A Temperature-sensitive Calmodulin Mutant Loses Viability during Mitosis

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Abstract. Although rare, a recessive temperature-sensitive calmodulin mutant has been isolated in *Saccharomyces cerevisiae.* The mutant carries two mutations in *CMD1*, isoleucine 100 is changed to asparagine and glutamic acid 104 is changed to valine. Neither mutation alone conferred temperature sensitivity. A single mutation that allowed production of an intact but defective protein was not identified. At the nonpermissive temperature, the temperature-sensitive mutant displayed multiple defects. Bud formation and growth was delayed, but this defect was not responsible for the temperature-sensitive lethality. Cells synchronized in G1 progressed through the cell cycle and retained viability until the movement of the nucleus to the neck between the mother cell and the large bud. After nuclear movement, $<5\%$ of the cells survived the first mitosis and could form colonies when returned to permissive conditions. The duplicated DNA was dispersed along the spindle, extending from mother to daughter cell. Cells synchronized in G2/M lost viability immediately upon the shift to the nonpermissive temperature. At a semipermissive temperature, the mutant showed approximately a 10-fold increase in the rate of chromosome loss compared to a wild-type strain. The mitotic phenotype is very similar to yeast mutants that are defective in chromosome disjunction. The mutant also showed defects in cytokinesis.

Calmodulin is one of the mediators of Ca^{2+} signals induced by extracellular stimuli. It is known to regulate smooth muscle contraction (Stull, 1988) and glycogen breakdown in mammalian liver and skeletal muscle (Cohen, 1988), and has been implicated in many other processes including neurotransmitter release, regulation of cAMP levels, maintaining Ca^{2+} homeostasis, and regulation of microfilaments and microtubules (Cohen and Klee, 1988). A genetic analysis of calmodulin function has shown that calmodulin is also required for cellular proliferation. Deletion of the calmodulin gene in *Saccharomyces cerevisiae* (Davis et al., 1986), in *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987), or in *Aspergillus nidulans* (Rasmussen et al., 1990) is a lethal mutation.

The role calmodulin plays during cellular proliferation is unknown. Expression of calmodulin antisense RNA in mouse C127 cells causes a transient cell cycle arrest in G1 and mitosis (Rasmussen and Means, 1989). Consistent with a role in mitosis, calmodulin is localized to the mitotic apparatus in rat kangaroo PtK_2, Chinese hamster ovary cells (Welsh et al., 1979), and plant endosperm (Vantard et al., 1985). One hypothesis is that Ca^{2+}-calmodulin regulates a protein phosphorylation cascade that results in chromosome segregation (Dinsmore and Sloboda, 1988; Keith, 1987; Ratan and Shelanski, 1986). However, the Ca^{2+} transients proposed to trigger this cascade were recently found not to be required for chromosome segregation (Kao et al., 1990). Ca^{2+} transients do induce nuclear envelope breakdown (Kao et al., 1990) via Ca^{2+}-calmodulin-dependent protein kinase (Baitinger et al., 1990).

Calmodulin is also implicated in regulation of microfilaments. Calmodulin is associated with the actin-based stress filaments during interphase (Welsh et al., 1978). Calmodulin binds several actin-binding proteins including spectrin (Tanaka et al., 1991), caldesmon (Sobue et al., 1981) and myosin I (Howe and Mooseker, 1983). In proliferating liver cells, calmodulin may regulate a nuclear contractile system that includes actin (Bachs et al., 1990).

S. cerevisiae offers an excellent system for studying calmodulin function during cell proliferation. Calmodulin mutants can be readily constructed and the effects of mutant proteins on cell proliferation can be assessed in the absence of interference from wild-type calmodulin. Vertebrate calmodulin is an effective substitute for yeast calmodulin in vivo (Davis and Thorner, 1989; Ohya and Anraku, 1989*a*; Persechini et al., 1991) suggesting the essential function in yeast may represent a conserved role found in many mitotically active cells. The available wealth of information about the yeast cell cycle provides a large framework within which new results can be interpreted.

Previously, we undertook a mutational analysis of calmodulin in *S. cerevisiae* to explore the structural requirements for calmodulin function. Several mutant calmodulins were constructed by site-directed mutagenesis. Surprisingly, mutants in which all the Ca^{2+}-binding sites have been inactivated can support the growth of yeast cells (Geiser et al.,

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1991), suggesting that calmodulin can perform its required function without binding Ca^{2+}. A mutant form of vertebrate calmodulin in which all the Ca^{2+}-binding loops were inactivated (Geiser et al., 1991) can also support the growth of yeast cells. Thus, the ability to perform the Ca^{2+}-independent function required for cell proliferation has been conserved. Another mutational analysis has shown that half-calmodulins can support growth (Sun et al., 1991).

In this and the accompanying paper (Brockerhoff and Davis, 1992), we describe two approaches to identify the functions of calmodulin during cell proliferation. Characterization of a temperature-sensitive calmodulin mutant indicates calmodulin is required for chromosome segregation and may specifically participate in disjunction. The immunolocalization of calmodulin implicates calmodulin in the polarized growth required to form a bud (Brockerhoff and Davis, 1992).

Materials and Methods

Media and Buffers

SD complete medium is SD medium (Sherman et al., 1986) supplemented with 0.1% casamino acids (Difco Laboratories Inc., Detroit, MI), 50 μg/ml adenine, 50 μg/ml tryptophan, and 25 μg/ml uracil. S complete medium is SD complete lacking glucose. SD −ura is SD complete medium lacking uracil. Other media for growth of *S. cerevisiae* and *E. coli* were as described (Geiser et al., 1991). λdil contains 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM $MgSO_4$, and 0.01% gelatin. PBS contains 12 mM phosphate buffer, pH 7.0, 0.17 M NaCl, and 3.4 mM KCl.

Plasmids and Strains

Plasmid pTD55 is a yeast integrating vector containing *URA3* and *ade3Δ-100* and was constructed from plasmid pTD54. In plasmid pTD54, a 5.4-kb BamHI-SalI fragment from plasmid pDK202 (Koshland et al., 1985) containing the *ADE3* gene (Staben and Rabinowitz, 1986) was inserted into plasmid pTD53 (Geiser et al., 1991). To construct plasmid pTD55, plasmid pTD54 was digested with XhoI and ClaI, and the ends were filled in and ligated such that a 2.2-kb internal fragment of the *ADE3* gene extending from the XhoI site to the first ClaI site is deleted. (The second ClaI site in the *ADE3* gene is methylated.)

Plasmid pTD56 is a pBR322-based plasmid that carries the 2-μm origin of replication, *CMD1*, *LYS2*, *ADE3*, and *bla*. Plasmid pTD59 contains *CMD1*, *ARS1*, *CEN4*, *URA3*, and *bla*. The construction of both plasmids was described previously (Geiser et al., 1991).

E. coli K-12 strain AB1886 has the genotype F⁻, *uvrA6*, *thr-1*, *leu-6*, *proA2*, *his-4*, *thi-1*, *argE3*, *lacY1*, *galK2*, *ara-14*, *xyl-5*, *mtl-1*, *tsx-33*, *str31*, *supE44*, λ- and was received from A. J. Clark (University of California, Berkeley, CA). The *S. cerevisiae* strains are listed in Table I. All yeast strains are derivatives of strain W303 (Wallis et al., 1989). Construction of the strains is described below. Strain TDY49 is a diploid formed by crossing W303-1A with W303-1B and then integrating the *cmd1Δ::TRP1* construct (Davis et al., 1986) and the *lys2Δ::HIS3* construct (Davis and Thorner, manuscript in preparation) as described (Davis et al., 1986). Homozygosis of the *lys2Δ::HIS3* allele was selected on plates containing α-aminoadipate (Sherman et al., 1986) and confirmed by a Southern blot analysis.

Strains carrying a deletion of the *ADE3* gene were constructed as follows. TDY49 was transformed with plasmid pTD55 linearized with EcoRI, and

