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A chromosome breakage assay to monitor mitotic forces in budding yeast

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SUMMARY

During the eukaryotic cell cycle, genetic material must be accurately duplicated and faithfully segregated to each daughter cell. Segregation of chromosomes is dependent on the centromere, a region of the chromosome which interacts with mitotic spindle microtubules during cell division. Centromere function in the budding yeast, *Saccharomyces cerevisiae*, can be regulated by placing an inducible promoter adjacent to centromere DNA. This conditional centromere can be integrated into chromosome III to generate a conditionally functional dicentric chromosome. Activation of the dicentric chromosome results in a transient mitotic delay followed by the generation of monocentric derivatives. The propagation of viable cells containing these monocentric derivative chromosomes is dependent upon the DNA repair gene *RAD52*, indicating that double-strand DNA breaks are structural intermediates in the dicentric repair pathway.

We have used these conditionally dicentric chromosomes to monitor the exertion of mitotic forces during cell division. Analysis of synchronized cells reveal that lethality in dicentric, *rad52* mutant cells occurs during G_2/M phase and is concomitant with the transient mitotic delay. The delay is largely dependent upon the cell cycle checkpoint gene *RAD9*, which is involved in monitoring DNA damage. These data demonstrate that DNA lesions resulting from dicentric activation are responsible for signalling the mitotic delay. Since the delay precedes the decline of $p34^{cdc28}$ kinase activity, mitotic forces sufficient to result in dicentric chromosome breakage are generated prior to spindle elongation and anaphase onset in yeast.

Key words: mitosis, dicentric chromosomes, mitotic forces, budding yeast, *RAD9*

INTRODUCTION

The dynamics of chromosome movement during mitosis have been studied extensively in higher eukaryotes (for reviews, see McIntosh and Koonce, 1989; Salmon, 1989). Many studies have shown that force production involving the kinetochore is regulated throughout the cell cycle. During prometaphase, one kinetochore of a duplicated chromosome initially attaches to spindle microtubules and oscillates toward and away from the spindle pole. When both kinetochores of the duplicated chromosome become attached to microtubules emanating from opposite poles, the chromosome progresses to the metaphase plate. At the onset of anaphase, the kinetochores of sister chromatids separate and move along microtubules in a poleward direction (anaphase A), followed by separation of the spindle poles (anaphase B).

In both budding and fission yeast, analysis of centromere and kinetochore movements has been hindered by their small sizes, which preclude conventional microscopic detection. Recently however, the development of fluorescence in situ hybridization (FISH) in fission yeast *S. pombe* (Uzawa and Yanagida, 1992) has enabled the analysis of centromere movement during the cell cycle in this organism (Funabiki et al., 1993). During interphase, centromeres are located at the periphery of the nuclear membrane, associated with the spindle pole body. At the beginning of mitosis, centromeres are found at random positions within the nucleus, which is interpreted as evidence of prometaphase-like movements, similar to that observed in higher eukaryotes. By metaphase, centromeres become localized to the center of the nucleus at a position corresponding to the metaphase plate. Finally, during anaphase, sister chromatid separation (anaphase A) is apparent and occurs around the time of spindle elongation (anaphase B).

In *S. cerevisiae*, the centromeres are even smaller, and only recently have antibodies toward centromeric components been available to study centromere movements during the cell cycle. An antibody to ndc10p, encoded by the *NDC10* (*CBF2*, *CTF14*) gene, has been used to monitor the position of the centromere during the cell cycle (Goh and Kilmartin, 1993). During most of the cell cycle, ndc10p is localized to the spindle pole body at the nuclear periphery. Only for a short time can ndc10p be seen to migrate out toward the central spindle.

We have developed a chromosome breakage assay to determine when centromeres and/or chromosomes are active. While the presence of a single centromere on each chromosome is essential for accurate segregation of the genome, multiple centromeres on a single chromosome can be deleterious, typically resulting in unstable chromosome inheritance. The chromosome breakage assay is based on the ability to construct dicentric chromosomes and to follow their fate, physically and genetically, throughout the cell cycle. Dicentric chromosomes were first studied extensively in *Zea mays*

(McClintock, 1939, 1941). Cytogenetic observations of the mitotic behaviour of dicentric chromosomes demonstrate a sequence of events termed a breakage-fusion-bridge cycle. When the two centromeres of a single chromosome are pulled to opposite poles during mitosis, the resulting anaphase bridge that forms between them is often broken. The broken ends of these monocentric chromosome derivatives are highly reactive. By fusing with other chromosomes, they can cause a number of chromosomal anomalies, including deletions, translocations and the regeneration of dicentric chromosomes. This cycle persists until stable rearrangements are formed.

Several studies have revealed that both dicentric plasmids and chromosomes are mitotically unstable in yeast (Mann and Davis, 1983; Koshland et al., 1987; Haber et al., 1984; Surosky and Tye, 1985; Hill and Bloom, 1987; Jager and Phillipsen, 1989). These studies have been limited to the introduction of dicentrics by transformation and analysis of viable cells. The construction of a conditional centromere allows the stable propagation of a conditionally dicentric chromosome (Hill and Bloom, 1987). The centromere of yeast can be inactivated by inducing a strong transcriptional promoter adjacent to the centromere. A dicentric chromosome III has been generated that contains this conditional centromere as well as a normal *CEN3* sequence (Hill, 1988; Hill and Bloom, 1989). The conditional dicentric chromosome is unique in that it provides a means of regulating centromere function, and thereby chromosome segregation, in vivo.

Upon activation, cells harboring this dicentric chromosome exhibit a mitotic delay (Hill and Bloom, 1989; Neff and Burke, 1992). Cells are temporarily arrested as large budded cells with the nucleus localized to the neck of the bud. In addition, rearranged monocentric chromosomes are generated (Hill and Bloom, 1989). In this study, activation of dicentric chromosomes in synchronized cells was used to investigate centromere and chromosome movements in the cell cycle of budding yeast *S. cerevisiae*.

MATERIALS AND METHODS

Strains

All yeast strains used in this study are isogenic derivatives of J178-1D (*MATa, ade1, met14, ura3-52, leu2-3,112, his3*), which have been modified by fragment-mediated transformation. Strain names and modifications are shown in Table 1. Two conditional dicentric derivatives of each strain were generated, as described elsewhere (Hill and Bloom, 1989). In strains with the conditional centromere designation #4, the conditional centromere sequence is oriented opposite to that of the endogenous centromere (inverted), whereas in strains with the designation #7, the two centromeres are in similar orientations (direct) (Fig. 1). These conditional centromere constructions contain a partial duplication of the *HIS4* gene (1.56 kb *Sal*I fragment) flanking the site of integration. Two plasmids were constructed (pJB2#4 and pJB2#7) and used to generate dicentric chromosomes by integration at *HIS4* without sequence duplication (Fig. 2). The designations #24 and #27 refer to the inverted and direct conditional centromere orientation, respectively, of these dicentric constructions.

Table 1. List of strains

Strain	Modification
J178#4	his4::URA3,GALCEN3
J178#7	his4::URA3,GALCEN3
J178#4-20	his4::URA3,GALCEN3; rad52::LEU2
J178#7-20	his4::URA3,GALCEN3; rad52::LEU2
J178#24	his4Δ::URA3,GALCEN3
J178#27	his4Δ::URA3,GALCEN3
J178#24-20	his4Δ::URA3,GALCEN3; rad52::LEU2
J178#27-20	his4Δ::URA3,GALCEN3; rad52::LEU2
JB9	rad9Δ::hisG
JB9#4	rad9Δ::hisG; his4::URA3,GALCEN3
JB9#7	rad9Δ::hisG; his4::URA3,GALCEN3
JB9#4-20	rad9Δ::hisG; his4::URA3,GALCEN3; rad52::LEU2
JB9#7-20	rad9Δ::hisG; his4::URA3,GALCEN3; rad52::LEU2
JB45	bub2Δ::LEU2
JB45#4	bub2Δ::LEU2; his4::URA3,GALCEN3
JB45#7	bub2Δ::LEU2; his4::URA3,GALCEN3
JB50	mad2-1
JB50#4	mad2-1; his4::URA3,GALCEN3
JB50#7	mad2-1; his4::URA3,GALCEN3
JB46	rad9Δ::hisG; bub2Δ::LEU2
JB46#4	rad9Δ::hisG; bub2Δ::LEU2; his4::URA3,GALCEN3
JB46#7	rad9Δ::hisG; bub2Δ::LEU2; his4::URA3,GALCEN3
JB51	rad9Δ::hisG; mad2-1
JB51#4	rad9Δ::hisG; mad2-1; his4::URA3,GALCEN3
JB51#7	rad9Δ::hisG; mad2-1; his4::URA3,GALCEN3

