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 Ca²⁺ 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 Ca²⁺ 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 PtK₂, Chinese hamster ovary cells (Welsh et al., 1979), and plant endosperm (Vantard et al., 1985). One hypothesis is that Ca²⁺-calmodulin regulates a protein phosphorylation cascade that results in chromosome segregation (Dinsmore and Sloboda, 1988; Keith, 1987; Ratani and Shelanski, 1986). However, the Ca²⁺ transients proposed to trigger this cascade were recently found not to be required for chromosome segregation (Kao et al., 1990). Ca²⁺ transients do induce nuclear envelope breakdown (Kao et al., 1990) via Ca²⁺-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, 1989a; 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 Ca²⁺-binding sites have been inactivated can support the growth of yeast cells (Geiser et al.,...
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-calmodulin is required for chromosome segregation and may specifically participate in disjunction. The immunolocalization of calmodulin implicates calmodulin in 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 lacking uracil. Other media for growth of *S. cerevisiae* and *E. coli* were as described (Geiser et al., 1991). **Table 1. Strains**

| Strain | Genotype | Reference |
|--------|----------|-----------|
| CRY1   | MATα/ade2-loc; can1-100; his3-11, 15; leu2-3,112; trp1-1; ura3-1 | Robert Fuller |
| TDY49  | MATα/Mata; ade2-loc/ade2-loc; can1-100/can1-100; cmdlA::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-loc; can1-100; cmdlΔ::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 | MATα; ade2-loc; 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-loc; ade3Δ-100; can1-100; CMD1; leu2-3,112, lys2Δ::HIS3; trp1-1; ura3-1; unknown cs | This study |
| TDY55  | TDY52-3D x TDY49-5B | This study |
| TDY55-5D | MATα; ade2-loc; ade3Δ-100; can1-100; cmdlΔ::TRP1; his3-11,15; leu2-3,112, lys2Δ::HIS3; trp1-1; ura3-1 | This study |
| TDY57  | MATα; ade2-loc; ade3Δ-100; can1-100; cmdl1-1; leu2-3,112, lys2Δ::HIS3; trp1-1; ura3-1; unknown cs | This study |
| TDY62  | TDY57 x CRY1 | This study |
| TDY62-1A | MATα; ade2-loc; can1-100; his3-11, 15; leu2-3,112; trp1-1; ura3-1 | This study |
| TDY62-13A | MATα; ade2-loc; ade3Δ-100; can1-100; cmdl1-1; leu2-3,112; trp1-1; ura3-1 | This study |
| JGY44  | TDY62-13A x CRY1 | This study |
| JGY44-2A | MATα; ade2-loc; can1-100; cmdl1-1; his3-11, 15; leu2-3,112; trp1-1; ura3-1 | This study |
| TDY68  | TDY62-13A plus cyh² | This study |
| TDY70  | TDY68 x TDY62-1A | This study |
| TDY70-30A | MATα; ade2-loc; ade3Δ-100; can1-100; CMD1; cyh²; leu2-3,112; trp1-1; ura3-1 | This study |
| TDY70-30C | MATα; ade2-loc; ade3Δ-100; can1-100; cmdl1-1; cyh²; leu2-3,112; trp1-1; ura3-1 | This study |
| TDY92  | MATα/MATA; ade2-loc/ade2-loc; ADE3/ade3Δ-100; can1-100/can1-100; cmdl1-1; cyh²; his3-11.15/HIS3; HIS4/his4::CYH2; 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/cmdl1-1 and his4::CYH2 is on the same chromosome as MATα | 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-fluorouracil (Boeke et al., 1987) and grown on low aden plates. The genotypes of the strains were checked by a Southern blot analysis.

Strain TDY57 is strain TDY52-3D in which the cmdl-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 1. TDY68 was selected as a spontaneous mutant of strain TDY52-13A resistant to 1 μg/ml cycloheximide. To confirm the mutation to resistance was in the CYH2 gene, I crossed TDY68 with strain XII4-S19 (received from the Yeast Genetic Stock Center, University of California, Berkeley, CA), known to carry cyh2" (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". 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.

For flow cytometry, strains TDY70-30A and TDY70-30C were converted to a" 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 a" strains could not use glycerol as a carbon source (Sherman et al., 1986). The his4::CYH2 allele in strains TDY52, TDY93, TDY94, and TDY96 was previously described (Runge et al., 1991).

**Mutagenesis**

Plasmid pTD59 (10 ng/μl) was irradiated with 225 ergs/mm² or 450 ergs/mm² of 254 nm light using four 15W germicidal light bulbs (GIST; General Electric Co., Wilmington, MA) and then placed on ice.