Table I. Strains

Strain	Genotype	Reference
CRY1	*MATa; ade2-1oc; can1-100; his3-11, 15; leu2-3,112; trp1-1; ura3-1*	Robert Fuller
TDY49	*Mata/Matα; ade2-1oc/ade2-1oc; can1-100/can1-100; cmd1Δ::TRP1/CMD1; his3-11,15/his3-11,15; leu2-3,112/leu2-3,112; lys2Δ::HIS3/lys2Δ::HIS3, trp1-1/trp1-1, ura3-1/ura3-1*	This study
TDY49-5B	*MATα; ade2-1oc; can1-100; cmd1Δ::TRP1; his3-11, 15; leu2-3, 112, lys2Δ::HIS3; trp1-1, ura3-1*	This study
TDY52	TDY49 with plasmid pTD55 integrated at *ADE3*	This study
TDY52-2C	*MATa; ade2-1oc, ade3Δ-100; can1-100; his3-11,15; leu2-3,112; lys2Δ::HIS3; trp1-1; ura3-1*	This study
TDY52-2D	Same as TDY52-2C but *MATα*	This study
TDY52-3D	*MATα; ade2-1oc; ade3Δ-100; can1-100; CMD1; leu2-3,112, lys2Δ::HIS3; trp1-1; ura3-1; unknown cs*	This study
TDY55	TDY52-2C × TDY49-5B	This study
TDY55-5D	*MATa; ade2-1oc, ade3Δ-100; can1-100; cmd1Δ::TRP1; his3-11,15; leu2-3,112; lys2Δ::HIS3; trp1-1; ura3-1*	This study
TDY57	*MATα; ade2-1oc; ade3Δ-100; can1-100; cmd1-1; leu2-3,112, lys2Δ::HIS3; trp1-1; ura3-1; unknown cs*	This study
TDY62	TDY57 × CRY1	This study
TDY62-1A	*MATα; ade2-1oc; can1-100; his3-11, 15; leu2-3,112; trp1-1; ura3-1*	This study
TDY62-13A	*MATα; ade2-1oc; ade3Δ-100; can1-100; cmd1-1; leu2-3,112; trp1-1; ura3-1*	This study
JGY44	TDY62-13A × CRY1	This study
JGY44-2A	*MATa; ade2-1oc; can1-100; cmd1-1; his3-11, 15; leu2-3,112; trp1-1; ura3-1*	This study
TDY68	TDY62-13A plus *cyh2ʳ*	This study
TDY70	TDY68 × TDY62-1A	This study
TDY70-30A	*MATα; ade2-1oc; ade3Δ-100; can1-100; CMD1; cyh2ʳ; leu2-3,112; trp1-1; ura3-1*	This study
TDY70-30C	*MATa; ade2-1oc; ade3Δ-100; can1-100; cmd1-1; cyh2ʳ; leu2-3,112; trp1-1; ura3-1*	
TDY92	*MATa/MATα; ade2-1oc/ade2-1oc; ADE3/ade3Δ-100; can1-100/can1-100; cmd1-1/cmd1-1; cyh2ʳ;/cyh2ʳ; his3-11,15/HIS3; HIS4/his4::CYH2ʳ; leu2-3,112/leu2-3,112; trp1-1/trp1-1, ura3-1/ura3-1*	This study
TDY93	Same as TDY92 except *CMD1/CMD1*	This study
TDY94	Same as TDY92 except *CMD1/cmd1-1* and *his4::CYH2ˢ* is on the same chromosome as *MATa*	This study
TDY96	Same as TDY92 but another isolate	This study

ura$^+$ transformants with pTD55 integrated at *ADE3* were selected to give strain TDY52. TDY52 was sporulated and haploid spores containing the integrated pTD55 were identified. Since these haploid cells had the entire plasmid pTD55 integrated, they carried both *ade3Δ* and *ADE3*. Strains TDY52-2C, TDY52-2D, and TDY52-3D, which carry only the *ade3Δ*, were derived from the haploid integrants and identified as strains resistant to 5-fluoroorotic acid (Boeke et al., 1987) and white on low ade plates. The genotypes of the strains were checked by a Southern blot analysis.

Strain TDY57 is strain TDY52-3D in which the *cmd1-1* gene was integrated by a two step transplacement procedure (Geiser et al., 1991). Strain TDY57 carries a cold-sensitive mutation of unknown origin. This mutation is not present in any of the strains listed below the TDY62 entry in Table I.

Strain TDY68 was selected as a spontaneous mutant of strain TDY62-13A resistant to 1 μg/ml cycloheximide. To confirm the mutation to resistance was in the *CYH2* gene, I crossed TDY68 with strain XS144-S19 (received from the Yeast Genetic Stock Center, University of California, Berkeley, CA), known to carry *cyh2^r* (Hawthorne and Mortimer, 1960). Resistance to cycloheximide is recessive. The diploid was resistant to 1 μg/ml cycloheximide as expected only if both haploids carry *cyh2^r*. The diploid was sporulated and 18 tetrads dissected. All 52 of the viable spores were resistant to cycloheximide indicating that the mutation conferring resistance to cycloheximide in strain TDY68 is closely linked to *cyh2*. This result combined with the resistance of the diploid strain led me to conclude that the mutation in TDY68 is *cyh2^r*.

For flow cytometry, strains TDY70-30A and TDY70-30C were converted to ρ^- strains, which lack detectable mitochondrial DNA, by treatment with ethidium bromide (Sherman et al., 1986). The lack of mitochondrial DNA was confirmed by fluorescence microscopy of cells stained with 4',6-diamidino-2-phenylindole (DAPI). As expected, the ρ^- strains could not use glycerol as a carbon source (Sherman et al., 1986).

The *his4::CYH2^s* allele in strains TDY92, TDY93, TDY94, and TDY96 was previously described (Runge et al., 1991).

Mutagenesis

Plasmid pTD59 (10 ng/μl) was irradiated with 225 ergs/mm^2 or 450 ergs/mm^2 of 254 nm light using four 15W germicidal light bulbs (G15T8; General Electric Co., Wilmington, MA) and then placed on ice. *E. coli* strain AB1886 (pGW249) (Gimble and Sauer, 1985; Langer et al., 1981) was grown in 10 ml LB containing 30 μg/ml kanamycin to 100 Klett units (approximately 5×10^8 cells/ml). Cells were pelleted at 6,000 rpm for 10 min in an SS-34 rotor (Sorvall Instruments, Wilmington, DE), washed in λdil, pelleted again and resuspended in 5 ml λdil. The suspension of cells was placed in an empty petri dish, swirled until the liquid covered the bottom of the dish and irradiated with 25 or 50 ergs/mm^2 UV light. These doses resulted in 50 and 5% survival, respectively. To allow induction of the enzymes required for error prone repair the cells were diluted 1:10 in 10 ml LB and grown for 2 h at 37°C. Competent cells were then prepared as follows. The cells were harvested, gently resuspended in cold 50 mM $CaCl_2$, incubated at 0°C for 20 min, harvested and resuspended in 1 ml cold 50 mM $CaCl_2$. 0.2 ml cells was incubated with 0.05 ml irradiated plasmid pTD59 for 30 min at 0°C and heat shocked at 37°C for 2 min. 3 ml LB was added and the cells were grown for 1 h at 37°C, harvested, resuspended gently in 1 ml of LB and plated on 5–10 LB plates containing 100 μg/ml ampicillin. The plates were incubated overnight at 37°C and then the colonies were scraped off and plasmid prepared. Each pool of mutagenized plasmid pTD59 contained DNA from 2,000 to 10,000 independent colonies.

Isolation of a Synchronous G1 Population of Cells

Yeast strains were grown to ~320 Klett units in YPD medium and 1 ml of cells was pelleted and resuspended in 0.32 ml YPD. To separate clumps of cells, the culture was sonicated for 5 s using a Braunsonic 1510 sonicator at 80 W. An aliquot (0.2 ml) was loaded on a 13 ml 4–10% gradient of ficoll (Sigma Chemical Co., St. Louis, MO) dissolved in YP. The gradients were sedimented in an HB-4 swinging bucket rotor in a high speed centrifuge (model RC-2B; Sorvall Instruments) for 5 min at 750 rpm. Fractions (0.5 ml) were collected and analyzed by phase-contrast microscopy. Except as noted, fraction 4 from the gradient with the wild-type cells, and fraction 5 from the gradient with the mutant cells, were selected for analysis because they were the first fractions with detectable turbidity ($1\text{–}2 \times 10^7$ cells/ml) and they contained between 93 and 100% unbudded cells. The mutant cells reproducibly sedimented faster than the wild-type cells. The cells in fraction 4 or 5 were pelleted, washed twice with YPD and resuspended in YPD at a concentration of $2\text{–}7 \times 10^6$ cells/ml.

Cytological Techniques

Yeast cells were prepared for flow cytometry and analyzed for DNA content as described (Muller, 1991). Cells were prepared for immunofluorescence as follows. Cells were fixed in 3.3% formaldehyde for >2 h, permeabilized as described (method 2 in Pringle et al., 1989), collected and washed in PBS. Slides were prepared as described (Pringle et al., 1989). Cells were stained with antibody prepared against a peptide containing the COOH-terminal 12 amino acids of yeast β-tubulin (Bond et al., 1986), at a dilution of 1:1,000 in PBS containing 0.5% BSA (Boehringer Mannheim Corp., Indianapolis, IN). The secondary antibody was goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Boehringer/Mannheim Corp.) diluted 1:1,000 in PBS containing 1.0% BSA. Cells were stained with DAPI (0.1 μg/ml, Sigma Chemical Co.) just before adding mounting medium (Citifluor glycerol, Ted Pella, Inc., Redding, CA). Stained cells were viewed with a Zeiss Axioplan fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed using Kodak T-MAX 400 professional film pushed to 1600 (Eastman Kodak Co., Rochester, NY).

Rate of Chromosome Loss

The rate of loss of a marked chromosome III in diploid strains TDY92, TDY93, TDY94, and TDY96 (Table I) was measured by a fluctuation analysis as described (Runge et al., 1991) except that cells were plated on YPD, and colonies were resuspended by sonication. The rates of loss and recombination were determined by the method of the median as described by Lea and Coulson (1949).

Calculation of the Percentage of Cells that Completed Cytokinesis

A population of cells in G1 were obtained by velocity sedimentation as described above. An important aspect of this G1 population is that it contains only individual cells and no clumps of cells. The culture was then incubated at 36°C for 4 h. Since yeast cells stick together even after they divide, any cell that had completed an entire cell cycle by 4 h appeared as a doublet, even if cytokinesis had occurred, and not as two unbudded cells. Any unbudded cells present in the culture at 4 h would be those that had not even started a bud. An aliquot of the culture removed at 4 h and analyzed without further sonication contained no unbudded cells ($n = 218$), 85% doublets, and 13% triplets or quadruplets, which represent cells that had completed a cell cycle and started one or two new buds. Thus, 98% of the mutant cells get at least as far as growing a large bud. Then an aliquot was treated with zymolyase to separate cells that had completed cytokinesis. Any unbudded cells or cells with small buds after zymolyase treatment were due to cells in the original culture that completed cytokinesis. The percentage of the cells in the original culture that had completed cytokinesis was calculated as $\{[(U + SB)/2]/[1 - [(U + SB)/2]]\} \times 100$ where U and SB represent the fraction unbudded and small budded cells present after treatment with zymolyase.

