect of the *rad9Δ::HIS3* mutation on kinetochore-induced delay (tested in two tetrad type tetrads) and no phenotypic anomalies were noted in six four-spored tetrads, no further studies were conducted.

MAD2. Congenic strains for the experiment were constructed by first introducing a cloned mutant allele into our laboratory strain background. A 5.5-kb *XhoI* fragment from pRL13 (*mad2-1* gift of R. Li and A. Murray) was cloned into *XhoI* site of pRS306 (Sikorski and Hieter, 1989) to generate pVM1. *mad2-1* yeast strains were obtained by two-step gene replacement methods (Rothstein, 1991) with this plasmid. The structure of intermediate and final products was verified by Southern blot analysis. The final *mad2-1* strain (YFS430) was Benomyl sensitive (10 μ g/ml) and exhibited rapid division kinetics as described in Li and Murray (1991).

The *ts* kinetochore mutant *ctf13-30* was introduced into *mad2-1* strains by standard genetic manipulations, which provided tetrad type tetrads for study. The strains characterized were YPH278, YFS451, YFS449, and s30. The chromosome fragments containing CDEIIΔ31 and wild-type CEN6 were introduced into *mad2-1* by chromosome transfer from donor strains in a *Kar1⁻* mating by methods essentially as previously described (Hugerat *et al.*, 1994; Spencer *et al.*, 1994). Analysis of the electrophoretic karyotype of chromoductants verified the presence of the marker chromosome and single chromosome transfer. Strains studied were YFS541 and YFS744. Strains YFS470 and YPH406 served as controls.

BUB1. A LEU2-marked null allele was constructed and introduced into yeast by one-step gene replacement methods (Rothstein, 1991). A 7-kb BUB1 encoding *Sall* to *SacI* genomic DNA fragment from pTR37 (kindly provided by T. Roberts and A. Hoyt) was cloned into pBluescript with the polylinker *Sall* and *SacI* sites to create pJP4. A 948 nucleotide *SmaI* to *XbaI* fragment was removed from the BUB1 gene and replaced with a 2.2-kb LEU2 fragment from pJJ283 (Jones and Prakash, 1990), generating pJP7. *Leu⁺* yeast were assayed for the presence of the deletion construct at the correct genomic locus by Southern blot analysis. *Bub⁻* phenotype of the transformants was verified by nocodazole treatment of liquid synchronous cultures as described in Hoyt *et al.* (1991). The strains studied were YPH278, s30, YFP2, and YFP3.

Chromosome fragments containing CDEIIΔ31 and wild-type CEN6 were introduced into a YFP2 derivative strain (*bub1Δ::LEU2 can^R*) by chromosome transfer in *Kar1⁻* mating. Strains studied were YFP17 and YFP53. Strains YFS470 and YPH406 served as controls.

RAD9. *ctf13-30* was introduced into the *rad9Δ::HIS3* background by crossing s30 (S288c) and YFS22 (W303 background). YFS22 is derived from a strain kindly provided by T. Weinert and differs by generation of a chromosome fragment. After sporulation and standard tetrad dissection, two tetrad type tetrads were independently characterized for all phenotypes. The original *rad9Δ::HIS3* strains and all derivatives were UV sensitive (120 J/m²). Radiation sensitivity segregated 2 resistant/2 sensitive spores from the S288c $\times$ W303 outcross. In this cross, inviability at 37°C (*ctf13-30*) segregated independently, and also clearly exhibited the expected 2 viable/2 inviable pattern. Data are shown from YFS719, YFS720, YFS721, and YFS722.

Chromosome fragments containing CDEIIΔ31 or wild-type CEN6 were introduced into congenic (W303) RAD9 and *rad9Δ::HIS3*

strains by DNA-mediated transformation with plasmids p466 (CEN6 wt) and p483 (CEN6 CDEIIΔ31), designed to yield newly formed marker chromosome fragments upon URA3 selection (Shero *et al.*, 1991). The electrophoretic karyotypes of the resulting strains were verified by pulsed-field gel and Southern blot analysis. Diploid derivatives (all of W303 background) were analyzed: YFS342, YFS344, YFS335, and YFS337.

BUB2. Chromosome fragments containing CDEIIΔ31 or wild-type CEN6 were created within congenic BUB2 and *bub2-1* strains by using DNA-mediated transformation with plasmids p466 (CEN6 wt) and p483 (CEN6 CDEIIΔ31) as described above for construction of the RAD9 congenic series. The electrophoretic karyotype of transformants was checked by pulsed-field gel analysis to verify presence of the intended chromosome fragments, and diploids were created by using standard genetic crosses among strains from the S288c background of the Bub mutagenesis study (Hoyt *et al.*, 1991). The strains analyzed were YFS786, YFS787, YFS349.1, and YFS350.1.

To test the function of *bub2* mutants in our own laboratory strain background, a LEU2-marked null allele (kindly provided on plasmid pJB5 by T. Roberts and A. Hoyt) was introduced into YPH277 by one-step gene replacement. The deletion allele removes 202 nucleotides of the BUB2 open-reading frame (from genomic *Bam*HI to *Hind*III sites), replacing them with a 2.2-kb LEU2 fragment. The *Bub* phenotype of *Leu⁺* transformants was verified by nocodazole treatment of α factor-synchronized cultures as described in Hoyt *et al.*, 1991. The transformant chosen for further study (YJP2) was crossed to s30, sporulated, and dissected to generate tetrad type tetrads containing *bub2Δ::LEU2* *ctf13-30* spores (YFP46, YFP47, YFP48, and YFP49). A standard genetic cross introduced the CDEIIΔ31 into the *bub2Δ::LEU2* mutant. Diploids (YFP24 and YFP25) were created by transformation with the yeast HO gene under the control of its own promoter on a 2- μ shuttle vector (pBF174; our unpublished observations). Diploidy was verified by spore formation and by flow cytometry.