Strain modifications

The generation of *rad52* mutants was accomplished by fragment mediated transformation (Ito et al., 1983). A 5 kb *Bam*HI restriction fragment of plasmid pSM20 (Schild et al., 1983), which contains a *LEU2* disruption of the *RAD52* gene, was used to generate the *rad52*

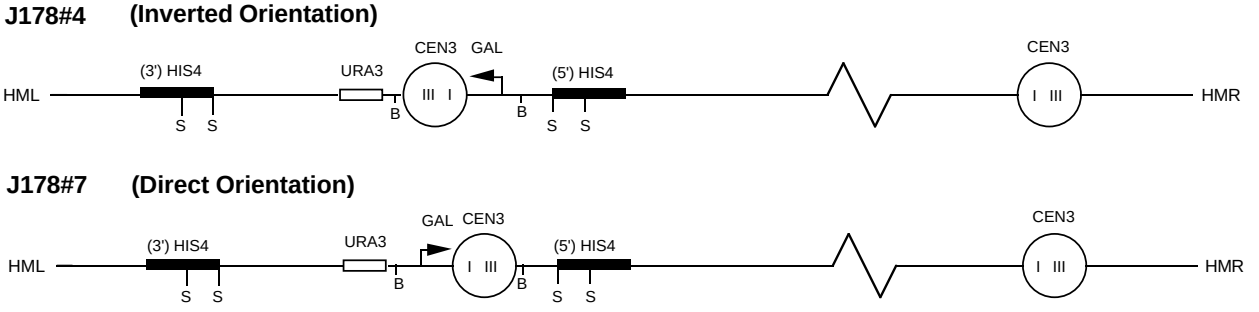

Fig. 1. Schematic representation of two conditional dicentric chromosomes. Integration of the conditional centromere at the *HIS4* locus of chromosome III results in a conditional dicentric chromosome. Two different dicentric chromosomes were generated: one in which the conditional centromere is in the opposite orientation relative to the wild-type centromere (Inverted Orientation); and one in which the centromeres are in the same orientation (Direct Orientation). The only difference between these two chromosomes is the orientation of the *Bam*HI fragment containing the conditional centromere. Also note that a portion of the *HIS4* gene is duplicated by integration of the conditional centromere, as indicated by the *Sal*I sites. B, *Bam*HI sites; S, *Sal*I sites.

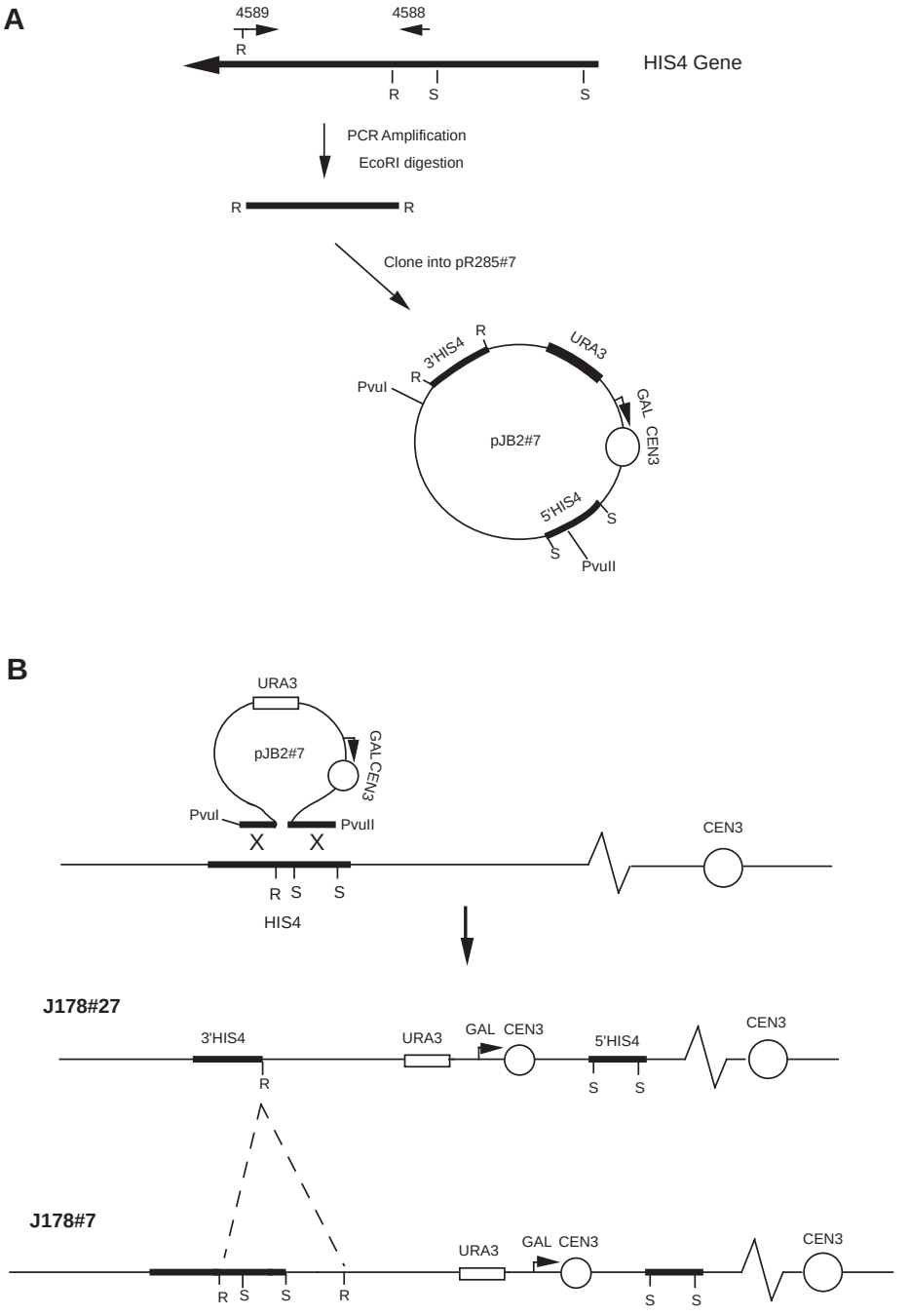

Fig. 2. Construction of plasmids pJB2#7 and pJB2#4 and dicentric strains J178#27 and J178#24. (A) A fragment of the *HIS4* gene, 3′ to the *Sal*I fragment in pR285, was amplified using the polymerase chain reaction (PCR). One oligonucleotide primer used for amplification (4588) hybridizes to a region 50 bp 3′ of the *Sal*I fragment and 240 bp 5′ of the *Eco*RI site (5′ CCATCGATTGGGTCG 3′); the second primer (4589) was located 3′ of the first primer and designed so that it generated a new *Eco*RI site (underlined), which could subsequently be used for cloning (5′ AGCCGAATTCGCAACGGCTTGGGC ACCACC 3′). The resulting *HIS4* fragment was cloned into the *Eco*RI site of both pR285#4 and pR285#7, generating plasmids pJB2#7 and pJB2#4, respectively (for simplicity only pR285#7 is shown). (B) These plasmids are used to generate dicentric chromosomes by integrating the *Pvu*I/*Pvu*II fragment into the *HIS4* site of chromosome III by fragment mediated transformation using *URA3* as a selectable marker. The strains generated by this integration (J178#27 and J178#24, not shown) result in a deletion of 248 bp of *HIS4* sequence. J178#7 is included for comparison.

deletion. Transformants were selected by their ability to grow on medium lacking leucine and generation of the *rad52* mutation was confirmed both by sensitivity to MMS and by Southern analysis.

Strain J178-1D was transformed to *rad9*Δ by fragment mediated transformation. A 7.1 kb *Sal*I/*Eco*RI fragment containing part of the 5′ end of the *RAD9* ORF, the *URA3* gene flanked by direct repeats of Salmonella *hisG* sequences (Alani et al., 1987) and a portion of the 3′ end of the *RAD9* ORF was isolated from plasmid pRR330 and used for transformation (Schiestl et al., 1989). Transformants were selected on the basis of uracil prototrophy and subsequently confirmed by sensitivity to UV irradiation and by Southern analysis.

A *ura3* mutant isolate was generated by selection on 5-fluoroorotic acid (5-FOA). Recombination between the *hisG* repeats which flank the *URA3* gene result in a *ura3*$^{-}$ cell which can grow in the presence of this compound.