Results

Isolation of a Temperature-sensitive Mutation in CMD1

To isolate temperature-sensitive and null mutations in the gene encoding calmodulin (*CMD1*) I developed a new procedure for mutagenizing cloned genes from a method used to mutagenize bacteriophage (Gimble and Sauer, 1985; Youderain and Susskind, 1980). Briefly, plasmid pTD59, which carries *CMD1*, was damaged by treatment with UV light and transformed into a sensitized strain of *E. coli*. The *E. coli* strain is deficient in excision repair (*uvrA$^-$*) and thus mutations were introduced during the error-prone repair of the damaged DNA. The efficiency of mutagenesis was enhanced by the mutator plasmid pGW249 (Gimble and Sauer, 1985; Langer et al., 1981), which is a derivative of plasmid pKM101 (Walker, 1977). Plasmid pKM101 enhances mutagenesis in *E. coli* 3–10-fold (Glickman, 1983). The doses of UV light were chosen such that between 0.5–2.6% of the

plasmids carried mutations that abolished the function of the URA3 gene, which is the selectable marker on plasmid pTD59.

The advantages of this easy procedure are severalfold. It does not involve the handling or disposal of hazardous chemical mutagens. Whereas chemical mutagens only give one or two of the many possible changes, UV mutagenesis gives a broad spectrum of mutations including transitions and transversions (Coulondre and Miller, 1977; LeClerc et al., 1988). Although changes tend to occur at or near pyrimidine pairs (LeClerc et al., 1988; Wood et al., 1984), analysis of *CMD1* revealed that only 16% of the nucleotides are not part of a pyrimidine pair on one strand or the other. Only 2/441 nucleotides are not next to a pyrimidine pair on one strand or the other. There are hotspots for UV mutagenesis, but 40-50% of the mutations occur at sites outside of these hotspots (Coulondre and Miller, 1977; Todd and Glickman, 1982).

Five pools of mutagenized plasmid pTD59 were prepared and transformed into the yeast indicator strain TDY55-5D (pTD56) and plated at 37°C. This strain was constructed such that colonies carrying a version of pTD59 in which the *CMD1* gene was functional would sector white. Colonies carrying a version of plasmid pTD59 in which the *CMD1* gene was not functional would remain a solid red (see Davis, 1990; and Geiser et al., 1991 for a more thorough discussion of this plasmid shuffling technique). Of the 35,000 colonies screened, 121 solid red colonies were isolated. Plasmid pTD59 could be rescued from 43 of the colonies. Sequencing of the *CMD1* gene revealed that 20 of the plasmids did not carry mutations in the coding region of *CMD1* and were not characterized further. Of the 23 plasmids with mutations in *CMD1*, at least 16 were independent isolates. 15 of the 16 carried null mutations in *CMD1*, and thus colonies of the indicator strain carrying any of these plasmids did not sector white at either 25 or 37°C. Of the 15 null mutations, nine were nonsense mutations, four were frameshift mutations, and two were missense mutations. The two independently isolated missense mutations were the same mutation and merely changed the initial ATG to AAG thereby preventing translation of the protein. A single missense mutation that resulted in production of an intact but inactive protein was not identified.

One plasmid from 35,000 colonies carried two missense mutations, which together conferred a temperature-sensitive phenotype. The mutations changed isoleucine 100 to asparagine (I100N) and glutamate 104 to valine (E104V). Growth of a strain with both mutations (*cmd1-1*) integrated at the *CMD1* locus is strongly temperature dependent. On rich solid medium, the *cmd1-1* strain formed colonies the same size as the wild-type strain at 21 and 25°C, 65% the size of wild-type colonies at 29°C, 20% at 32°C and did not form colonies at 34°C or above. A diploid heterozygous for *cmd1-1* forms colonies the same size as wild type at all temperatures tested (21-38°C), thus *cmd1-1* is recessive. Plasmids carrying either I100N or E104V were constructed and tested for their ability to support the growth of yeast. Neither mutation alone is lethal at 37°C although a strain carrying the I100N mutation formed colonies 80% the size of wild-type colonies at 37°C, and 60% the size of wild-type colonies at 38°C. A strain carrying the E104V mutation formed colonies the same size as a wild-type strain at all temperatures tested (21-38°C).

Characterization of the Temperature-sensitive Calmodulin Mutant

Asynchronous cultures of strains carrying the *cmd1-1* allele did not arrest with a uniform terminal morphology when incubated at a nonpermissive temperature. After 4 h at 37°C, cultures accumulated 85% budded cells, but the size of the bud varied from a third of the size to the same size as the mother cell. The cells were detectably larger than wild-type cells. After 10 h, the number of budded cells decreased to 70% of the cells; the size of the buds was still variable. After 22 h at the nonpermissive temperature, substantial cellular debris was present indicating that many cells had lysed.

Calmodulin Promotes Formation and Growth of a Bud

The fact that the terminal morphology is not uniform suggests that either calmodulin is required at many stages of the cell cycle or that response to lack of calmodulin is heterogeneous. To assess the requirement for calmodulin at each stage of the cell cycle, I examined the phenotype of cells as they synchronously progressed through the cell cycle at the nonpermissive temperature. In initial experiments, the mating pheromone α-factor was used to synchronize the cells at G1. After the α-factor was removed, the cells were incubated at the nonpermissive temperature and aliquots taken for analysis. α-factor arrested the mutant cells at G1 as expected, but depending on the dose of α-factor, between 10 and 50% of the cells did not start a bud or begin DNA synthesis within 4 h after release. The wild-type cells recovered normally. When corrected for the proportion of cells that did not recover, the results suggested that DNA synthesis occurred simultaneously in the mutant and wild-type cultures at the nonpermissive temperature, but bud emergence and bud growth was delayed in the mutant culture (data not shown). However, the fact that a large population of mutant cells did not recover indicated that α-factor was affecting the mutant cells in additional ways besides a transient arrest at G1 and thus is not ideal for obtaining synchronous cultures. As an alternative, a synchronous population of cells was obtained by subjecting a culture to velocity sedimentation through a ficoll gradient (see Materials and Methods). Cells in G1 are unbudded and smaller than cells at other stages of the cell cycle and thus remain near the top of the gradient.

Small unbudded *cmd1-1* cells progressed through S phase at the nonpermissive temperature. RNA synthesis occurred at an identical rate in the mutant and wild-type cultures (Fig. 1 *A*). DNA synthesis began and continued to completion in the mutant culture at a rate similar to the wild-type strain (Fig. 1 *B*). In three experiments, bud emergence was slightly delayed in the mutant when compared with the initiation of DNA synthesis (Fig. 2). In the mutant culture, bud emergence nearly coincided with the completion of DNA synthesis, whereas in the wild-type culture, bud emergence occurred ~10 min before the completion of DNA synthesis. Growth of the bud was significantly delayed in the mutant culture, since 50% of the cells had medium buds 23 min after the wild-type culture. Together with the results obtained using α-factor to synchronize cultures, these results suggest

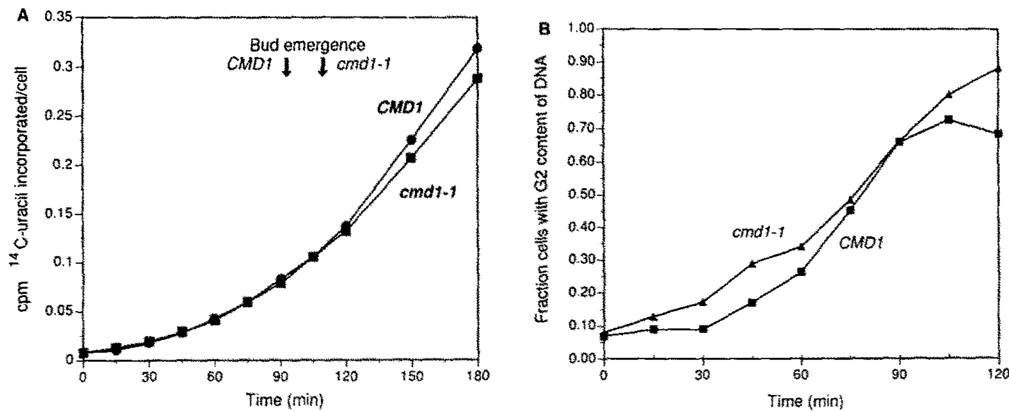

Figure 1. (*A*) RNA synthesis in *cmdl-1* mutant and wild-type cultures. A population of cells in G1 were obtained from cultures of strains JGY44-2A or CRY1 grown to 200 Klett units in SD complete by velocity sedimentation through a ficoll gradient as described in Materials and Methods except the ficoll was dissolved in S complete medium. The cells were washed two times with SD − ura and resuspended at a concentration of 1.5×10^6 viable cells/ml (*cmdl-1*) or 1.7×10^6 viable cells/ml (*CMD1*) in SD − ura containing 74 μM [2-^{14}C]uracil (54 mCi/mmol). Cells were grown for 20 min at 21°C and then shifted to 36°C. Every 20 min, aliquots (50 μl) were pipetted onto filter disks saturated with 5% TCA containing 0.25 mg/ml uracil. Filters disks were washed three times with 5% TCA and one time with 95% ethanol allowing for 20 ml per filter disk per wash. Filter disks were dried and counted in Ecolume (ICN Biomedicals, Cosa Mesa, CA). Time zero is the time of the shift to 36°C. Each arrow represents the time at which 50% of the cells in a culture had completed bud emergence. (*B*) Rate of completion of DNA synthesis in synchronous cultures of mutant and wild-type cells. A population of cells in G1 were obtained from cultures of strains JGY44-2A or CRY1 as described in Materials and Methods. The cells were washed and shifted to 36°C. Every 15 min, aliquots (40 μl) were mixed with 95% ethanol (0.1 ml). At the end of the experiment, samples were prepared for flow cytometry as described in Materials and Methods.