Temperature Shift Protocol for the Analysis of *ctf13-30* Double Mutants

Temperature shift experiments were repeated between two and five times for each congenic strain set, and representative data are shown. For each strain to be characterized, asynchronous logarithmically growing cultures maintained in YPD at 25°C were split, and one-half was shifted to 34°C. Aliquots taken at defined intervals after the shift were processed in parallel for flow cytometry, DNA and bud morphology, and cell viability. Processing for flow cytometry was performed as previously described (Hutter and Eipel, 1978). Samples to be stained with 4,6-diamidino-2-phenylindole (DAPI) at a final concentration of 300 ng/ml were first subjected to brief sonication, fixed in 3.7% formaldehyde for ≥ 2 h, and washed once in 1 M Sorbitol containing 50 mM potassium phosphate, pH 7.5, before adding dye. Uninucleate, very large budded cells were scored as those with one roughly spherical DAPI staining mass at the neck and with two near-equal cell bodies (the smaller with a diameter $\geq 75\%$ of the larger). Cell viability was scored in a microcolony formation assay on return to permissive temperature. In this assay, aliquots from the temperature shift experiment were subjected to brief sonication, plated on rich (YPD) medium at a density of 10^3 cells/cm², and cultured for a further 24 h at 25°C. Inviability cells were scored as those that failed to produce ≥ 32 cell bodies at this time, when most founder cells had produced >200 . Most inviable cells produced fewer than eight cell bodies.

RESULTS

Two strategies were used to test the role of mutants defective in G2/M checkpoint functions in kinetochore-induced delays. The first strategy takes advantage of a temperature-sensitive allele of the gene en-

coding a kinetochore protein in budding yeast, CTF13 (Doherty *et al.*, 1993). Limitation of *ctf13-30* temperature-sensitive function, imposed by culturing cells at semipermissive or nonpermissive temperatures, produces a pronounced delay before anaphase. This delay was evaluated in the presence of the G2/M checkpoint defect in three different spindle assembly checkpoint mutants—*mad2-1*, *bub1Δ::LEU2*, and *bub2Δ::LEU2*. A *rad9Δ::HIS3* mutant, previously characterized for defective checkpoint control in response to incomplete replication and DNA damage (Weinert and Hartwell, 1988; Lydall and Weinert, 1995), was also tested.

The *ctf13-30*-induced Delay Does Not Occur in *mad2-1* or *bub1Δ::LEU2* Strains

Analysis of *mad2-1* Mutants. To ascertain whether MAD2 was required for the preanaphase accumulation of *ctf13-30* cells at semipermissive temperature, a *MAD2/mad2-1* CTF13/*ctf13-30* diploid was sporulated, and two tetrads with tetratype segregation for

benomyl sensitivity (*mad2-1*) and *ts* phenotype (*ctf13-30*) were characterized. Logarithmically growing asynchronous cultures in rich medium were analyzed after temperature shift from 25°C to 34°C (at *t* = 0 h). Aliquots taken at 0, 2.5, and 4.5 h were characterized for delay characteristics and cell viability. Results from the two tetrads were essentially identical, and data from one are shown.

The mitotic delay in *ctf13-30* cells at 34°C can be observed as a marked accumulation of cells with G2/M DNA content in flow cytometry profiles (Figure 1, row A). Although the *mad2-1* mutation itself does not produce an altered cell cycle profile under these experimental conditions, clearly the *ctf13-30 mad2-1* double mutants lack G2/M accumulation at 34°C (Figure 1, row B). Delay in anaphase entry can also be detected, because the frequency of uninucleate cells with bud growth advanced beyond that normally seen in wild-type control cells (Spencer and Hieter, 1992), scored as a morphological class referred to as “very

Figure 1. Flow cytometry profiles of *ctf13-30* double mutants after temperature shift. Logarithmically growing cells cultured at 25°C were processed for flow cytometry or shifted to 34°C for 2.5 h before processing. The strains shown are YPH278 (CTF13), s30 (*ctf13-30*), YFS451 (*mad2-1*), YFS449 (*mad2-1 ctf13-30*), YFP2 (*bub1Δ::LEU2*), YFP3 (*bub1Δ::LEU2 ctf13-30*), YFP48 (*bub2Δ::LEU2*), YFP47 (*bub2Δ::LEU2 ctf13-30*), YFS722 (*rad9Δ::HIS3*), and YFS719 (*rad9Δ::HIS3 ctf13-30*).

large budded uninucleate cells." This morphological class includes only cells in which the bud has achieved $\geq 75\%$ the diameter of the mother cell and in which there is a single, unelongated nucleus at the mother/bud neck. This class of cells is observed in wild-type controls at frequencies $\sim 1\%$ (Spencer and Hieter, 1992; Figure 2). After shift to 34°C , the *ctf13-30* MAD2 strain rapidly accumulates very large budded uninucleate cells (Figure 2A), whereas the *CTF13* MAD2, *CTF13* *mad2-1*, and, importantly, the congenic *ctf13-30* *mad2-1* cells do not. Thus, by flow cytometry and cell morphology assays, the *ctf13-30*-induced delay does not occur in the absence of MAD2 function.

Viability of *ctf13-30* cells held at nonpermissive temperature decreases with time, but slowly (Doheny *et al.*, 1993). Aliquots of the same logarithmically growing cultures used for flow cytometric and morphological analysis in the temperature shift protocol described above were plated for single colonies on rich medium and returned to 25°C to assay cell viability. The frequency of cells incapable of supporting colony formation was determined by direct observation of the behavior of 200 founder cells after 24 h. *ctf13-30* cell populations held at 34°C for 2.5 and 4.5 h were 92 and

59% viable, respectively. In contrast, *ctf13-30* *mad2-1* double mutants were 48 and 22% viable at the same time points (Figure 2E). Thus, viability of the double mutant more rapidly decreases under conditions that are semipermissive for *ctf13-30* MAD2 cells.

Population viability of *ctf13-30* MAD2 and *ctf13-30* *mad2-1* cells was also compared under conditions of constant culturing at a semipermissive temperature. *ctf13-30* cells do not form single colonies visible to the naked eye at 34°C , although dense patches of cells do exhibit growth. Therefore, we chose a less stringent condition (30°C) for the constant culturing assay. Saturated cultures were diluted serially in 10-fold steps in a microtiter format, transferred to solid medium, and incubated at 25 or 30°C to allow visible colony formation. In this assay, viability of *ctf13-30* *mad2-1* strain at 30°C is decreased ~ 10 -fold in comparison with the *ctf13-30* and *mad2-1* controls (Figure 3).