Strains were transformed to *bub2Δ* using a 4.2 kb *Sst*I-*Xho*I fragment from plasmid pJB5, which contains a *LEU2* deletion disruption of the *BUB2* gene. pJB5 was constructed by replacing a *Bam*HI-*Hin*dIII fragment of the *BUB2* gene in plasmid pTR24 (Hoyt et al., 1991) with a *Bam*HI-*Hin*dIII fragment containing the *LEU2* gene. The result is a 202 bp deletion of the *BUB2* gene. Transformants were confirmed by Southern analysis and by sensitivity to benomyl, a microtubule-destabilizing drug.

Transformation to *mad2-1* was accomplished with plasmid pRL11 (Li and Murray, 1991). The entire plasmid was targeted into the *MAD2* gene by cutting with the restriction enzyme *Kpn*I. This results in duplication of the *MAD2* gene (a wild-type copy and a *mad2-1* mutant copy) flanking the *URA3* gene at the site of integration. Selection for *ura3* mutants on 5-FOA regenerates a single copy of the *MAD2* gene, which may be wild type or mutant, depending on site of

recombination. The *mad2-1* derivatives were identified by testing for sensitivity to benomyl.

Media

Yeast rich medium (YPGal) contained 2% galactose, 2% peptone and 1% yeast extract. Yeast minimal media contained 0.67% yeast nitrogen base and 2% glucose (or galactose) supplemented with 0.5% casamino acids and either adenine alone or adenine and uracil (16.5 μg/ml each). These are abbreviated YCaglu, YCagal, YCauglu and YCaugal, accordingly.

Timecourse on glucose

Cells were grown to mid-logarithmic phase in 50 ml of selective medium (YCagal) to maintain the structural integrity of the dicentric chromosome. A 10 ml sample was taken for phenotypic characterization of cells and restriction analysis of DNA (timepoint 0). An additional 25 ml sample was harvested by centrifugation (3500 ***g*** for 5 minutes) and washed once in sterile water. They were then resuspended in 25 ml of nonselective glucose medium (YCauglu) to activate the dicentric chromosome and to recover monocentric derivatives. After 1 hour, the culture was diluted with 25 ml of YCauglu medium. Subsequent dilutions were made by subculturing a sample of cells into fresh sterile medium. At the indicated timepoints, 10 ml samples were collected for phenotypic characterization and restriction analysis.

Preparation of cells for immunofluorescence

Cells were prepared for indirect immunofluorescence as described by Kilmartin and Adams (1984). Cells were incubated in primary antibody (monoclonal antitubulin, Sera Lab) for 14 hours, washed and incubated in secondary antibody (rhodamine-labeled goat anti-rat). After washing off the secondary antibody, cells were stained with the DNA dye Hoechst 33258.

Flow cytometry

Preparation of samples for flow cytometry was carried out essentially as described by Nash et al. (1988). Approximately 1×10^7 cells were collected and sonicated for 4-5 seconds with the microtip at setting 6. Cells were washed once in ice-cold distilled water, then fixed in 70% ethanol for 8 hours. After fixing, cells were resuspended in 5 ml of 50 mM sodium citrate, pH 7.0, sonicated briefly, then resuspended in 1 ml of sodium citrate solution. 25 μl of RNaseA (10 mg/ml) was added to each sample and incubated at 50-55˚C for 30 minutes. Before staining with propidium iodide, cell concentrations were adjusted to 5×10^6 cells/ml in 50 mM sodium citrate, pH 7.0. Propidium iodide was added to a final concentration of 40 mg/ml. Cells were incubated at room temperature in the dark for 1-2 hours and filtered through 35 μm mesh.

A Becton Dickenson FACS 440 was used to analyze cells by flow cytometry. Excitation of propidium iodide bound to DNA was provided by the 488 nm line of an Innova 90-5 argon laser (Coherent, Inc. Palo Alto, CA) operating at an output power of 550 mW. Fluorescence emission was collected through a 625/35 nm band-pass filter. The resulting signals were processed through a linear amplifier and stored on a DEC (Digital Equipment Corporation, Maynard, MA) 11/73-based microcomputer. Consort 40 software (Becton Dickinson, Mountain View, CA) was used for both data acquisition and subsequent data analysis.

H1 kinase assay

Galactose-grown cells J178-1D and J178#7, switched to glucose for 4.5 hours were used for histone H1 kinase assays. 10^8 cells of each culture were washed once in ice-cold water and lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.2, 1 mM sodium pyrophosphate). Cells were resuspended in 120 μl lysis buffer with protease inhibitors (1.0 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A) and lysed by vortexing with sterile glass beads (150 μg of 0.45 mm beads) in six 30 second pulses. Supernatant from lysed cells was clarified by centrifugation twice for 15 minutes. 50 μl of each sample was incubated with 10 μl of p13-Agarose beads (Oncogene Products) for two hours on ice. Beads were then washed three times with lysis buffer containing 250 mM NaCl, twice in lysis buffer without SDS or sodium pyrophosphate and once in 2× reaction buffer (15 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.4).

For the kinase assay, beads were resuspended in 10 μl of 2× reaction buffer with 2 μg of histone H1 and 1 μl of [γ-^{32}P]ATP (10 μCi) and water to final volume of 20 μl. The reaction was incubated at 37˚C for 30 minutes. Samples were boiled for 5 minutes and electrophoresed on a 12% SDS-polyacrylamide gel. Relative amounts of histone H1 kinase activity among the samples were determined by scanning an autoradiograph and by scintillation counting of the histone H1 band excised from the polyacrylamide gel.

Alpha factor synchronization experiment

Synchronization experiments were carried out using strain J178#7-20. Cells were grown exponentially in selective medium (YCagal) to an optical density A_{660} of 0.25. Cultures were harvested, washed once in sterile water and resuspended in YPRaf (1% yeast extract, 2% bactopeptone, 2% raffinose). Alpha factor was added immediately to a concentration of 20 μg/ml and cells were incubated for 4 hours at 32˚C. By this method, 80-85% of the culture were reproducibly schmoos or single cells and were poised for release from alpha factor with dicentric chromosomes. Cells were incubated in alpha factor for 4 hours, then washed out of medium containing alpha factor and resuspended in YPRaf. At 30 minute intervals, cells were counted with a haemocytometer and plated onto YCaugal (monocentric conditions) to determine plating efficiency. Samples were also stained with Hoechst 33258 to monitor the cell cycle stage. Raffinose was used as a permissive carbon source for dicentric activation. The *GAL* promoter is strongly activated by galactose, even in the presence of raffinose, minimizing any lag time when the cells are switched from dicentric back to monocentric conditions.

RESULTS

Two conditional dicentric chromosomes have been constructed by inserting a conditional centromere (*CEN3* under control of the *GAL1* promoter) at the *HIS4* locus of chromosome III, 45 kb away from the existing centromere (Hill and Bloom, 1989; Fig. 1). In one strain (J178#4), the conditional centromere sequence is inverted relative to the endogenous centromere; in the second strain (J178#7), the two centromeres are in the same orientation. Growth on galactose inactivates the conditional centromere and the chromosome remains functionally monocentric. Growth on medium containing glucose activates the conditional centromere and results in a dicentric chromosome.

When the conditional centromere is activated by exposure to glucose, monocentric derivatives arise in the population. The recovery of viable cells following dicentric activation in several different strains is presented in Table 2. The percentage of cells able to generate colonies on glucose (dicentric) is expressed relative to the number of colonies on galactose (monocentric). In both conditional centromere orientations, about 58% of the cells with an active dicentric chromosome can give rise to colony forming units.

The colony morphology of monocentric derivatives is heterogeneous in size and shape. In addition, individual colonies, when replica-plated onto selective medium (lacking uracil), show a significant amount of sectoring for the URA phenotype.

These observations indicate that multiple rearrangements can occur in a single colony, either because the dicentric chromosome does not break in every cell cycle, or because the dicentric chromosome can be regenerated after it has broken.

Generation of monocentric derivatives is dependent on the RAD52 gene product

We have constructed dicentric strains that are deficient in the *RAD52* gene product (J178#4-20 and J178#7-20), which is required for double strand break repair during mitosis (Orr-Weaver et al., 1981). Dicentric viability is decreased dramatically in the absence of *RAD52* (Table 2, 55-60% to 1-2% in both centromere orientations).