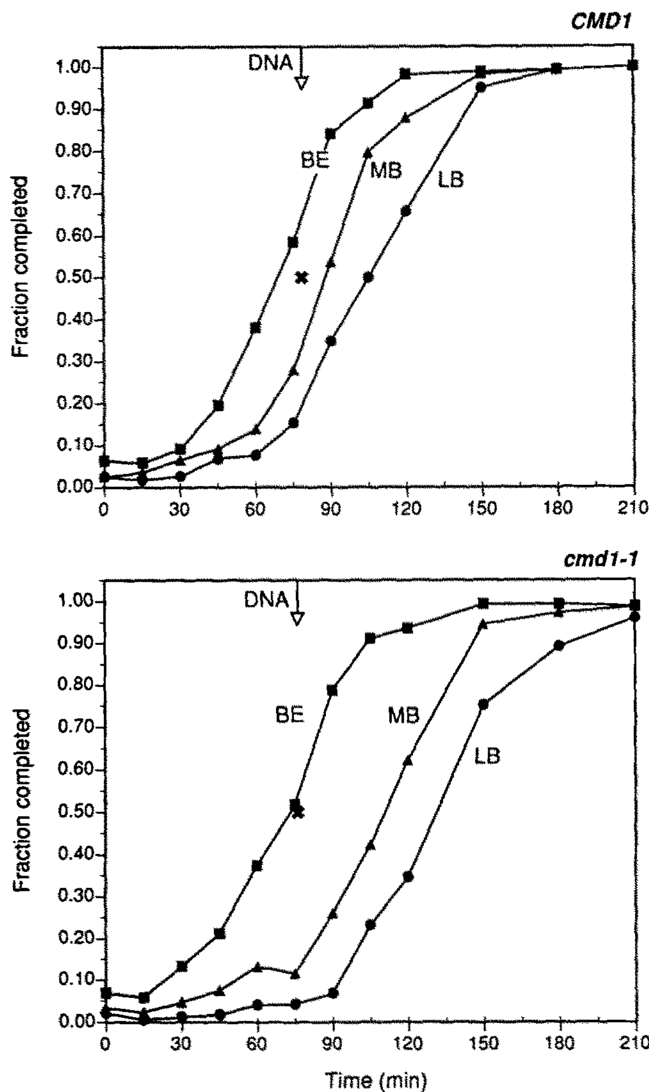

Figure 2. Progression through the cell cycle of synchronous cultures of mutant and wild-type cells. A population of cells in G1 were obtained from cultures of strains JGY44-2A or CRY1 as described in Materials and Methods except that the ficoll gradient was only centrifuged for 3.5 instead of 5 min. The cells were washed and shifted to 36°C. Every 15 min, formaldehyde was added to aliquots (40 μl) to a final concentration of 4.2%. The morphology of the cells was assessed by phase-contrast microscopy. 150–200 cells were counted for each time point. A small bud is less than one third the size of the mother cell, a medium bud is less than two thirds the size of the mother cell and a large bud is two thirds or equal to the size of the mother cell. *BE*, bud emergence; *MB*, medium bud; *LB*, large bud. The arrow and the x mark the point at which 50% of the cells had a G2 content of DNA as determined in Fig. 1 *B*.

that calmodulin is not required for DNA or RNA synthesis but facilitates bud emergence and growth.

Mutant Cells Synchronized in G1 Traverse the Cell Cycle and Accumulate in Mitosis at the Nonpermissive Temperature

An analysis of the morphology of the DNA and the spindle was performed in synchronous cultures of a *cmdl-1* strain and a *CMD1* strain that lacked detectable mitochondrial DNA (see Material and Methods). The nuclear DNA, as revealed by DAPI staining of fixed cells, first appeared in the neck 120 min after the shift to 36°C in both the mutant and wild-type cultures. At 210 min after the shift, 70% of the *cmdl-1* cells had the DNA stretched through the neck. In contrast, the wild-type cells rapidly proceeded through mitosis; the number of wild-type cells with the DNA in the neck never exceeded 12%. In 90% of the mutant cells at 210 min, the DAPI staining had an unusual appearance. The DNA was more diffuse (Fig. 3 *E*) or more elongated (Fig. 3, *A–E*) than in a wild-type cell (Fig. 3 *G*) or had a spur extending from the bulk of the DNA (Fig. 3, *B* and *E*, top cell). Of the few

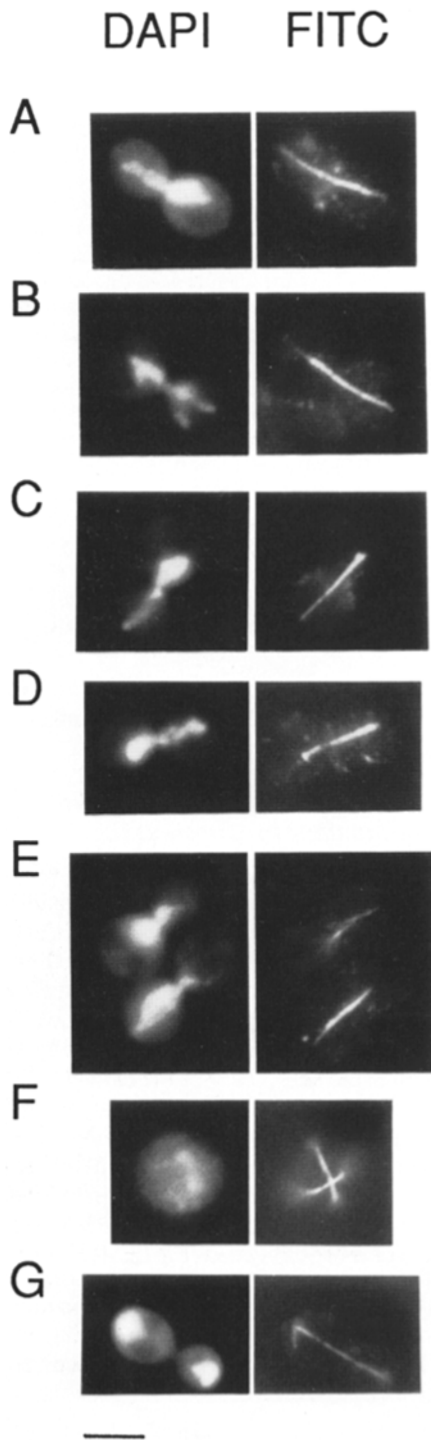

Figure 3. Morphology of the DNA and spindle in mutant and wild-type cells. A population of cells in G1 were obtained from cultures of strains TDY70-30Cρ^- or TDY70-30Aρ^- as described in Materials and Methods and shifted to 36°C. Samples were removed and stained for immunofluorescence as described in Materials and Methods. *A–E*, TDY70-30C (*cmdl-l*) 210 min after the shift to 36°C; *F*, TDY70-30C 280 min after the shift, G TDY70-30A (*CMDl*) 210 min of the shift. Bar, 5 μm.

unbudded mutant cells present at 4 h, 20% (n = 61) had little or no DAPI-staining material (Fig. 3 *F*). In mutant cells containing mitochondrial DNA, the nuclear DNA also appeared to be extended, but because of its unusual morphology it was difficult to distinguish from the mitochondrial DNA in some cells. The timing of nuclear migration was very similar in mutant strains with and without mitochondrial DNA.

The spindle was visualized by staining with anti-tubulin antibody and FITC conjugated secondary antibody as described in Materials and Methods. The mutant cells delayed in mitosis contained extensive arrays of microtubules (Fig. 3, *A–E*). In 45% (n = 102) of the cells at 210 min (Fig. 3, *A–B*), the spindle was approximately the length of a fully elongated spindle in a wild-type cell (Fig. 3 *G*). In 40% of the mutant cells (Fig. 3, *C–E*), the spindle was approximately two-thirds the length of a fully elongated spindle in a wild-type cell (Fig. 3 *G*), but longer than a wild-type spindle before elongation. In 85% of the large budded cells the spindle was linear and in 15% the spindle was branched (Fig. 3 *E*, top cell). A similar branched spindle was never observed in a wild-type cell. Cytoplasmic microtubules that formed an array around the end of the spindle of the wild-type cells were not visible in the mutant cells. In general, the DNA extended along the microtubules. Half of the unbudded cells without DNA contained detectable microtubules (Fig. 3 *F*).

Flow cytometry of a synchronous culture of mutant cells labeled with propidium iodide revealed that after 270 min at 36°C, there was substantial heterogeneity in the amount of DNA per cell (Fig. 4). The DNA content varied from none to 2× the G2 content of DNA with an increase in cells with less than a G1 content of DNA and a broad peak centering at 1.3× the G2 content. The extra DNA is not due to mitochondrial DNA synthesis since the strains lack detectable mitochondrial DNA. Flow cytometry of cells that contain mitochondrial DNA gave similar results. These results suggest significant aneuploidy in the mutant culture incubated at the nonpermissive temperature.

Calmodulin Is Required to Complete Mitosis

To determine at what stage (or stages) of the cell cycle calmodulin is required to maintain viability, I assayed the viability of cells as they synchronously traversed the cell cycle. Small unbudded cells in G1 were collected from a ficoll gradient and incubated at the nonpermissive temperature (36°C). Samples were taken every 15 min and titered for viability at the permissive temperature (21°C) (Fig. 5). 100% of the mutant cells remained viable for 120 min after the shift to high temperature. Loss of viability occurred after the appearance of a large bud (compare Figs. 2 *B* and 5), but the exact time that elapsed between the two events was different in each of four different experiments.