Analysis of *bub1 Δ ::LEU2* Mutants. For these experiments, a congenic strain set (*CTF13* *BUB1*, *CTF13* *bub1 Δ* , *ctf13-30* *BUB1*, *ctf13-30* *bub1 Δ*) was created by DNA-mediated transformation of yeast strains YPH278 (*CTF13* *BUB1*) and s30 (*ctf13-30* *BUB1*) with a *bub1 Δ ::LEU2* disruption plasmid, which replaced 948

Figure 2. Preanaphase delay and cell viability in *ctf13-30* double mutants after temperature shift. Logarithmically growing asynchronous cultures growing at 25°C were split ($t = 0$ h), and one-half were shifted to 34°C . Each graph shows the data from four strains (a "tetra-type" set) at two temperatures. Data are shown for preanaphase delay, as measured by the frequency of very large budded uninucleate cells (A, B, C, and D) and by cell viability (E, F, G, and H). The relevant strain genotypes are indicated by the following symbols: *ctf13-30*, triangle; the surveillance mutant shown above the graph, cross; *ctf13-30* surveillance double mutant, circle; wild-type control, square. Solid lines represent data from temperature shift samples that were subjected to the temperature shift; dashed lines indicate data from the same cultures followed at 25°C . Specific strain designations for each experiment are given in the text (see MATERIALS AND METHODS).

Figure 3. Population viability of *ctf13-30* double mutants grown at constant semipermissive temperature. A saturated culture of each strain grown at 25°C was diluted in 10-fold increments in a micro-titer format, and cells were transferred to solid rich medium in 2–5 μ l drops with the use of a multiprong device. The plates were incubated at the indicated temperatures for 2 (30°C) or 4 (25 and 37°C) d. The strains tested were red segregants (without a chromosome fragment) from the following: YPH278 (CTF13), s30 (*ctf13-30*), YFS451 (*mad2-1*), YFS449 (*mad2-1 ctf13-30*), YFP2 (*bub1Δ*), YFP3 (*bub1Δ ctf13-30*), YFP48 (*bub2Δ*), and YFP47 (*bub2Δ ctf13-30*).

nucleotides of the BUB1 open-reading frame with the prototrophic marker LEU2 (see MATERIALS AND METHODS). Logarithmically growing cultures were analyzed in the same temperature shift protocol as above. At 34°C, the *ctf13-30 bub1Δ::LEU2* double mutant did not accumulate in G2/M as assayed by flow cytometry (Figure 1, row C), did not generate cells with very large budded uninucleate morphology (Figure 2B), and exhibited a sharp decline in viability

(Figure 2F). In addition, the *ctf13-30 bub1Δ::LEU2* double mutant exhibited an increase (2–3 orders of magnitude) in lethality with continuous growth at 30°C (Figure 3).

These results parallel those observed in the *ctf13-30 mad2-1* double mutant. They demonstrate clearly that both MAD2 and BUB1 are essential for the *ctf13-30*-induced delay and that this delay is important for cell viability.

The ctf13-30-induced Delay Is Reduced in bub2Δ::LEU2 Strains

A controlled congenic strain set (CTF13 BUB2, CTF13 *bub2Δ*, *ctf13-30* BUB2, *ctf13-30 bub2Δ*) was generated as follows. A *bub2Δ::LEU2* mutant (removing 202 nucleotides of the BUB2 open-reading frame) was introduced into yeast by DNA-mediated transformation of YPH277 (CTF13 BUB2). *ctf13-30* was introduced in a genetic cross with congenic strain s30 (*ctf13-30* BUB2). A tetatype tetrad from this cross was subjected to the same experimental protocols used in the analysis of *mad2-1* and *bub1Δ::LEU2* mutants. However, results obtained for this double mutant study were quite different. In the 34°C temperature shift protocol, the *ctf13-30 bub2Δ::LEU2* double mutant did exhibit a G2/M accumulation as assayed by flow cytometry quite similar to that seen for *ctf13-30* cells (Figure 1, row D). In addition, the very large budded uninucleate cell frequency rose with time, although not to the same level as seen in the *ctf13-30* control strain (Figure 2C). Five repetitions of this experiment indicate that the difference between the *ctf13-30 bub2Δ::LEU2* and *ctf13-30* strain is significant: calculated means $\pm$ SD for the data at 4.5 h post-shift yield 15.9 ± 1.9 and $27.4 \pm 2.7\%$ very large budded uninucleate cells, respectively. We conclude that the *ctf13-30*-induced delay response in *bub2Δ::LEU2* cells is present but reduced.

Although the flow cytometry profile indicated a delay fully equivalent to the *ctf13-30* control strain, the morphological analysis indicated a partial delay. The scoring of very large budded uninucleate cells specifically measures a cell cycle position immediately before anaphase, whereas the G2/M peak of the flow cytometry profile includes cells post-S phase up to cell separation. We attempted to determine whether there was a secondary delay later in this G2/M window but did not find another morphologically scorable class with significant accumulation (our unpublished observations). This suggests that progress after anaphase is altered, but not in a way we can visualize by following DNA and bud morphologies.

Despite the presence of an attenuated delay, the viability of the *ctf13-30 bub2Δ::LEU2* mutant declined sharply (Figure 2G), indicating that the delay experienced by the double mutant cells does not support

viability. This result is also supported by results from five repetitions. At 4.5 h post-shift, the frequency of colony-forming cells in the *ctf13-30 bub2Δ::LEU2* strain was $19.8 \pm 8.3\%$, whereas the frequency in the *ctf13-30* strain was $58.4 \pm 10\%$. Surprisingly, the viability of the same *ctf13-30 bub2Δ::LEU2* strain cultured at 30°C was indistinguishable from the *ctf13-30 BUB2* control (Figure 3). *ctf13-30* strains do exhibit a preanaphase delay in logarithmically growing cultures at 30°C, visible by flow cytometry (our unpublished observations) and as an increase in very large budded uninucleate cells in logarithmically growing cultures (to ~10% of the culture). Loss of BUB2 function under these conditions apparently does not compromise cell viability.