Physical analysis of monocentric derivatives confirms that the predominant chromosome repair pathway is absent in the *rad52* mutant strain. Recombination between the two centromeres of the dicentric chromosome is the primary event in generating monocentric derivatives and can be detected by monitoring centromeric restriction fragments (Hill and Bloom, 1989; Fig. 3A). Digestion of DNA with the restriction enzyme *Bam*HI produces two *CEN3*-containing fragments. One of these is an 865 bp fragment containing *GALCEN3*, while the

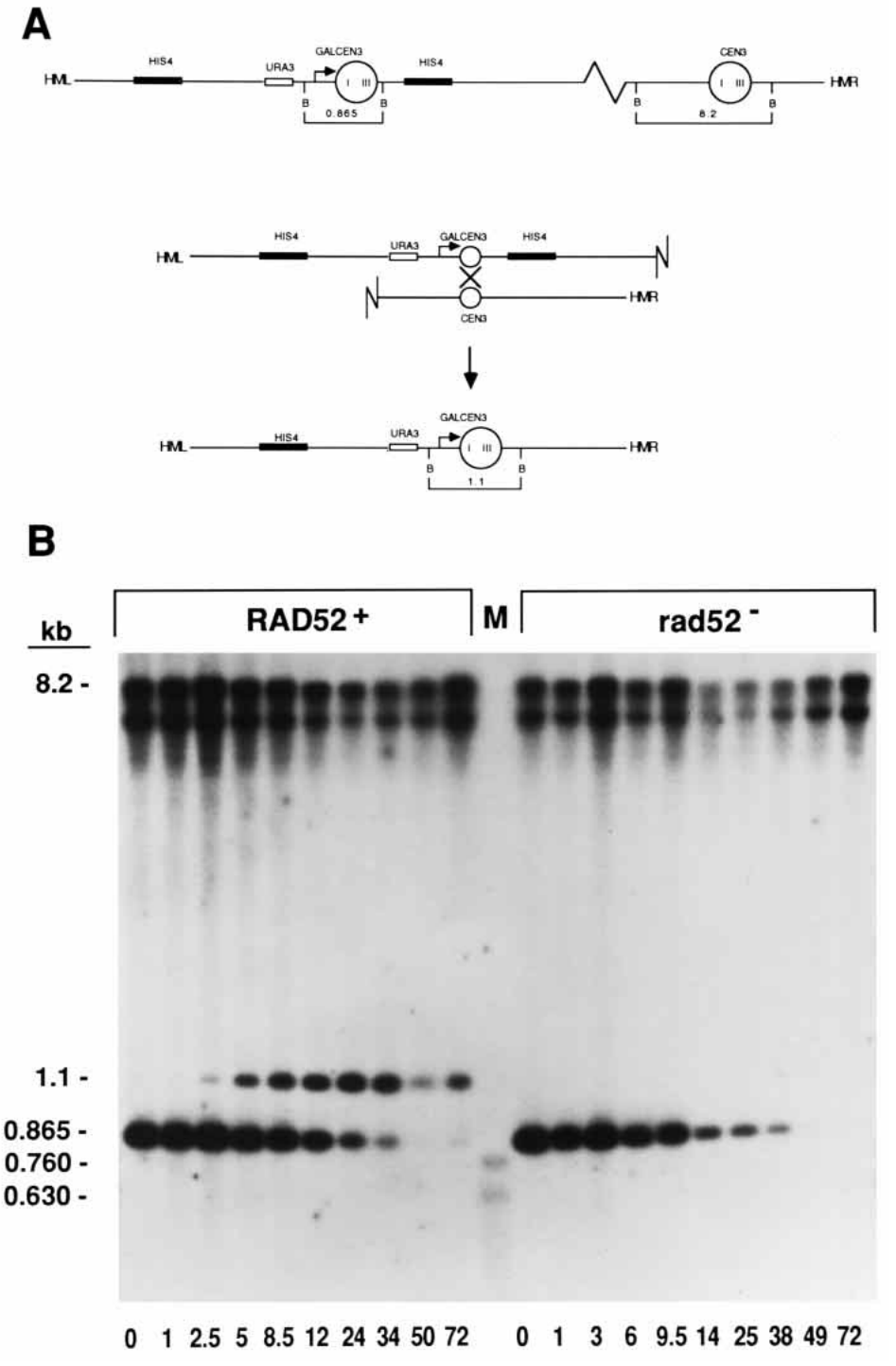

Fig. 3. Monocentric derivatives generated by recombination between *CEN3* repeats. (A) Recombination between the centromeres is depicted after a chromosome break has occurred. In the dicentric parental chromosome, *Bam*HI fragments containing the *CEN3* sequence are 865 bp and 8.2 kb. After recombination, the derivative chromosome fragment of 1.1 kb containing the *CEN3* sequence is generated. (B) A Southern analysis of chromosomal DNA over time after activating the dicentric chromosome. DNA was digested with the restriction enzyme *Bam*HI and probed with a radiolabelled fragment containing the *GALCEN3* sequence. Unrearranged chromosomes contain two *CEN3* fragments of 8.2 kb and 865 bp. Centromere recombination produces a *CEN3* fragment of 1.1 kb. A genomic *GAL* fragment of about 7 kb is also detected. Numbers indicate the time, in hours, after activation of the dicentric chromosome. M, DNA size standards (kb).

Table 2. Dicentric viability in wild-type and *rad52* mutant strains

Strain	Centromere orientation	RAD52 allele	HIS4* popout	Viability (%)
J178#7	Direct	+	+	58
J178#4	Inverted	+	+	58
J178#7-20	Direct	–	+	1
J178#4-20	Inverted	–	+	2
J178#27	Direct	+	–	55
J178#24	Inverted	+	–	64
J178#27-20	Direct	–	–	0.04
J178#24-20	Inverted	–	–	0.04

* + and – refer to the presence and absence, respectively, of duplicated *HIS4* sequences that mediate the ability of the conditional centromere to popout at the *HIS4* locus.

Dicentric viability is measured by comparing the number of colony forming units on glucose (dicentric) relative to galactose (monocentric) plates.

other is an 8.2 kb fragment containing the wild-type copy of *CEN3*. Monocentric recovery via centromere recombination results in the generation of a 1.1 kb fragment containing the *CEN3* sequence. The results of *Bam*HI restriction analysis of samples taken during a time-course of dicentric activation are shown in Fig. 3B. In the *RAD52* strain, the 1.1 kb derivative is evident after 2.5 hours on glucose. This band increases in intensity, accompanied by gradual loss of the 865 bp (conditional centromere) fragment. In the *rad52* mutant strain, loss of the 865 bp *GALCEN3* fragment also occurs. In contrast to RAD^+ however, this loss is not accompanied by generation of the 1.1 kb *CEN3* fragment. These data provide physical evidence that centromere recombination relies on a *RAD52* dependent repair pathway.

The low fraction of viable cells in the *rad52* mutant strains (1-2%) results primarily from recombination between the duplicated *HIS4* fragment at the site of centromere integration (see Fig. 1). The product of this event is evident in both RAD^+ and rad^- strains (Fig. 4B) and is consistent with previous demonstrations of *rad52* independent recombinatorial pathways (Jackson and Fink, 1981). This event leads to the generation of monocentric chromosomes that may not have experienced dicentric breakage, but simply reflect alternative pathways of genetic recombination.

To confirm that chromosome breakage is the primary lesion incurred upon dicentric activation, a dicentric chromosome was constructed in which there was no duplication of the *HIS4* gene (Fig. 2). In J178#24 and J178#27, viability of cells upon