To determine if loss of viability correlated with an identifiable event, small unbudded cells and slightly larger unbudded cells were collected from two different fractions of a single ficoll gradient from a mutant culture, and incubated at 36°C. Every 30 min, samples were collected and assayed for viability, DNA content, bud size, and location of the nucleus in the cell. As expected because the smaller unbudded cells must grow for a longer time before they become large enough to begin the cell cycle, bud emergence and DNA synthesis occurred ~15 min later in the fraction of the smallest unbudded cells than in the fraction of the larger cells. The cells that began as smaller unbudded cells also lost viability later than the cells that began as larger unbudded cells. This result argues that the cells lose viability at a

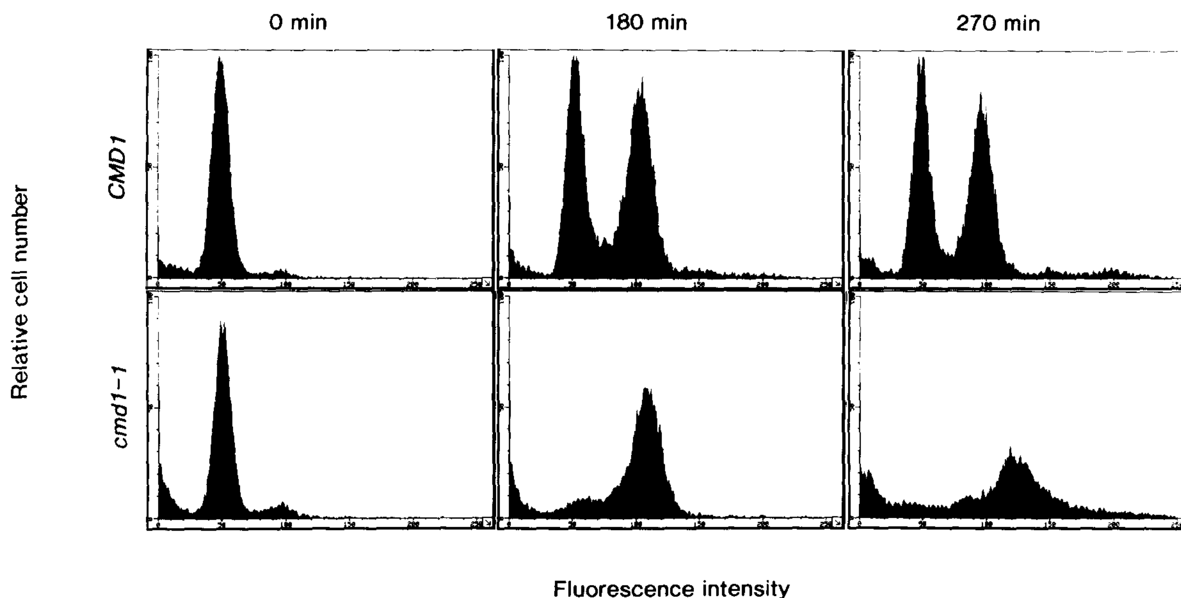

Figure 4. Flow cytometry of *cmdl-1* mutant. A population of cells in G1 were obtained from cultures of strains TDY70-30Cρ^- or TDY70-30Aρ^- as described in Materials and Methods and shifted to 36°C. At the given times, samples were removed and prepared for flow cytometry as described in Materials and Methods. Material that gave a forward scattering signal less than one third that of an unbudded cell was not included in the analysis.

specific stage of the cell cycle and not just at a given time after the shift to 36°C. Loss of viability directly coincided with the appearance of the nuclear DNA in the neck (Fig. 6), an event that just precedes chromosome segregation in *S. cerevisiae.*

The previous results strongly suggest that calmodulin is required to perform an essential function near the time of chromosome segregation. Microtubules are also required at this stage of the cell cycle. To directly relate the timing of the step that requires calmodulin to the timing of the step that requires microtubules I analyzed the phenotype of mutant and

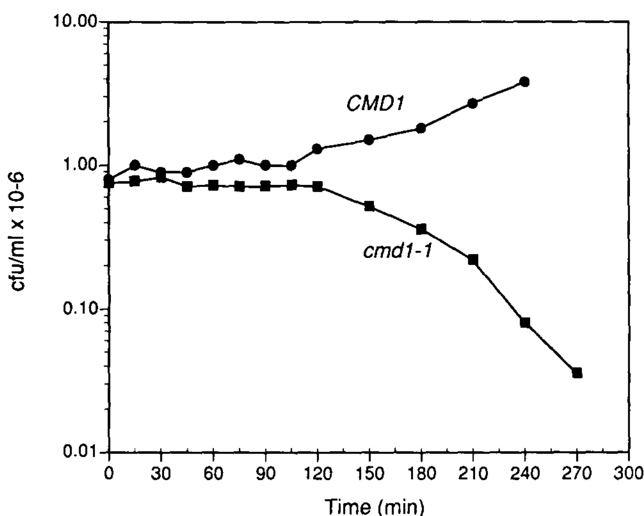

Figure 5. Loss of viability in mutant and wild-type cultures synchronized at G1. The experiment was performed as described in Fig. 2, except that after the shift to the 36°C, aliquots were removed, sonicated, and then titered for colony forming units at 21°C on YPD.

wild-type cells treated with nocodazole at 21°C for 1.5 generations and then released at either 21 or 36°C. Cells treated with nocodazole, which promotes depolymerization of microtubules, arrest with large buds before the nucleus migrates to the neck (Jacobs et al., 1988). When released from a nocodazole arrest at 36°C, the mutant culture rapidly lost viability (Fig. 7), within 70 min only 13% were viable. The timing of loss of viability is in sharp contrast to the results obtained with a culture synchronized at G1, which does not begin to lose viability until 150 min after the shift to 36°C (see Fig. 5). Together the experiments indicate that calmodulin is specifically required during mitosis. Calmodulin is not required for microtubule polymerization per se because under nonpermissive conditions, the microtubules repolymerized in the mutant cells after release from nocodazole (data not shown). 60 min after release at 36°C, 38% (n = 300) of the unbudded and small budded cells in the mutant culture and 11% (n = 211) in wild-type culture contained little if any nuclear DAPI-staining material, implying that defective chromosome segregation had occurred.

Calmodulin May Be Required for Cytokinesis

After a shift to nonpermissive temperature, the calmodulin mutant gave a different arrest phenotype depending on which stage of the cell cycle the cells began. Only 18% of *cmdl-1* cells synchronized early in G1 and then shifted to 36°C completed cytokinesis within 4 h after the shift to nonpermissive temperature (see Materials and Methods for the method of calculation). 50% of the cells had the nucleus in the neck at 90 min after the shift, and thus the 4-h time point represents 150 min after G2/M. The same result was obtained whether or not the cells had mitochondrial DNA. 35% of the *cmdl-1* cells synchronized later in G1 (obtained from fraction 7 of a ficoll gradient [Fig. 6]) completed cytokinesis. In *cmdl-1* cells synchronized at G2/M with nocodazole at the permis-

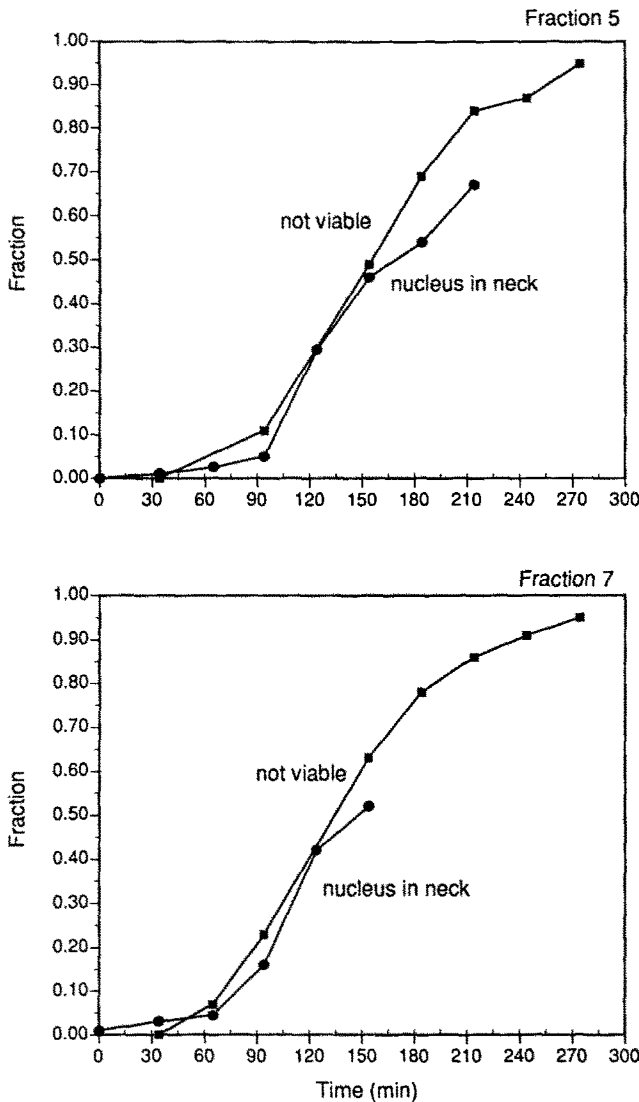

Figure 6. Correlation between loss of viability and appearance of the nuclear DNA in the neck. Cells from a culture of JGY44-2A (*cmdl-1*) were subjected to velocity sedimentation as described in Materials and Methods. The cells were collected from fractions 5 and 7, washed, and then shifted to 36°C. Every 30 min, samples were taken and either fixed with formaldehyde or sonicated and titered for viable cells at 21°C. The fixed cells were permeabilized and stained with DAPI as described in Materials and Methods. The location of the nuclear DNA was assessed by fluorescence microscopy in at least 200 cells for each time point. Phase-contrast microscopy of the fixed cells revealed that 50% of the cells in fraction 5 completed bud emergence by 67 min after the shift; whereas, 50% of the cells in fraction 7 completed bud emergence by 51 min after the shift.

sive temperature and then released at 36°C, 68% of the mutant cells completed cytokinesis in only 75 min. Thus, cells that traversed early G1 at the nonpermissive temperature showed a defect in cytokinesis not seen in cells that only progressed S and G2 at the nonpermissive temperature. Loss of viability is independent of whether the cells proceed through cytokinesis, since the mutant cells lost viability whether they were shifted to the nonpermissive temperature in either G1 or G2.