To summarize these experiments, preanaphase delay does occur in *ctf13-30 bub2Δ::LEU2* cells but occurs in fewer cells or is not maintained for so long a period as in *ctf13-30 BUB2* cells. Under conditions in which preanaphase delay is strongly induced (on shift to 34°C), *ctf13-30 bub2Δ::LEU2* cells exhibit a marked decrease in viability. Under conditions that induce a more modest preanaphase delay (30°C), *ctf13-30 bub2Δ::LEU2* cells are fully viable. Clearly, the relationship between BUB2 activity and the *ctf13-30*-induced delay suggests that the role for BUB2 in the kinetochore surveillance pathway may be distinctly different from the function(s) of the MAD2 and BUB1 genes.

The ctf13-30-induced Delay Is Unaffected in rad9Δ::HIS3 Strains

Genetic analysis of DNA damage and spindle assembly checkpoint functions indicates that the two pathways are distinct, depending on at least some gene products that function uniquely in each. In theory, however, the two may use shared functions in overlapping or converging pathways not yet connected by current experimental evidence. It was therefore of interest to determine whether RAD9 was required for kinetochore-induced delays.

Two tetratype tetrads derived from a *CTF13/ctf13-30 RAD9/rad9Δ::HIS3* diploid were analyzed. These gave essentially identical results, and the data from one are presented. Logarithmically growing cultures shifted to 34°C were examined for anaphase delay and for cell viability upon return to permissive temperature, as described above. Clearly, the *ctf13-30*-induced anaphase delay was not affected by the presence of the *rad9Δ::HIS3* allele, whether assayed by flow cytometry (Figure 1, row E) or cell morphology (Figure 2D). In addition, cell viability was unaffected and remained a function solely of the *ctf13-30* genotype (Figure 2H). These results clearly indicate that the absence of RAD9 gene function has no consequence for the *ctf13-30*-induced delay.

A Test Chromosome Containing a Delay-inducing CEN DNA Mutation Exhibits Extreme Instability in mad2-1 and bub1Δ::LEU2 Strains

Although the primary defect engendered by *ctf13-30* occurs at kinetochores, the preanaphase delay that is induced could, in principle, include a response to secondary defects elsewhere in the spindle. We have not noted grossly abnormal microtubule morphologies in *ctf13-30* mutants cultured under semipermissive conditions (our unpublished observations), but because all chromosomal kinetochores may be abnormal at high temperatures, it is quite possible that the simple "bar-like" mitotic spindle of budding yeast may suffer disorganization not observed by the microscopic techniques used. In contrast, the delay induced by a single mutant kinetochore is not expected to cause general spindle disorganization. Therefore, strains containing the same surveillance deficient mutations were assayed for their response to single chromosomes containing a centromere DNA mutation that induces a preanaphase delay (Spencer and Hieter, 1992).

Yeast strains containing single mutant kinetochores were constructed by introducing single-copy test chromosomes engineered to contain chromosome 6 centromeres of wild-type DNA sequence (CEN6 WT) or mutant DNA sequence (CEN6 CDEIIΔ31). The CEN6 CDEIIΔ31 DNA mutation (Panzeri *et al.*, 1985; Shero *et al.*, 1991) is a deletion of 31 nucleotides from element II of the yeast centromere. The test chromosomes used were chromosome fragments (CFs) created by DNA-mediated transformation and homologous recombination and contain large tracts of natural yeast chromosomal DNA (Shero *et al.*, 1991). The chromosome fragment in each case was a 90-kb chromosome III left arm disome (Hegemann *et al.*, 1988) containing the URA3 marker for maintenance and SUP11 marker for determination of test chromosome stability by colony color assay (Hieter *et al.*, 1985). Briefly, the SUP11 marker encodes an ochre-suppressing tyrosyl tRNA that allows expression of productive enzyme from the *ade2-101* ochre allele. Cells blocked at the ADE2 step of adenine biosynthesis accumulate a red pigment, and, thus, cells without the test chromosome are red. However, cells containing the test chromosome SUP11 marker are not blocked in adenine synthesis and are white. Therefore, the degree of red sectoring within colonies indicates the frequency of loss of the SUP11-marked test chromosome.

Congenic strain sets were constructed for comparison. *mad2-1* and *bub2Δ::LEU2* strains containing a CEN6 CDEIIΔ31 chromosome fragment (CFΔ31) or CEN6 wild-type chromosome fragment (CF WT) were generated through chromosome transfer in *Kar1⁻* matings (Dutcher, 1981; Spencer *et al.*, 1994). This method was used after standard matings failed to

yield desired spore products containing the delaying test chromosome. The haploid karyotypes of the strains used in this analysis were verified by pulsed-field gel electrophoresis, followed by Southern blot analysis. This characterization confirmed the presence of the expected 90-kb chromosome fragment and the absence of cotransferred natural yeast chromosomes (Figure 4).

Unfortunately, the frequency of uninucleate very large budded cells in *mad2-1* and *bub1Δ::LEU2* strains containing CDEIIΔ31 could not be determined by microscopic analysis. These strains grew extremely slowly under selection in liquid medium and accumulated large numbers of bloated and dead cells that exhibited uninformative DAPI staining patterns. However, Figure 5A shows the stability of the chromosome fragment in colonies from these strains. The visual colony color assay provides a very accurate and reproducible measure of relative stabilities, as long as colony density and culture times are well controlled in the plates to be compared. Each strain was first grown