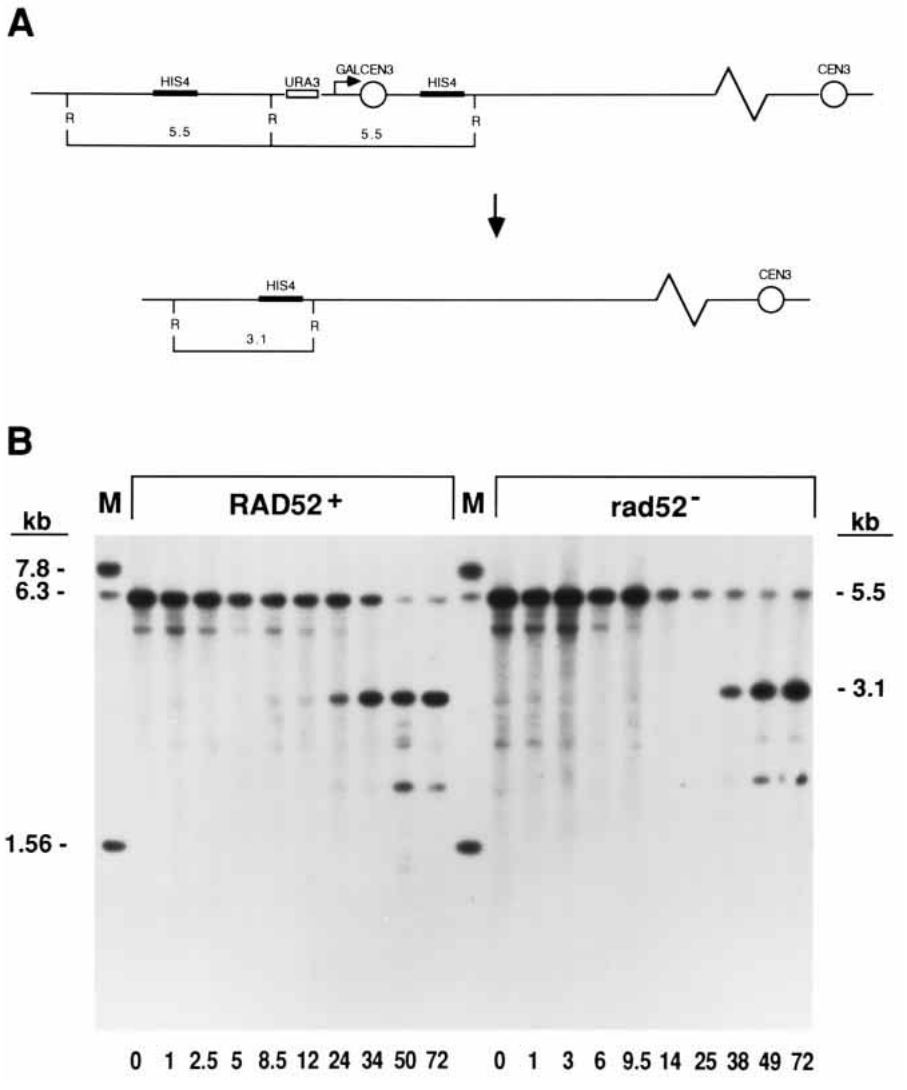

Fig. 4. Monocentric derivatives generated by recombination between *HIS4* repeats. (A) Recombination between HIS4 repeats generates a monocentric chromosome containing the wild-type centromere. (B) The same time-course as in Fig. 3B. Chromosomal DNA was cut with the restriction enzyme *Eco*RI and probed with the 1.56 kb *Sal*I fragment of *HIS4*. The unrearranged chromosome contains two comigrating *HIS4* fragments both of which are 5.5 kb. Recombination between the *HIS4* repeats produces a *HIS4* fragment of 3.1 kb. Numbers indicate the time, in hours, after activation of the dicentric chromosome. M, DNA size standards (kb).

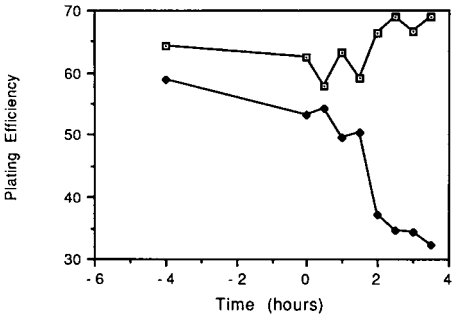

Fig. 5. Time-course of dicentric lethality in a *rad52* mutant strain. The decrease in viability after dicentric activation of dicentric strain J178#7-20 was measured over the course of the cell cycle by monitoring plating efficiency. Cells were synchronised by the addition of alpha factor at −4 hours, as described in Materials and Methods. Cells were released from alpha factor at the 0 hour timepoint. (□) is the control culture, maintained in galactose; (♦) is the experimental culture, grown in raffinose.

activation of the dicentric chromosome is similar to that of strains in which the *HIS4* recombination event can occur. However, when these strains are deficient in the *RAD52* repair gene, viability of cells upon activation of the dicentric chromosome drops to 0.04% (Table 2). This is a 50- to 100-fold decrease compared with strains in which the *HIS4* duplication is intact, and indicates that DNA double-strand breakage accounts for more than 99% of the genetic events in these cells. The molecular rearrangements which give rise to the 0.04% of viable colonies were examined to determine the nature of the rearrangement event. Restriction mapping of isolated DNA by Southern analysis indicated that in most derivatives, one of the two centromeres had been deleted. From a total of 245 isolates, 243 had suffered deletions of either the wild-type *CEN3* or the conditional *GALCEN3* centromere. These deletions are diverse in position and size, ranging from 230 bp to over 4 kb. In both direct and inverted centromere orientations, these deletions preferentially involve the conditional centromere, which accounts for approximately 80% of these rearrangements. Sequence analysis of the repair junctions of some of these deletions indicates that they are mediated by non-homologous repair mechanisms (Kramer et al., 1994). Taken together, these data indicate that the dicentric chromosomes are broken, resulting in excision of the one of the two centromeres. The repair events may involve centromere recombination (as shown in Fig. 3) or excision and healing by non-homologous mechanisms (Kramer et al., 1994). That these monocentric products are not observed until the dicentric chromosome is activated indicates that the exertion of mitotic forces at both centromeres is responsible for the chromosome lesions. The ability to synchronize cells and regulate centromere activity allows us to harness these spindle forces and determine their time of action.

Commitment to chromosome breakage occurs during G_2/M phase

The lethality of dicentric chromosomes in a *rad52* mutant provides a simple viability assay for examining the commitment to chromosome breakage during the cell cycle. The *rad52*

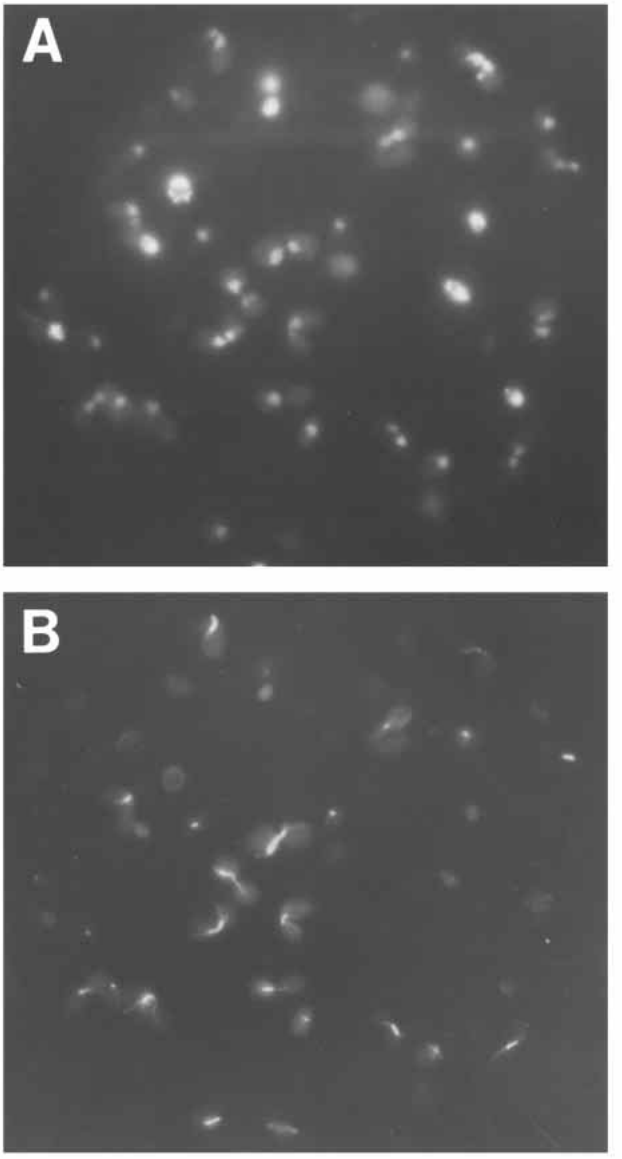

Fig. 6. Staining of tubulin and DNA in cells after activation of the dicentric chromosome. Cells were stained for tubulin and DNA as described in Materials and Methods. (A) DNA staining with Hoechst 33258 shows many cells with single large buds and the nucleus localized in or very near the neck of the bud. (B) Tubulin staining reveals the presence of short to mid-sized spindles traversing the aperture of the budding cell.

mutant strain J178#7-20 was synchronized with alpha factor, which arrests cells in G_1, and simultaneously transferred to raffinose, to activate the dicentric chromosome. Raffinose was used as the carbon source in place of glucose to minimize the lag time for centromere inactivation upon plating back to galactose (see Materials and Methods). After a 4 hour incubation, alpha factor was washed out of the culture and cells were released into raffinose (dicentric conditions). At 30 minute intervals, cells were switched to monocentric conditions and plating efficiencies on galactose were determined. A control culture was treated with alpha factor, but maintained with a functionally monocentric chromosome over the time-course