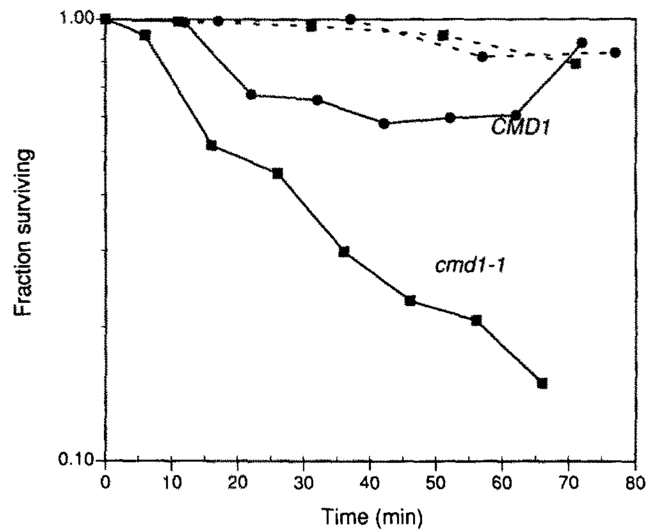

Figure 7. Loss of viability in mutant and wild-type cultures synchronized with nocodazole. Strains JGY44-2A and CRY1 were grown at 21°C in YPD to 30 Klett units. Nocodazole was added to a final concentration of 5 μg/ml and the cultures were incubated for 1.5 generations at 21°C. (The concentration of nocodazole was chosen because it gave 85–95% large budded cells, which showed few if any microtubules as visualized by immunofluorescence.) The cells were collected by filtration and washed twice with YPD and resuspended in 10 ml of YPD. Each culture was split in half and one half was incubated at 21°C and the other half was shifted to 36°C. At the given times, aliquots were removed, sonicated, and titered for colony forming units on YPD at 21°C. (——) 37°C; (– – –) 21°C.

Under Semipermissive Conditions, the Calmodulin Mutant Shows a Modest Increase in the Rate of Chromosome Loss

The immunofluorescence and flow cytometry results suggest that under nonpermissive conditions the DNA is dispersed along the microtubules and the chromosomes are not equally segregated between the mother and daughter cell. Fidelity of chromosome segregation was directly assayed as the rate of loss of a marked chromosome III in a *cmdl-1/cmdl-1* diploid strain grown under semipermissive conditions (Table II). At 30°C, the *cmdl-1* mutant showed a 7–16-fold increase in chromosome loss as compared with a wild-type strain. The rate of recombination was the same in the mutant and wild-type cells. The rate of loss and recombination in the wild-type cell was very similar to that observed previously (Hartwell and Smith, 1985; Runge et al., 1991). Even at 21°C, the permissive temperature, the mutant strain showed a threefold increase rate of chromosome loss (Table II).

Discussion

Mutations in calmodulin that allow production of an intact but defective protein are surprisingly rare. Only one temperature-sensitive mutant was isolated from 35,000 colonies, a frequency at least 10-fold lower than obtained using the same procedure to mutate other genes (Schulz et al. 1992; Levin and Bartlett-Heubusch, 1992). The temperature-sensitive mutant carries two mutations, I100N and

*Table II. Rate of Chromosome Loss in Mutant and Wild-Type Strains**

Strain	Temperature	Loss rate/10^5 cell divisions	Recombination rate/10^5 cell divisions	Recombination + Loss/10^5 cell divisions
	°C			
Day 1				
cmd1-1/cmd1-1	30	32	0.84	33
CMD1/CMD1	30	4.5	0.67	5.2
cmd1-1/CMD1	30	3.0	0.76	3.8
Day 2				
cmd1-1/cmd1-1	21	ND	ND	13
CMD1/CMD1	21	ND	ND	3.6
cmd1-1/cmd1-1	30	ND	ND	38
CMD1/CMD1	30	ND	ND	2.4

* Rate of loss and recombination was measured as described in Materials and Methods. The strains used were *cmd1-1/cmd1-1*, TDY92 on day 1 and TDY96 on day 2; *CMD1/CMD1*, TDY92; *cmd1-1/CMD1*, TDY94.

E104V. Both mutations are required to confer a temperature-sensitive phenotype. I100N alone inhibits but does not prevent growth at 37°C. I100N also increases the sensitivity of the third Ca^{2+}-binding loop to proteolysis (Brockerhoff et al., 1992) suggesting it decreases the stability of that region. E104V removes two of the seven ligands to the Ca^{2+} ion in the third loop and substantially decreases the affinity of that loop for Ca^{2+} (Geiser et al., 1991 and T. N. Davis, unpublished data). It also decreases the stability of the third loop (Brockerhoff et al., 1992), but alone confers no phenotype. We have shown previously that calmodulin does not require a high affinity for Ca^{2+} to perform the essential function (Geiser et al., 1991).

When incubated at the nonpermissive temperature, the temperature-sensitive mutant did not show any defects in macromolecular synthesis, but bud emergence and bud growth were delayed. When synchronized in G1, mutant cells showed severe defects in cytokinesis. A gradual depletion of calmodulin from *S. cerevisiae* cells leads to an accumulation of cells with buds of different sizes and with a G2/M content of DNA (Ohya and Anraku, 1989*b*) consistent with the conclusion that calmodulin is required for efficient growth of a bud but not for DNA synthesis. These results are discussed further in the accompanying paper, where we present evidence that calmodulin concentrates at sites of cell growth and is required for organization of actin (Brockerhoff and Davis, 1992).

Mitosis in the mutant cells was severely affected at the nonpermissive temperature. During mitosis in a wild-type yeast cell, the chromosomes are primarily separated during anaphase B when the spindle pole bodies move to opposite ends of the cell at a rate similar to such movements in mammalian cells (Palmer et al., 1989). The DNA largely stays as two compact entities each associated with a spindle pole body (Huffaker et al., 1988; Palmer et al., 1989). In contrast, in the calmodulin mutant at the nonpermissive temperature, the DNA was dispersed along the spindle. In the mutant, the DNA was usually associated with at least one end of the spindle, but much of the DNA was stretched between the two ends. The spindle was typically linear and stretched through the neck. Thus, the spindle pole bodies apparently had separated, but all the DNA did not stay associated with the end of the spindle. In addition, a force was apparently exerted on the DNA resulting in the extended appearance. The cells did not survive mitosis in the absence of calmodulin; starting with a culture synchronized in either G1 or G2 of the cell cycle, >85% of the mutant cells died during the first mitosis at the nonpermissive temperature. Under semipermissive conditions, the mutant showed a modest increase in the rate of chromosome loss. In cells depleted of calmodulin, the spindle does not elongate (Ohya and Anraku, 1989*b*) suggesting that there is a different response to the slow depletion of wild-type calmodulin (14 h) as opposed to the inactivation of a temperature-sensitive calmodulin. Preliminary results (Ohya and Anraku, 1989*b*) suggested that calmodulin-depleted cells show an increase in chromosome loss.

Even though the calmodulin temperature-sensitive mutant produces some aploid cells, a primary defect either in the spindle pole bodies or in the attachment of the DNA to the spindle seems unlikely for two reasons. First, the DNA appears stretched along an elongated spindle implying the DNA is attached to both spindle pole bodies for at least a portion of anaphase. Second, mutants known to be defective in DNA attachment or spindle pole body morphogenesis have phenotypes distinct from the calmodulin mutant. A mutant (*ndc1-1*) that has a complete defect in attachment of the DNA to one of the spindle pole bodies does not die as it proceeds through mitosis, but instead goes through an asymmetric cell division in which one daughter cell doubles in ploidy and the other inherits no chromosomes (Thomas and Botstein, 1986). Mutants that form monopolar spindles, such as *cdc31* (Baum et al., 1986) and *mps2* (Winey et al., 1991), do not proceed through mitosis and instead arrest as large budded cells and remain viable. One exception is the *mps1* mutant (Winey et al., 1991), which proceeds through mitosis and dies, but the DNA remains compact and not elongated as in the *cmd1-1* mutant.

The phenotype of the *cmd1-1* mutant at the nonpermissive temperature is very similar to that of mutants defective in chromosome disjunction. In the *dis* mutants of *S. pombe*, the spindle elongates but the chromosomes do not separate. The result is a random arrangement of the chromosomes along the spindle (Ohkura et al., 1989). The *dis2*$^+$ gene encodes a type 1 protein phosphatase (Ohkura et al., 1989).