Figure 4. Electrophoretic karyotypes of strains created by $Kar1^-$ mating. The ethidium bromide-stained gel shown in the top panel shows a representative donor (lane 1), the recipient parental strains (lanes 2, 4, and 6) and chromoductants selected after $Kar1^-$ mating (lanes 3, 5, and 7). The bottom panel shows hybridization of the chromosome fragment band after DNA was transferred to a nylon membrane and hybridized with pBluescript radiolabeled probe. The karyotypes of these strains show no evidence of increased copy number of yeast natural chromosomes from cotransfer. Strains shown: lane 1, YHP2 (representative donor); lane 2, YFS540 (*mad2-1*); lane 3, YFS541 (*mad2-1* + CF Δ31); lane 4, YFP6 (*bub1Δ::LEU2*); lane 5, YFP17 (*bub1Δ::LEU2* + CF Δ31); lane 6, YFP7 (*bub1Δ::LEU2*); and lane 7, YFP15 (wild-type recipient + CF WT).

under selection for the chromosome fragment in liquid medium lacking uracil and subsequently plated on uracil-supplemented medium to allow the development of red-sectoring colonies at a density of ~200 colonies per plate. The chromosome loss rate engendered by the CDEIIΔ31 mutation on this chromosome fragment has been quantitatively determined to be 1 in 100 divisions, or 50-fold more frequent than a wild-type control (Shero *et al.*, 1991). *mad2-1* and *bub1Δ::LEU2* mutations induce loss of the chromosome fragment with a wild-type centromere, with increases in the range of 10- to 50-fold (by visual comparison of the colony sectoring phenotypes). For the *mad2-1* strain, this is in agreement with previously reported results (Li and Murray, 1991). Clearly, the chromosome fragments segregating with the CDEIIΔ31 mutation are extremely unstable in *mad2-1* and *bub1Δ::LEU2* genetic backgrounds. While sectors are barely, if at all, visible in colonies grown in the absence of selection. This level of chromosome fragment loss is equal to or greater than that observed for test chromosomes with centromere DNA mutations that render them nearly nonfunctional (Hegemann *et al.*, 1988), such as a transversion from G to C in nucleotide 15 of CDEIII. This mutation confers a loss event in 1 of every 12 divisions, on average, or 440-fold more frequently than the wild-type CEN DNA control. The CF instability in the *mad2-1* and *bub1Δ::LEU2* backgrounds is, therefore, not simply at the level of additive loss because of the *mad2-1* or *bub1Δ::LEU2* and CDEIIΔ31 mutations (which would have been 60- to 100-fold) but clearly represents a synergistic effect of the simultaneous presence of two defects.

From these observations we conclude that *MAD2* and *BUB1* are important for the mitotic stability of test chromosomes containing normal centromeres in unperturbed cultures. The wild-type function of these genes is also exceedingly important for the stable maintenance of CDEIIΔ31 containing test chromosomes. The known functions of *MAD2* and *BUB1* strongly suggest that loss of anaphase initiation control is responsible for the dramatic effect on chromosome transmission.

The Accumulation of Large Budded Uninucleate Cells Induced by CEN6 CDEIIΔ31 Occurs in bub2 Mutant Cells

Kinetochore-induced delay competence for *bub2* mutant cells was tested in two different mutant alleles present in different laboratory strain backgrounds. The *bub2-1* allele was tested in a congenic strain set created within the strain background of the original *bub* mutagenesis (Hoyt *et al.*, 1991). This group of strains was produced by generating chromosome fragments containing wild-type CEN6 or CEN6 CDEIIΔ31 in *BUB2* and *bub2-1* strains by DNA-mediated

Figure 5. Chromosome stability in surveillance mutants. Each strain was grown under selection for the chromosome fragment with either wild-type CEN6 sequence (CF[WT]) or delaying mutant CEN6 sequence (CF $\Delta 31$) and plated on minimal medium with limiting adenine. (A) Strains: YPH406 (wild-type CF[WT]), YFS470 (wild-type CF($\Delta 31$)), YFS744 (mad2-1 CF[WT]), YFS541 (mad2-1 CF($\Delta 31$)), YFP53 (bub1 Δ ::LEU2 CF[WT]), YFP 17 (bub1 Δ ::LEU2 CF($\Delta 31$)). (B) Strains: YFS342 (RAD9/rad9 Δ ::HIS3 CF[WT]), YFS344 (RAD9/RAD9 CF($\Delta 31$)), YFS335 (rad9 Δ ::HIS3/rad9 Δ ::HIS3 CF[WT]), YFS337 (rad9 Δ ::HIS3/rad9 Δ ::HIS3 CF($\Delta 31$)). (C) Strains: YFS786 (BUB2/BUB2 CF[WT]), YFS 787 (BUB2/BUB2 CF($\Delta 31$)), YFS 349 (bub2-1/bub2-1 CF[WT]), YFS 350 (bub2-1/bub2-1 CF($\Delta 31$)).

ated transformation. In addition, a bub2 Δ ::LEU2 allele was introduced into the same laboratory strain in which the mad2-1 and bub1 Δ ::LEU2 alleles were characterized above, and a chromosome fragment with CDEII $\Delta 31$ was introduced by Kar1⁻ mating (Figure 4). Diploid strains were desirable for this analysis, because the anaphase delay induced by the mutant kinetochore is observed with greater signal-to-noise ratio in diploid than in haploid cells, and appropriate diploid strains were generated by methods that maintained congenic backgrounds (see MATERIALS AND METHODS). Logarithmically growing asynchronous populations were scored for very large budded uninucleate cells.

In both genetic backgrounds, the bub2 mutant cell populations containing the delaying centromere DNA mutation did exhibit preanaphase delay (Table 2). Surprisingly, a new phenotypic class appeared: cells with "excess buds" (Table 2 and Figure 6). These cells possessed bud spheres in excess of the number allowed per nucleus in a normal cell cycle ($n + 1$); that is, they

had rebudded in the absence of nuclear division. This is the phenotype for which the bub mutants were isolated: the "budding uninhibited by benzimidazole" phenotype. In the original mutant screen, bub2-1 mutants did not recover from high concentration Benomyl treatment, developed an extra bud although nuclear division could not occur, and initiated a new round of DNA synthesis. (However, the original mutant screen used very high concentrations of drug sufficient to completely prevent spindle assembly, whereas in our experiment there is no drug present.) It is tempting to speculate that cells that develop "excess buds" reveal the effects of prolonged preanaphase delay experienced by a subset of cells in the population.