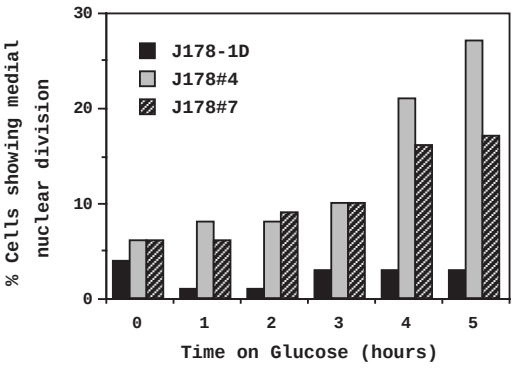

Fig. 7. Increase of cells in medial nuclear division upon activation of the dicentric chromosome. Strain J178-1D and both dicentric derivatives, J178#4 and J178#7, were switched from galactose (monocentric) to glucose (dicentric) medium and analysed at hourly intervals. Cells were fixed in 70% ethanol at room temperature for 20 minutes, stained with the fluorescent DNA stain DAPI and scored for medial nuclear division (budded cells with DNA spanning the neck of the bud). Between 250 and 500 cells were counted for each timepoint.

examined. The results from six independent experiments were pooled and are shown in Fig. 5.

Plating efficiency of the control culture remained at 60-70% over the course of the experiment. However, plating efficiency of the dicentric culture decreased precipitously from 1.5 hours to 2.5 hours (73% of the total decline over a 3.5 hour period). During this time, most cells had single large buds, with DNA localized at or near the neck of the mother/bud, corresponding to the G_2/M phase of the cell cycle and characteristic of the dicentric mitotic delay (see below; also Hill and Bloom, 1989; Neff and Burke, 1992).

The remaining viable cells (34%) indicate that not every cell suffers a lethal event upon activation of the dicentric chromosome. This suggests that dicentric chromosomes are not broken in every cell cycle and is consistent with an analysis of *rad52*, dicentric strains grown on glucose. Most of the cells (86%) formed microcolonies, ranging in size from 2 to 16 cells (1-3 cell divisions). These results confirm that a lethal event does not occur in every cell cycle and that in some cases the dicentric chromosome may be able to segregrate without breakage.

Activation of a dicentric chromosome results in a cell cycle delay that coincides with the commitment to lethality

The phenotype observed upon commitment of *rad52*, dicentric cells to lethality is reminiscent of the mitotic delay reported for wild-type cells containing an active dicentric chromosome (Hill and Bloom, 1989; Bloom et al., 1989; Neff and Burke, 1992). In wild-type, dicentric cells a transient large-budded phenotype is observed upon 1.5 to 2 generations of growth following dicentric activation. To assess the precise mitotic stage coincident with the mitotic delay and hence the commitment to lethality, several morphological and biochemical parameters of cell cycle position were examined.

The dicentric chromosome in strains J178#4 and J178#7 was activated by switching a logarithmically growing culture from selective galactose into glucose medium for 4.5 hours (approximately 2 cell-cycle doublings). Cells were fixed and stained with DAPI (4,6-diamidino-2-phenylindole), a fluorescent dye for DNA, and stained with a fluorescein-conjugated antibody for tubulin (Fig. 6). In many of the large budded cells, the nucleus is located in or at the neck of the budding cell, accompanied by mid-sized spindles. This stage of the cell cycle is classically known as medial nuclear division (Pringle and Hartwell, 1981). For each of the dicentric strains, there was a 3- to 4-fold increase in the percentage of cells in medial nuclear division (Fig. 7). To further delimit the phase in the cell cycle in which the delay occurs, cultures were analysed by flow cytometry. The budded phenotype is correlated with an increase in the number of cells that contain G_2 levels of DNA, indicating that the delay follows DNA replication.

The primary biochemical indicator of cell cycle stage is the protein kinase p34^{cdc28}. The activity of this protein kinase is elevated during M phase, when it is complexed with M-phase cyclins, and drops precipitously at the onset of anaphase, when

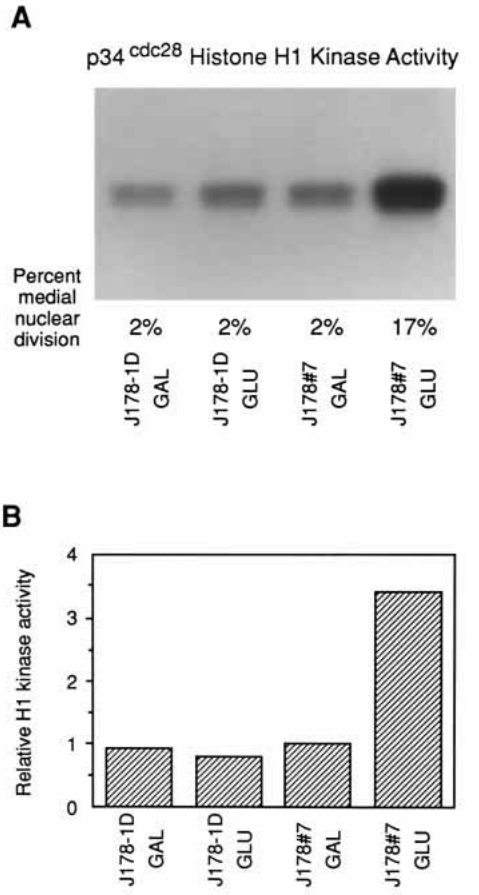

Fig. 8. Relative p34^{cdc28} histone H1 kinase activity after activation of the dicentric chromosome. Comparison of p34^{cdc28} histone H1 kinase activity in J178-1D and J178#7 in galactose, and after a 4 hour incubation in medium containing glucose (active dicentric), at 32˚C. (A) Sample assay, demonstrating the transfer of phosphate from [γ-^{32}P]ATP by p34^{cdc28}. Numbers below show the percentage of cells from the corresponding samples in medial nuclear division. (B) The values of three experiments (derived from scintillation counting) were pooled and expressed relative to that of J178#7 on galactose, which was arbitrarily defined as 1.0.