Temperature-sensitive mutants in topoisomerase II in *S. cerevisiae* (Holm et al., 1985) or *S. pombe* (Uemura and Yanagida, 1986) die at the nonpermissive temperature as they attempt to separate sister chromatids that are still entwined. The similarities between the *top2* mutants in *S. cerevisiae* and the *cmdl-1* mutant are especially striking. Mutants in topoisomerase (Holm et al., 1985) or calmodulin lose viability specifically as they proceed through mitosis at the nonpermissive temperature. When synchronized with nocodazole, both mutants lose viability immediately after release with nearly identical kinetics (Holm et al., 1989). Neither display a uniform terminal morphology and instead arrest with a variety of bud morphologies. In both mutants during mitosis, the DNA appears to be dispersed along the spindle (Holm et al., 1985), although this phenotype is more pronounced in the calmodulin mutant. The rate of chromosome loss in the *top2* strain incubated for 2 h at 35°C is 40-fold greater than in the wild-type strain (Holm et al., 1989). The rate of chromosome loss in the *cmdl-1* strain grown at the semipermissive temperature of 30°C is 7–16-fold greater than in the wild-type strain. These numbers are of the same order of magnitude for a characteristic that can vary over three orders of magnitude among the different mutants that show mitotic defects (Hartwell and Smith, 1985). Neither the *top2* nor the *cmdl-1* mutation have an effect on the rate of recombination.

Calmodulin could be acting by regulating protein phosphatase type I or topoisomerase II, but there is little precedence for direct interactions. Although topoisomerase II from *Drosophila* can be phosphorylated by Ca^{2+}-calmodulin–dependent protein kinase (Sahyoun et al., 1986), the bulk of the phosphorylation in vivo depends on casein kinase II (Ackerman et al., 1988). Recent results suggesting that topoisomerase II function is modulated by calmodulin-mediated events were based on the effects of trifluoperazine (Banapathi et al., 1991) an inhibitor that is not specific for calmodulin (Schatzman et al., 1984). Thus, direct regulation of topoisomerase II or protein phosphatase type I by calmodulin seems unlikely.

The phenotype of the calmodulin mutant differs from that of mutants defective in other steps of mitosis. Mutants that produce multiple spindle pole bodies, such as *espl* in *S. cerevisiae* (Baum et al., 1988) and *cutl⁻* (Uzawa et al., 1990) in *S. pombe*, show a triangular spindle very different from the spindle observed in *cmdl-1* mutants at the nonpermissive temperature. Another *cut* gene in *S. pombe*, *cut7⁺*, encodes a novel mitotic motor with similarity to kinesin. Unlike the *cmdl-1* mutant, in a *cut7⁻* mutant, the two spindle pole bodies are unable to separate resulting in a distinctive V-shaped spindle (Hagan and Yanagida, 1990).

Vertebrate calmodulin can support the growth of yeast cells (Davis and Thorner, 1989; Ohya and Anraku, 1989; Persechini et al., 1991), and thus the ability to perform the essential mitotic function in yeast has been conserved. Consistent with a role during chromosome disjunction, depletion of calmodulin from C127 cells using anti-sense RNA leads to a delay at metaphase suggesting that the chromosomes can line up but not separate (Rasmussen and Means, 1989). Conidia from an *Aspergillus nidulans* strain carrying a deletion of the gene encoding calmodulin cannot complete more than a single mitosis. Some of the conidia arrest with fragmented nuclear DNA (Rasmussen et al., 1990). Calmodulin colocalizes with the kinetochore microtubules in plant endosperm (Vantard et al., 1985) and rat kangaroo PtK_2 cells (Welsh et al., 1979). Immunogold staining of the plant endosperm cells shows calmodulin associated with the microtubules during anaphase (Vantard et al., 1985). The localization seems different in yeast cells in that calmodulin is not detected on the mitotic spindle (Brockerhoff and Davis, 1992). However, in yeast there is only one kinetochore microtubule per chromosome (Peterson and Ris, 1976), as opposed to many in higher eukaryotic cells. Thus, even if calmodulin had a similar mitotic distribution in yeast as in plant cells, it probably could not be detected.

In yeast cells, the mitotic function can be performed by mutant calmodulins that do not bind detectable levels of Ca^{2+} (Geiser et al., 1991). Even mutant forms of vertebrate calmodulin defective in Ca^{2+}-binding can perform the essential function in yeast. Thus, the essential mitotic function does not require Ca^{2+}-binding. In agreement with this conclusion in yeast, the ability of calmodulin to bind to the mitotic apparatus in PtK_1 cells is not dependent on the presence of Ca^{2+}. Even a modified calmodulin unable to activate cyclic nucleotide phosphodiesterase can stabilize the kinetochore microtubules (Sweet et al., 1988). Thus, a possible Ca^{2+}-independent function is stabilization of the kinetochore microtubules. Future studies will continue to dissect the functions of calmodulin during chromosome segregation.

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References

Ackerman, P., C. V. C. Glover, and N. Osheroff. 1988. Phosphorylation of DNA topoisomerase II in vivo and in total homogenates of *Drosophila* Kc cells. *J. Biol. Chem.* 263:12653–12660.

Bachs, O., L. Lanini, J. Serratosa, M. J. Coll, R. Bastos, R. Aligue, E. Rius, and E. Carafoli. 1990. Calmodulin-binding proteins in the nuclei of quiescent and proliferatively activated rat liver cells. *J. Biol. Chem.* 265:18595–18600.

Baitinger, C., J. Alderton, M. Poenie, H. Schulman, and R. A. Steinhardt. 1990. Multifunctional Ca^{2+}/calmodulin-dependent protein kinase is necessary for nuclear envelope breakdown. *J. Cell Biol.* 111:1763–1773.

Banapathi, R., N. Kamath, A. Constantinou, D. Grabowski, J. Ford, and A. Anderson. 1991. Effect of the calmodulin inhibitor trifluoperazine on phosphorylation of P-glycoprotein and topoisomerase II: relationship to modulation of subcellular distribution, DNA damage and cytotoxicity of doxorubicin in multidrug resistant L1210 mouse leukemia cells. *Biochem. Pharmacol.* 41:R21–26.

Baum, P., C. Furlong, and B. Byers. 1986. Yeast gene required for spindle pole body duplication: homology of its product with Ca^{2+}-binding proteins. *Proc. Natl. Acad. Sci. USA.* 83:5512–5516.

Baum, P., C. Yip, L. Goetsch, and B. Byers. 1988. A yeast gene esential for regulation of spindle pole duplication. *Mol. Cell. Biol.* 8:5386–5397.

Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* 154: 164–175.

Bond, J. F., J. L. Fridovich-Keil, L. Pillus, R. C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules in vivo. *Cell.* 44:461–468.

Brockerhoff, S. E., and T. N. Davis. 1992. Calmodulin concentrates in regions of cell growth in *Saccharomyces cerevisiae. J. Cell Biol.* 118:619–629.

Brockerhoff, S. E., C. G. Edmonds, and T. N. Davis. 1992. Structural analysis of wild-type and mutant yeast calmodulins by limited proteolysis and electrospray ionization mass spectrometry. *Protein Science* 1:504–516.

Cohen, P. 1988. The regulation of phosphorylase kinase activity by calmodulin and troponin. *In* Calmodulin. P. Cohen and C. B. Klee, editors. Elsevier