The mitotic stability of the chromosome fragment-containing wild-type CEN6 in the bub2-1/bub2-1 diploids analyzed above is indistinguishable from BUB2 controls (Figure 5). This stability is also observed in bub2 Δ ::LEU2/bub2 Δ ::LEU2 strains and in haploids (our unpublished observations). Thus, in contrast to

Table 1. Yeast strain genotypes

Name	Genotype	Source or Ref
s30	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 cf13-30 +CFIII (CEN3.L.YPH278) URA3 SUP11	Doheney <i>et al.</i> , 1993
YPH277	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 +CFVII (RAD2.d.YPH277) URA3 SUP11	Spencer <i>et al.</i> , 1990
YPH278	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 +CFIII (CEN3.L.YPH278) URA3 SUP11	Spencer <i>et al.</i> , 1990
YPH406	MAT α ura3-52 lys2-801 ade2-101 trp1 Δ 1 +CFIII (D8B.d.YPH281) URA3 SUP11	Hieter Laboratory
YF470	MAT α ura3-52 lys2-801 ade2-101 trp1 Δ 1 +CFIII (D8B.d.YPH429) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFP2	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 bub1 Δ ::LEU2 +CFIII (CEN3.L.YPH278) URA3 SUP11	this study
YFP3	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 bub1 Δ ::LEU2 ctf13-30 +CFIII (CEN3.L.YPH278) URA3 SUP11	this study
YFP15	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 can ^R +CFIII (D8B.d.YPH281) URA3 SUP11 LEU2	this study
YFP53	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 can ^R bub1 Δ ::LEU2 +CFIII (D8B.d.YPH281) URA3 SUP11 LEU2	this study
YFP17	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 can ^R bub1 Δ ::LEU2 +CFIII (D8B.d.YPH429) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFP24	<u>MATα ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ leu2Δ1 bub2Δ::LEU2</u> MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 trp1 Δ leu2 Δ 1 bub2 Δ ::LEU2 +CFIII (D8B.d.YPH429) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFP25	<u>MATα ura3-52 lys2-801 ade2-101 trp1Δ leu2Δ1</u> MAT α ura3-52 lys2-801 ade2-101 trp1 Δ leu2 Δ 1 +CFIII (D8B.d.YPH429) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFP46	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 trp1 Δ leu2 Δ 1 +CFIII (D8B.d) URA3 SUP11 LEU2	this study
YFP47	MAT α ura3-52 lys2-801 ade2-101 trp1 Δ leu2 Δ 1 bub2::LEU2 ctf13-30 +CFIII (D8B.d) URA3 SUP11 LEU2	this study
YFP48	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 bub2::LEU2 +CFIII (D8B.d) URA3 SUP11 LEU2	this study
YFP49	MAT α ura3-52 lys2-801 ade2-101 leu2 Δ 1 ctf13-30 +CFIII (D8B.d) URA3 SUP11 LEU2	this study
YFS744	MAT α ura3-52 lys2-801 ade2-101 trp1 leu2 Δ 1 mad2-1 can ^R +CFIII (D8B.d.YPH281) URA3 SUP11	this study
YFS541	MAT α ura3-52 lys2-801 ade2-101 trp1 leu2 Δ 1 mad2-1 can ^R +CFIII (D8B.d.YPH429) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFS342	<u>MATα ura3-52 lys2-801 ade2-101 trp1Δ1 his3Δ200 RAD9</u> MAT α ura3-52 lys2-801 ade2-101 trp1 Δ 1 his3 Δ 200 rad9 Δ ::HIS3 +CFIII (D8B.d.YPH322) URA3 SUP11	this study
YFS344	<u>MATα ura3-52 lys2-801 ade2-101 trp1Δ1 his3Δ200 RAD9</u> MAT α ura3-52 lys2-801 ade2-101 trp1 Δ 1 his3 Δ 200 RAD9 +CF III (D8B.d.YPH318) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFS335	<u>MATα ura3-52 lys2-801 ade2-101 trp1Δ1 his3Δ200 rad9Δ::HIS3</u> MAT α ura3-52 lys2-801 ade2-101 trp1 Δ 1 his3 Δ 200 rad9 Δ ::HIS3 +CFIII (D8B.d.YPH322) URA3 SUP11	this study
YFS337	<u>MATα ura3-52 lys2-801 ade2-101 trp1Δ1 his3Δ200 rad9Δ::HIS3</u> MAT α ura3-52 lys2-801 ade2-101 trp1 Δ 1 his3 Δ 200 rad9 Δ ::HIS3 +CFIII (D8B.d.YPH324) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFS786	<u>MATα ura3-52 ade2-101 his3Δ200 leu2-3,112</u> MAT α ura3-52 ade2-101 his3 Δ 200 leu2-3,112 +CFIII (D8B.d.YFS299.1) URA3 SUP11	this study
YFS787	<u>MATα ura3-52 ade2-101 his3Δ200 leu2-3,112</u> MAT α ura3-52 ade2-101 his3 Δ 200 leu2-3,112 +CFIII (D8B.d.YFS299.1) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFS346.1	<u>MATα ura3-52 ade2-101 his3Δ200 leu2-3,112 BUB2</u> MAT α ura3-52 ade2-101 his3 Δ 200 leu2-3,112 bub2-1 +CFIII (D8B.d.YFS299.1) URA3 SUP11	this study
YFS347.1	<u>MATα ura3-52 ade2-101 his3Δ200 leu2-3,112 BUB2</u> MAT α ura3-52 ade2-101 his3 Δ 200 leu2-3,112 bub2-1 +CFIII (D8B.d.YFS299.1) URA3 SUP11 [CEN6 CDEII Δ 31]	this study

Table 1 (cont).