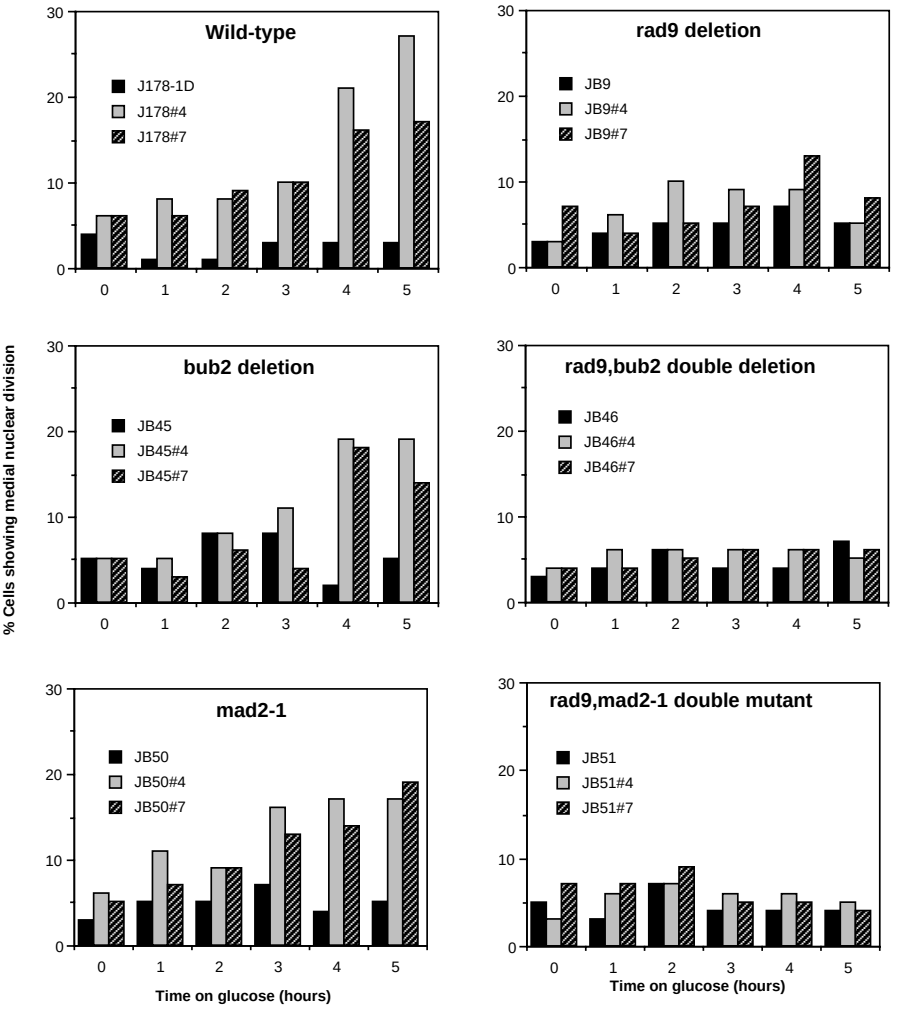

Fig. 9. Percent cells in medial nuclear division after activation of the dicentric chromosome in cell cycle checkpoint mutants. Asynchronous cultures in logarithmic growth were switched from galactose (monocentric) to glucose (dicentric) medium to activate the dicentric chromosome. For each strain, the nondicentric parental control and both conditional dicentric derivatives were tested. Cells were collected at hourly intervals, fixed in 70% ethanol at room temperature for 20 minutes, stained with the fluorescent DNA stain DAPI and scored for percentage of cells in medial nuclear division (budded cells with DNA spanning the neck of the bud). At least 500 cells were counted for each timepoint. The wild-type strain J178-1D and its two conditional dicentric derivatives were included for ease of comparison.

cyclins are degraded. The kinase activity of this enzyme was examined in cells in which the dicentric chromosome had been activated. Cells were grown to logarithmic phase and switched to glucose to activate the dicentric chromosome. After 4 hours, a sample of cells from each culture was fixed and stained with DAPI to monitor the percentage of cells in medial nuclear division. The remaining cells were used to isolate $p34^{cdc28}$. Results of the kinase assay and cell analysis are shown in Fig. 8. For the J178-1D parental control strain, the level of kinase activity was similar for cultures grown on both galactose and glucose. For the dicentric strain J178#7, however, a threefold increase in $p34^{cdc28}$ Histone H1 kinase activity was detected after the 4 hour incubation in glucose (activation of the dicentric chromosome). There was also an increase in the number of cells in medial nuclear division, as indicated in Fig. 8. The short mitotic spindles and elevated $p34^{cdc28}$ kinase levels observed following dicentric activation are typical of cells blocked prior to anaphase onset, which in yeast is characterized by dramatic elongation of the spindle apparatus into the bud.

The dicentric delay is dependent upon the RAD9 checkpoint gene

The transient mitotic delay following dicentric activation may represent the response to a number of cellular aberrations, including DNA damage, spindle abnormalities or chromosome attachment defects. The ability of cells to delay their cyclic progression in response to DNA damage or microtubule aberrations has been well documented and checkpoint genes responsible for these delays have been identified (*RAD9*, Weinert and Hartwell, 1988; *MAD1*,*2* and *3*, Li and Murray, 1991; *BUB1*,*2* and *3*, Hoyt et al., 1991). We have used mutations in these checkpoint pathways to identify the genetic lesions responsible for the mitotic delay. The role of the *RAD9*, *BUB2* and *MAD2* response pathways was investigated by constructing conditional dicentric chromosomes in strains that are mutant for these genes. These strains were tested for viability and cells were examined for the pause after activating the dicentric chromosome.

As shown in Table 3, viability in the *rad9* mutant strain, after activating the dicentric chromosome, was not impaired. In both centromere orientations, between 60 to 70% of the cells were able to generate colonies on glucose. The viability of *bub2* and *mad2* mutant strains, containing the active dicentric chromosome, is slighty reduced but comparable to the isogenic wild-type strains. In *rad9*,*bub2* and *rad9*,*mad2* double mutants, viability of cells containing the active dicentric chromosome was similar to the *rad9* mutant alone. Thus, upon activation of a dicentric chromosome, viability is not severely impaired in any of these checkpoint mutants.

Upon activation of the dicentric chromosome, cells were examined for the characteristic mitotic delay. Cultures in mid-logarithmic growth were shifted from galactose to glucose medium and examined microscopically at hourly intervals to determine the percentage of cells in medial nuclear division (Fig. 9). Neither the *mad2-1* point mutant nor the *bub2* deletion had a dramatic affect on generating the delay. Within 4 hours of activating the dicentric chromosome, a 3- to 4-fold increase of cells in medial nuclear division was observed, similar to levels in wild-type dicentric strains. Alternatively, the *rad9* deletion mutant exhibited a less pronounced delay after activating the dicentric chromosome. Except for one (4 hour) timepoint, the number of cells in medial nuclear division for both dicentric strains remained below 10%. The *rad9,bub2* and *rad9,mad2* double mutants appeared to completely abolish the delay.

The single mutants were also tested for changes in $p34^{cdc28}$ kinase activity after a 4 hour incubation in glucose and the results are shown in Fig. 10. Both the *bub2*Δ and the *mad2-1* point mutants showed a threefold increase in kinase activity after activation of the dicentric chromosome, as well as an increase of cells in medial nuclear division, similar to the wild-type strain. The *rad9*Δ mutation, in contrast, showed a much smaller increase in kinase activity and again, a very slight increase in cells at medial nuclear division. These data suggest that the dicentric phenotype is predominantly dependent on the *RAD9* gene and may be effected through the elevation of $p34^{cdc28}$ kinase activity. In addition, this result indicates that monocentric derivatives are generated through DNA lesions which are monitored by the *RAD9* checkpoint pathway.

The effect of the *RAD9* gene in response to dicentric chromosome activation was further investigated by examining the viability of cells after activating the dicentric chromosome in *rad9,rad52* double mutants. As indicated in Table 3, viability of this strain (1%) is similar to that of the *rad52* single mutant (Table 2). The formation of microcolonies was also examined in the double mutant. In the *rad9,rad52* double mutant, microcolonies are generally larger than in the *rad52* single mutant and greater than 50% have more than 16 cells in each colony (compared to 13% for the *rad52* mutant alone). In the *rad52* single mutant, a broken dicentric chromosome cannot be repaired and is lethal to the cell. In the double mutant, the *rad9* defect would allow cells with the broken chromosome to continue through the cell cycle. However, since the cells cannot repair the damaged DNA, due to absence of the *rad52* gene, this will eventually lead to lethality in subsequent cell cycles.

Table 3. Dicentric viability in cell cycle checkpoint mutants

Strain	Relevant genotype	% Viability of different centromere orientations	
		Direct	Inverted
J178	Wild type	58	58
JB9	*rad9*Δ	63	65
JB45	*bub2*Δ	46	52
JB50	*mad2-1*	47	45
JB46	*rad9*Δ,*bub2*Δ	72	73
JB51	*rad9*Δ,*mad2-1*	73	72
JB9-20	*rad9*Δ,*rad52*Δ	1	1

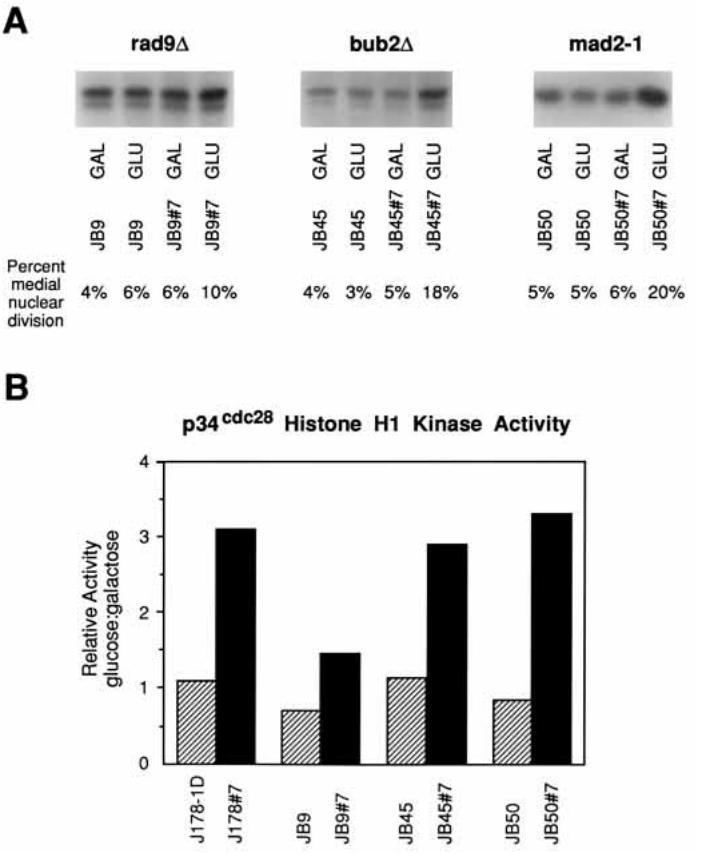

Fig. 10. Relative $p34^{cdc28}$ histone H1 kinase activity after dicentric activation in cell cycle checkpoint mutants. Comparison of $p34^{cdc28}$ histone H1 kinase activity of various checkpoint mutant strains in galactose and after a 4 hour incubation in medium containing glucose (active dicentric). (A) Sample assays, demonstrating the transfer of phosphate from [γ-^{32}P]ATP by $p34^{cdc28}$. Numbers below show the percentage of cells in medial nuclear division from the corresponding samples. (B) At least two experiments were done for each strain and the values derived from scintillation counting were pooled. For each strain, the change in kinase activity on glucose relative to galactose was determined. Hatched bars represent relative kinase activity of the parental strains, which do not contain the conditional centromere; solid bars indicate the relative kinase activity of strains containing the conditional dicentric chromosome. JB9(*rad9*Δ); JB45(*bub2*Δ); JB50(*mad2-1*). The wild-type strain J178-1D and its dicentric derivative J178#7 were included for ease in comparison.