Science Publishers, Amsterdam. 123–144.
Cohen, P., and C. B. Klee. 1988. Molecular Aspects of Cellular Regulation. Elsevier Science Publishers, Amsterdam. 371 pp.
Coulondre, C., and J. H. Miller. 1977. Genetic studies of the *lac* repressor: IV. Mutagenic specificity in the *lacI* gene of *Escherichia coli*. *J. Mol. Biol.* 117:577–606.
Davis, T. 1990. Genetic analysis of calcium-binding proteins in yeast. *In* Stimulus Response Coupling: The Role of Intracellular Calcium-binding proteins. V. L. Smith and J. R. Dedman, editors. CRC Press, Boston, MA. 237–249.
Davis, T. N., and J. Thorner. 1989. Vertebrate and yeast calmodulin, despite significant sequence divergence, are functionally interchangeable. *Proc. Natl. Acad. Sci. USA.* 86:7909–7913.
Davis, T. N., M. S. Urdea, F. R. Masiarz, and J. Thorner. 1986. Isolation of the yeast calmodulin gene: calmodulin is an essential protein. *Cell.* 47:423–431.
Dinsmore, J. H., and R. D. Sloboda. 1988. Calcium and calmodulin-dependent phosphorylation of a 62 kd protein induces microtubule depolymerization in sea urchin mitotic apparatuses. *Cell.* 53:769–780.
Geiser, J. R., D. van Tuinen, S. E. Brockerhoff, M. M. Neff, and T. N. Davis. 1991. Can calmodulin function without binding calcium? *Cell.* 65:949–959.
Gimble, F. S., and R. T. Sauer. 1985. Mutations in bacteriophage λ repressor that prevent RecA-mediated cleavage. *J. Bacteriol.* 162:147–154.
Glickman, B. 1983. Mutational specificity of UV light in *E. coli:* influence of excision repair and the mutator plasmid pKM101. *In* Induced Mutagenesis: Molecular Mechanisms and Their Implications for Environmental Protection. C. W. Lawrence, editor. Plenum Publishing Corp., New York. 135–170.
Hagan, I., and M. Yanagida. 1990. Novel potential mitotic motor protein encoded by the fisson yeast *cut7*$^+$ gene. *Nature (Lond.).* 347:563–566.
Hartwell, L. H., and D. Smith. 1985. Altered fidelity of mitotic chromosome transmission in cell cycle mutants of *S. cerevisiae*. *Genetics.* 110:381–395.
Hawthorne, D. C., and R. K. Mortimer. 1960. Chromosome mapping in *Saccharomyces* centromere-linked genes. *Genet.* 45:1085–1110.
Holm, C., T. Goto, J. C. Wang, and D. Botstein. 1985. DNA topoisomerase II is required at the time of mitosis in yeast. *Cell.* 41:553–563.
Holm, C., T. Stearns, and D. Botstein. 1989. DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. *Mol. Cell. Biol.* 9:159–168.
Howe, C. L., and M. S. Mooseker. 1983. Characterization of the 110-kdalton actin- calmodulin- and membrane-binding protein from microvilli of intestinal epithelial cells. *J. Cell Biol.* 97:974–985.
Huffaker, T. C., J. H. Thomas, and D. Botstein. 1988. Diverse effects of beta-tubulin mutations on microtubule formation and function. *J. Cell Biol.* 106:1997–2010.
Jacobs, C. W., A. E. M. Adams, P. J. Szaniszlo, and J. R. Pringle. 1988. Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* 107:1409–1426.
Kao, J. P. Y., J. M. Alderton, R. Y. Tsien, and R. A. Steinhardt. 1990. Active involvement of Ca^{2+} in mitotic progression of Swiss 3T3 fibroblasts. *J. Cell. Biol.* 111:183–196.
Keith, C. H. 1987. Effect of microinjected calcium-calmodulin on mitosis in PtK2 cells. *Cell Motil. Cytoskeleton.* 7:1–9.
Koshland, D., J. C. Kent, and L. H. Hartwell. 1985. Genetic analysis of the mitotic transmission of minichromosomes. *Cell.* 40:393–403.
Langer, P. J., W. G. Shanabruch, and G. C. Walker. 1981. Functional organization of plasmid pKM101. *J. Bacteriol.* 145:1310–1316.
Lea, D. E., and C. A. Coulson. 1949. The distribution of the numbers of mutants in bacterial populations. *J. Genet.* 49:264–284.
LeClerc, J. E., J. R. Christensen, P. V. Tata, R. B. Christensen, and C. W. Lawrence. 1988. Ultraviolet light induces different spectra of *lacI* sequence changes in vegetative and conjugating cells of *Escherichia coli*. *J. Mol. Biol.* 203:619–633.
Levin, D., and E. Bartlett-Heubusch. 1992. Mutants in the *S. cerevisiae PKC1* gene display a cell cycle specific osmotic stability defect. *J. Cell Biol.* 116:1221–1229.
Muller, E. G. D. 1991. Thioredoxin deficiency in yeast prolongs S phase and shortens the G1 interval of the cell cycle. *J. Biol. Chem.* 266:9194–9202.
Ohkura, H., N. Kinoshito, S. Miyatani, T. Toda, and M. Yanagida. 1989. The fission yeast *dis2*$^+$ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. *Cell.* 57:997–1007.
Ohya, Y., and Y. Anraku. 1989*a*. Functional expression of chicken calmodulin in yeast. *Biochem. Biophys. Res. Commun.* 158:541–547.
Ohya, Y., and Y. Anraku. 1989*b*. A galactose-dependent *cmd1* mutant of *Saccharomyces cerevisiae:* involvement of calmodulin in nuclear division. *Curr. Genet.* 15:113–120.
Palmer, R. E., M. Koval, and D. Koshland. 1989. The dynamics of chromosome movement in the budding yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 109:3355–3366.
Persechini, A., R. H. Kretsinger, and T. N. Davis. 1991. Calmodulins with deletions in the central helix functionally replace the native protein in yeast cells. *Proc. Natl. Acad. Sci. USA.* 88:449–452.
Peterson, J. B., and H. Ris. 1976. Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. *J. Cell. Sci.* 22:219–242.
Pringle, J. R., R. A. Preston, A. E. Adams, T. Stearns, D. G. Drubin, B. K. Haarer, and E. W. Jones. 1989. Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31:357–435.
Rasmussen, C. D., and A. R. Means. 1989. Calmodulin is required for cell-cycle progression during G1 and mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:73–82.
Rasmussen, C. D., R. L. Means, K. P. Lu, G. S. May, and A. R. Means. 1990. Characterization and expression of the unique calmodulin gene of *Aspergullus nidulans*. *J. Biol. Chem.* 265:13767–13775.
Ratan, R. R., and M. L. Shelanski. 1986. Calcium and the regulation of mitotic events. *Trends Biochem. Sci.* 11:456–459.
Runge, K. W., R. J. Wellinger, and V. A. Zakian. 1991. Effects of excess centromeres and excess telomeres on chromosome loss rates. *Mol. Cell. Biol.* 11:2919–2928.
Sahyoun, N., M. Wolf, J. Besterman, T. Hsieh, M. Sander, H. Le Vine, K. J. Chang, and P. Cuatrecasas. 1986. Protein kinase C phosphorylates topoisomerase II: topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc. Natl. Acad. Sci. USA.* 83:1603–1607.
Schatzman, R. C., R. S. Turner, and J. F. Kuo. 1984. Phospholipid-sensitive Ca^{2+}-dependent protein phosphorylation. *In* Calcium and Cell Function. Vol. V. W. Y. Cheung, editor. Academic Press Limited, London. 33–66.
Schultz, M. C., R. H. Reeder, and S. Hahn. 1992. Variants of the TATA-binding protein can distinguish subsets of RNA polymerase I, II, and III promoters. *Cell.* 69:697–702.
Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 186 pp.
Sobue, K., Y. Muramoti, M. Fujita, and S. Kakiuchi. 1981. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. *Proc. Natl. Acad. Sci. USA.* 78:5652–5655.
Staben, C., and J. C. Rabinowitz. 1986. Nucleotide sequence of the *Saccharomyces cerevisiae ADE3* gene encoding C_1-tetrahydrofolate synthase. *J. Biol. Chem.* 261:4629–4637.
Stull, J. T. 1988. Myosin light chain kinases and caldesmon:biochemical properties and roles in skeletal and smooth muscle contractions. *In* Calmodulin. P. Cohen and C. B. Klee, editors. Elsevier Science Publishers, Amsterdam. 91–122.
Sun, G., Y. Ohya, and Y. Anraku. 1991. Half-calmodulin is sufficient for cell proliferation. *J. Biol. Chem.* 266:7008–7015.
Sweet, S. C., C. M. Rogers, and M. J. Welsh. 1988. Calmodulin stabilization of kinetochore microtubule structure to the effect of nocodazole. *J. Cell Biol.* 107:2243–2251.
Takeda, T., and M. Yamamoto. 1987. Analysis and in vivo disruption of the gene coding for calmodulin in Schizosaccharomyces pombe. *Proc. Natl. Acad. Sci. USA.* 84:3580–3584.
Tanaka, T., K. Kadowaki, E. Lazarides, and K. Sobue. 1991. Ca^{2+}-dependent regulation of the spectrin/actin interaction by calmodulin and protein 4.1. *J. Biol. Chem.* 266:1134–1140.
Thomas, J. H., and D. Botstein. 1986. A gene required for the separation of chromosomes on the spindle apparatus in yeast. *Cell.* 44:65–76.
Todd, P. A., and B. W. Glickman. 1982. Mutational specificity of UV light in *Escherichia coli:* indications for a role of DNA secondary structure. *Proc. Natl. Acad. Sci. USA.* 79:4123–4127.
Uemura, T., and M. Yanagida. 1986. Mitotic spindle pulls but fails to separate chromosomes in typeII DNA topoisomerase mutants: uncoordinated mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1003–1010.
Uzawa, S., I. Samejima, T. Hirano, K. Tanaka, and M. Yanagida. 1990. The fission yeast *cut1*$^+$ gene regulates spindle pole body duplication and has homology to the budding yeast *ESP1* gene. *Cell.* 62:913–925.
Vantard, M., A. Lambert, J. De Mey, P. Picquot, and L. J. Van Eldik. 1985. Characterization and immunocytochemical distribution of calmodulin in higher plant endosperm cells: localization in the mitotic apparatus. *J. Cell Biol.* 101:488–499.
Walker, G. C. 1977. Plasmid (pKM101)-mediated enhancement of repair and mutagenesis: dependence on chromosomal genes in *Escherichia coli* K-12. *Mol. & Gen. Genet.* 152:93–103.
Wallis, J. W., G. Chrebet, G. Brodsky, M. Rolfe, and R. Rothstein. 1989. A hyper-recombination mutation in S. cerevisiae identifies a novel eukaryotic topoisomerase. *Cell.* 58:409–419.
Welsh, M. J., J. R. Dedman, B. R. Brinkley, and A. R. Means. 1978. Calcium-dependent regulator protein: localization in mitotic apparatus of eukaryotic cells. *Proc. Natl. Acad. Sci. USA.* 75:1867–1871.
Welsh, M. J., J. R. Dedman, B. R. Brinkley, and A. R. Means. 1979. Tubulin and calmodulin: effects of microtubule and microfilament inhibitors on localization in the mitotic apparatus. *J. Cell Biol.* 81:624–634.
Winey, M., L. Goetsch, P. Baum, and B. Byers. 1991. *MPS1* and *MPS2*: novel yeast genes defining distinct steps of spindle pole body duplication. *J. Cell Biol.* 114:745–754.
Wood, R. D., R. R. Skopek, and F. Hutchinson. 1984. Changes in DNA base sequence induced by targeted mutagenesis of lambda phage by ultraviolet light. *J. Mol. Biol.* 173:273–291.
Youderain, P., and M. M. Susskind. 1980. Identification of the products of bacteriophage P22 genes, including a new late gene. *Virology.* 107:258–269.