Name	Genotype	Source or Ref
YFS349.1	<u>MATα ura3-52 ade2-101 his3Δ200 leu2-3,112 bub2-1</u> MAT α ura3-52 ade2-101 his3 Δ 200 leu2-3,112 bub2-1 +CFIII (D8B.d.YFS306.1) URA3 SUP11	this study
YFS350.1	<u>MATα ura3-52 ade2-101 his3Δ200 leu2-3,112 bub2-1</u> MAT α ura3-52 ade2-101 his3 Δ 200 leu2-3,112 bub2-1 +CFIII (D8B.d.YFS307.1) URA3 SUP11 [CEN6 CDEII Δ 31]	this study
YFS719	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 rad9 Δ ::HIS3 ctf13-30 +CFIII (D8B.d.YF322) URA3 SUP11	this study
YFS720	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 trp1 Δ 1 leu2 Δ 1	this study
YFS721	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 ctf13-30 +CFIII (D8B.d.YF322) URA3 SUP11	this study
YFS722	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 trp1 Δ 1 leu2 Δ 1 rad9 Δ ::HIS3	this study
YFS451	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 trp1 leu2 Δ 1 mad2-1 +CFIII (D8B.d.YPH278) URA3 SUP11	this study
YFS449	MAT α ura3-52 lys2-801 ade2-101 his3 Δ 200 trp1 leu2 Δ 1 ctf13-30 mad2-1 +CFIII (D8B.d.YPH278) URA3 SUP11	this study

All chromosome fragments contain wild-type centromere DNA sequences, unless indicated otherwise.

the effect of loss of MAD2 or BUB1 function, loss of BUB2 function does not decrease chromosome transmission fidelity in unperturbed populations. Interestingly, the CF-containing CDEII Δ 31 is specifically destabilized in the bub2-1/bub2-1 mutant. Two sources of instability may be envisioned. The reduced preanaphase delay may lead to increased missegregation of the test chromosome. Alternatively, there may be increased cell lethality in CDEII Δ 31 cell lineages, because the "excess budded" phenotype observed in the

presence of high concentrations of antimicrotubule drugs is associated with a lethal event (Hoyt *et al.*, 1991).

The Accumulation of Large Budded Uninucleate Cells Induced by CEN6 CDEII Δ 31 Is Unaffected in rad9 Δ ::HIS3 Strains

Congenic experimental and control strains were created by introduction of chromosome fragments con-

Table 2. Preanaphase delay induced by a single-copy abnormal kinetochore

A. bub2 mutant strains

Strain	Genotype	CF CEN	N	Delay ^a	Extra budded ^b
YFS349.1	bub2-1/bub2-1	WT	3	2.0 $\pm$ 0.9	0.3 $\pm$ 0.3
YFS350.1	bub2-1/bub2-1	CDEII Δ 31	8	7.8 $\pm$ 2.9	5.9 $\pm$ 3.6
YFS346.1	BUB2/bub2-1	WT	3	0.7 $\pm$ 0.3	0.
YFS347.1	BUB2/bub2-1	CDEII Δ 31	3	6.9 $\pm$ 1.5	0.
YFP24	bub2 Δ ::LEU2/bub2 Δ ::LEU2	CDEII Δ 31	8	6.2 $\pm$ 1.2	3.9 $\pm$ 2.0
YFP25	BUB2/BUB2	CDEII Δ 31	7	8.2 $\pm$ 5.1.8	0.

B. rad9 mutant strains

Strain	Genotype	CF CEN	N	Delay ^a
YFS335	rad9 Δ ::HIS3/rad9 Δ ::HIS3	WT	3	0.6 $\pm$ 0.2
YFS337	rad9 Δ ::HIS3/rad9 Δ ::HIS3	CDEII Δ 31	2	9.6 $\pm$ 0.6
YFS342	RAD9/rad9 Δ ::HIS3	WT	4	0.9 $\pm$ 0.5
YFS344	RAD9/RAD9	CDEII Δ 31	7	10.2 $\pm$ 1.4

^aThe frequency (%) of very large budded uninucleate cells in logarithmically growing cultures.

^bThe frequency (%) in the same cultures.

The data are shown as mean $\pm$ SD for the number of independent trials (N) indicated.

Figure 6. Cells exhibiting excess budded morphology from a log-phase culture of *bub2Δ::LEU2/bub2Δ::LEU2* (upper left panel) or *bub2-1/bub2-1* (remaining three panels) yeast strains containing CF(Δ 31).

taining either a wild-type CEN6 or CEN6 CDEII Δ 31 into RAD9 and *rad9Δ* yeast strains by DNA-mediated transformation. Diploid strains were created by standard genetic crosses, and CDEII Δ 31-induced preanaphase delay was analyzed by cell morphology. Asynchronous logarithmically growing cultures were analyzed for the frequency of very large budded uninucleate cells (Table 2). The presence of the CEN6 CDEII Δ 31 mutant induced a 10-fold increase in large budded uninucleate cells in the presence or absence of the RAD9 gene product, indicating that RAD9 function has no role in the CDEII Δ 31-induced delay. As expected, stability of the test chromosome as assayed by red sectoring (Figure 5) was also not affected by the presence or absence of the RAD9 gene.

DISCUSSION

We have previously observed that mutations leading to alterations in kinetochore structure are associated with preanaphase delays in budding yeast (Spencer and Hieter, 1992; Doheny *et al.*, 1993) and have proposed that this delay is due to a spindle surveillance activity. Mutations identifying three MAD genes (Li and Murray, 1991) and three BUB genes (Hoyt *et al.*, 1991) were isolated in studies that sought to identify mutants that failed to delay or arrest under conditions that prevented normal spindle assembly by interfering with microtubule polymerization dynamics. This strategy defined spindle assembly checkpoint mutants in budding yeast. Subsequent molecular analysis of the six gene products identified in MAD and BUB screens has indicated that BUB1 encodes a protein kinase (Roberts *et al.*, 1994) and that MAD1 is a phosphoprotein whose phosphorylation depends on BUB1 (Hardwick and Murray, 1995). Molecular roles of BUB2, BUB3, and MAD2 are not apparent from their primary amino acid sequences (Hoyt *et al.*, 1991; Li *et al.*, 1994). BUB2 shows striking similarity to CDC16 from *Schizosaccharomyces pombe* at the primary amino

acid level and in functional studies (Fankhauser *et al.*, 1993), providing evidence supporting the existence of a conserved function.