DISCUSSION

We have utilized a dicentric chromosome breakage assay to monitor mitotic forces in the budding yeast *S. cerevisiae*. Several dicentric strains have been constructed in which the *RAD52* gene, which is required for repair of DNA double-strand breaks, has been deleted. In these strains, the ability of cells to recover from the dicentric insult and generate colonies is decreased up to one-thousandfold relative to wild-type dicentric strains. In addition, centromere recombination, a major repair event which generates monocentric derivatives, is completely abolished. Thus the generation of monocentric

derivatives is mediated through DNA double-strand break intermediates.

The lethality of dicentric activation in a *rad52* mutant has been used to monitor the time of spindle force production. Activation of the conditional centromere at start (alpha factor arrest point) leads to a decline in cell viability 1.5 to 2.5 hours after start. Cells exhibit a large budded phenotype with DNA localized to the neck of the bud, similar to the phenotype observed after the delay induced by activation of the dicentric chromosome in an asynchronous population. Since these cells are delayed with elevated $p34^{cdc28}$ H1 kinase activity, it is suggestive that centromere function is activated during late G_2/M phase, prior to anaphase onset.

The viability assay used to monitor recovery from activation of the dicentric chromosome is not a direct measure of chromosome breakage, but monitors the commitment to a lethal event. It is possible, for instance, that centromere function and/or attachment of kinetochores to spindle microtubules becomes irreversible by transcriptional activation of the *GAL* promoter during G_2 or M phase. This would then result in cell lethality via chromosome breakage or nondisjunction at a later time in the cell cycle, such as anaphase.

We have used mutations in several cell cycle checkpoint genes to distinguish whether the mitotic delay in response to activation of a dicentric chromosome originates from the conditional centromere itself or reflects downstream events involving chromosome and/or spindle anomalies. Several genes which are responsible for cell cycle delays in response to DNA damage (*RAD9*) and microtubule defects (*BUB2* and *MAD2*) have been examined to determine whether they are involved in activation of the dicentric delay. Dicentric strains mutant in either the *BUB2* or *MAD2* genes are competent to induce the dicentric delay. However, in *rad9* mutant strains, activation of the dicentric chromosome does not result in the characteristic increase of cells in medial nuclear division. In addition, the increase in $p34^{cdc28}$ kinase activity following dicentric activation is less than 1.5-fold in *rad9* mutants, compared to a threefold increase in activity observed in wild-type strains. Since the *RAD9* gene acts by monitoring DNA damage, at least part of the response pathway is likely to result from a broken dicentric chromosome. In addition, the fact that both the increase of cells in medial nuclear division, as well as the elevated $p34^{cdc28}$ activity, are under control of the *RAD9* gene, indicates that the dicentric delay is induced by chromosomes that are broken prior to anaphase onset.

Interestingly, the cell cycle delay induced upon activating the dicentric chromosome does not appear to be required for cell viability. In *rad9* mutants, as well as the double mutants, *rad9,bub2* and *rad9,mad2*, the delay is reduced significantly, yet there is no affect on viability upon activating the dicentric chromosome. Neff and Burke (1992) have also reported no decrease in viability upon dicentric activation in a *rad9*, dicentric mutant (40% viability in *rad9*, dicentric strains versus 11% in wild-type, dicentric strains). Their *rad9,bub2* double mutant strains exhibited slower growth upon activation of the dicentric chromosome, but only a twofold decrease in viability. The high viability upon activation of dicentric chromosomes in *rad9* mutants may indicate that the broken dicentric chromosome is repaired quickly and does not require the delay, or that repair of broken dicentric chromosomes may occur during the subsequent cell cycle.

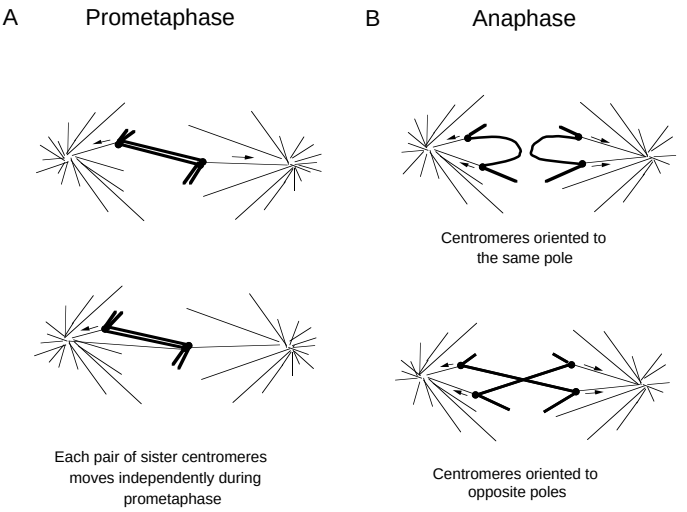

Fig. 11. Dicentric chromosome breakage resulting from centromere movements during prometaphase or anaphase. A schematic diagram representing possible centromere attachments of a dicentric chromosome to the mitotic spindle during prometaphase and anaphase. (A) During prometaphase, the two centromeres will independently form bipolar attachments to the mitotic spindle. Upper panel: a dicentric chromosome in which the two centromeres have formed initial monopolar attachments to opposite spindle poles. Lower panel: a dicentric chromosome in which one centromere pair has formed a bipolar spindle attachment, while the other still has only a monopolar attachment. Centromere movements toward opposite spindle poles, as indicated by the arrows, may subsequently result in chromosome breakage. (B) When the two centromeres of a dicentric chromosome have successfully formed bipolar attachments, centromere movement at anaphase may result in chromosome breakage. When the two centromeres of each chromosome are oriented toward the same spindle pole (upper panel), chromosome segregation is normal. However, when they are oriented toward opposite spindle poles (lower panel), anaphase bridges are formed and chromosome breakage can occur.

The results presented herein are consistent with a model in which yeast centromeres may actually be subject to mitotic forces during prometaphase, resulting in chromosome breakage prior to sister chromatid separation. Recent microscopic observations have documented extensive prometaphase chromosomal movements in yeast (Palmer et al., 1989). The two centromeres of a single dicentric chromosome could move independently during the formation of bipolar spindle attachments. Tension along the intervening chromatin, as illustrated in Fig. 11A, could then result in chromosome breakage. For instance, if the initial attachments of the two centromeres are made to separate spindle poles (top panel, Fig. 11A), the two centromeres may move in opposite directions, resulting in tension and chromosome breakage. Similarly, if one of the centromeres has already formed a stable bipolar attachment while the other is undergoing prometaphase movements (lower panel, Fig. 11A), tension and chromosome breakage may occur. Chromosomes which form bipolar attachments may also break during anaphase, if the centromeres of a single chromatid are attached to opposite spindle poles, as shown in Fig. 11B.

In higher eukaryotes extensive movements of centromeres can be visualized during mitosis. During prometaphase, the

centromeres of duplicated chromosomes form bipolar attachments to the mitotic spindle. Initially, one of the sister centromeres becomes attached to spindle fibers of a single spindle pole. The chromosome then oscillates toward and away from the spindle pole to which this centromere is attached. Eventually, the second centromere of the duplicated chromosome becomes attached to spindle fibers emanating from the opposite spindle pole and the chromosome then migrates to the metaphase plate. The breakage of dicentric chromosomes prior to anaphase onset suggests that the machinery which produces such oscillatory movements of the centromere may also occur in budding yeast.

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