In this report, we find that kinetochore-induced delays are altered in *mad2*, *bub1*, and *bub2* mutant yeast strain backgrounds, whether the delay is induced by the temperature-sensitive mutant protein encoded by *ctf13-30* or by a single chromosome containing the CEN6 DNA mutation CDEII Δ 31. The data we present clearly demonstrate that MAD2 and BUB1 protein functions are each absolutely required for the preanaphase delay induced by mutant kinetochore structures, in agreement with recently published independent studies (Wang and Burke, 1995; Wells and Murray, 1996). Furthermore, we find that loss of delay capability in *mad2* and *bub1* mutants is associated with chromosome instability in unperturbed cells and with chromosome instability or cell lethality under conditions in which kinetochore structure is altered by mutation of either DNA or protein components. Because proteins that are required for the spindle assembly checkpoint (originally defined by microtubule disruption) play a role in kinetochore surveillance, we conclude that the budding yeast spindle assembly checkpoint monitors kinetochore structure.

In contrast, the budding yeast RAD9 gene is clearly not involved. Both DNA damage and spindle assembly checkpoints impose a preanaphase delay. Our results make it clear that if the two control pathways are related, it must be after the point at which RAD9 acts. Consistent with this observation, a recent report has proposed that RAD9 may act at the level of generation of a signal that triggers the DNA damage response (Lydall and Weinert, 1995).

Although our results indicate that kinetochore structure is an inducer of the delay imposed by the spindle assembly checkpoint, it does not rule out the possibility that other spindle structures are subject to surveillance. Recent work has demonstrated that the spindle pole defects caused by *cdc31* and *mps2* mutants lead to a MAD1-dependent preanaphase arrest (Weiss and Winey, 1996). In addition, both benzimidazole and spindle pole-induced preanaphase arrests were shown to require MPS1, an essential kinase that functions in spindle pole body duplication as well as mitotic checkpoint control (Weiss and Winey, 1996). Moreover, preanaphase accumulation is seen in the presence of mutations in spindle-associated kinesin-like proteins (Hoyt *et al.*, 1992; Roof *et al.*, 1992). A delay-inducing mutant allele of one of these (*kar3*) exhibits synthetic lethality in combination with *mad2-1* (Roof *et al.*, 1991). It is formally possible that these nonkinetochore defects and drug-associated perturbations in microtubule structure all have the effect of inducing activity of a spindle assembly checkpoint through the generation of abnormal kinetochore configuration. Yet it seems equally likely that other aspects of spindle structure

may be monitored, and future work is required to adequately address this issue.

Recent work has suggested that BUB2 is not required for the *ctf13-30*-induced preanaphase accumulation (Wang and Burke, 1995). In contrast, we find that although *ctf13-30 bub2Δ::LEU2* cells do delay after shift to 34°C, the frequency of preanaphase cells is lower than that observed in the *ctf13-30* control strain. We also find that the *ctf13-30 bub2Δ::LEU2* double mutant exhibits markedly increased inviability within 4.5 h after the shift. Moreover, *bub2* mutants show decreased transmission fidelity of a chromosome fragment containing a delay-inducing, partially functional kinetochore and develop a significant number of extra-budded cells that have apparently arisen from misregulation of cell cycle events. Thus, we conclude that BUB2 does play a role in the kinetochore-induced delay.

Taken together, our results suggest that the role of the BUB2 protein in the surveillance pathway may be qualitatively different from that of MAD2 and BUB1. We currently favor the hypothesis that BUB2 plays a role in a late delay function, such as delay maintenance, for two reasons. First, *bub2* mutants do exhibit preanaphase delay in the presence of kinetochore abnormalities, showing an attenuated response in the *ctf13-30 bub2Δ::LEU2* double mutants and a delay comparable to BUB2 controls in the single-delaying kinetochore assay. Therefore, the *bub2* mutants we have studied can initiate events required for anaphase delay. Second, the delay observed in *bub2* mutant cells has characteristics distinct from the delay that occurs in wild-type (BUB2) cells: It does not rescue cells from rapid lethality in *bub2 ctf13-30* double mutants and induces "extra budded" cells in the presence of the CF containing CDEIIΔ31. Taken together, these observations point toward a role for BUB2 in delay maintenance or recovery from delay. However, our results are also consistent with a partial failure in the induction of preanaphase delay in a scenario in which the surveillance pathway is branched and modification of a subset of targets is defective in *bub2* mutants. It is worth pointing out that the *bub2* deletion mutation used is predicted to allow expression of the N-terminal 91 amino acids encoded by the BUB2 gene, and, thus, the question of whether or not it is a true null is open. Further work is clearly required to determine the nature of the partial function we have observed.

What is the molecular nature of the abnormal kinetochore structure that triggers activity of the spindle assembly checkpoint? This is not directly addressed by our data, but clearly the delay-inducing structure is effective at single copy (or at very low copy number). We have speculated that kinetochore-induced delays may be caused by a lack of bipolar spindle attachment of one or more yeast chromosomes in metaphase, as has been observed in cultured animal cells. Our results provide strong evidence linking kinetochore structure

in yeast to the activities responsible for the spindle assembly checkpoint, currently defined by a widely conserved cellular response to microtubule disruption. The combined power of yeast and animal cell systems, and recent progress in both experimental platforms, holds promise for a deeper understanding of how it is that cells ensure proper partitioning of chromosomal information at each cell division.

ACKNOWLEDGMENTS

Plasmids and strains were kindly provided by T. Roberts and A. Hoyt (Johns Hopkins University, Baltimore), R. Li, J. Minshull, and A. Murray (University of California, San Francisco), and T. Weinert (University of Arizona). We are grateful for technical assistance in plasmid and strain construction from V. Madike, J. Pongsiri, and H. Perlick and in flow cytometry from J. Flook. This work was supported by funding from the Predoctoral Training Program in Human Genetics (NIGMS07814) and the Ford Foundation to F.P., and the American Cancer Society (CB112A and JFRA562) and the National Institutes of Health (GM-50842) to F.S.

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