**Isolation of a Synchronous G1 Population of Cells**

Yeast strains were grown to ~320 Klett units in YPD medium and 1 nil of yeast cells was added to 10 ml LB containing 30 μg/ml kanamycin to 100 Klett units of turbidity. Yeast cultures were then grown in 10 ml LB containing 30 μg/ml kanamycin to 100 Klett units. Yeast cells had reached a density of 1-2 × 10⁷ cells/ml. Cells were pelleted at 6,000 rpm for 10 min in an SS-34 rotor (Sorvall Instruments, Wilmington, DE), washed in 3 X 10⁵ cells/ml. 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/mm² UV light. These doses resulted in 50 and 5 % survival, respectively. To allow induction of mutations in TDY68 is cyh2.

Flow cytometry, strains TDY70-30A and TDY70-30C were converted to a" 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 a" strains could not use glycerol as a carbon source (Sherman et al., 1986). The his4::CYH2 allele in strains TDY52, TDY93, TDY94, and TDY96 was previously described (Runge et al., 1991).

**Plasmid pTD59** (10 ng/μl) was irradiated with 225 ergs/mm² or 450 ergs/mm² of 254 nm light using four 15W germicidal light bulbs (GIST; General Electric Co., Wilmington, MA) and then placed on ice.

**Flow Cytometry**

For flow cytometry, strains TDY70-30A and TDY70-30C were converted to a" 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 a" strains could not use glycerol as a carbon source (Sherman et al., 1986). The his4::CYH2 allele in strains TDY52, TDY93, TDY94, and TDY96 was previously described (Runge et al., 1991).

**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 with triton-X-100 as described (method 2 in Pringle et al., 1989), 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 1:10,000 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 GI 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 as not two un budded 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)) × 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; Youserain 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 (uvrΔ-) 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; LeClere et al., 1988). Although changes tend to occur at or near pyrimidine pairs (LeClere 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 (cmdl-1) integrated at the CMD1 locus is strongly temperature dependent. On rich solid medium, the cmdl-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 cmdl-1 forms colonies the same size as wild type at all temperatures tested (21–38°C), thus cmdl-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 cmdl-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 GI. 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 GI 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 GI 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 GI are unbudded and smaller than cells at other stages of the cell cycle and thus remain near the top of the gradient.

Small unbudded cmdl-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
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 × 10⁶ viable cells/ml (cmdl-I) or 1.7 × 10⁶ viable cells/ml (CMDI) in SD − ura containing 74 μM [2-¹⁴C]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, Costa 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.

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-I strain and a CMDI 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-I 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 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.**
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.

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 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-

Davis Temperature-sensitive Calmodulin Mutant

613
Figure 6. Correlation between loss of viability and appearance of the nuclear DNA in the neck. Cells from a culture of JGY44-2A (cmd/-) 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 titrated 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.

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 titrated 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/10^9 cell divisions | Recombination rate/10^9 cell divisions | Recombination of Loss/10^9 cell divisions |
|-------------------|-------------|-------------------------------|----------------------------------------|------------------------------------------|
| cmdl-1/cmdl-1     | 30          | 32                            | 0.84                                   | 33                                       |
| CMD1/CMD1         | 30          | 4.5                           | 0.67                                   | 5.2                                      |
| cmdl-1/CMD1       | 30          | 3.0                           | 0.76                                   | 3.8                                      |
| cmdl-1/cmdl-1     | 21          | ND                            | ND                                     | 13                                       |
| CMD1/CMD1         | 21          | ND                            | ND                                     | 3.6                                      |
| cmdl-1/cmdl-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 cmdl-1/cmdl-1, TDY92 on day 1 and TDY96 on day 2; CMD1/CMD1, TDY92; cmdl-1/CMD1, TDY94.

E104V. Both mutations are required to confer a temperature-sensitive phenotype. II00N alone inhibits but does not prevent growth at 37°C. II00N also increases the sensitivity of the third Ca²⁺-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 Ca²⁺ ion in the third loop and substantially decreases the affinity of that loop for Ca²⁺ (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 Ca²⁺ 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) 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, 1989b) 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, 1989b) 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 (ndcl-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 cmdl-1 mutant.

The phenotype of the cmdl-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 Ca2+-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 (Bana-pathi 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 cut1* (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 fragmental nuclear DNA (Rasmussen et al., 1990). Calmodulin colocalizes with the kinetochore microtubules in plant endosperm (Vantard et al., 1985) and rat kangaroo PTK 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 Ca2+ (Geiser et al., 1991). Even mutant forms of vertebrate calmodulin defective in Ca2+-binding can perform the essential function in yeast. Thus, the essential mitotic function does not require Ca2+-binding. In agreement with this conclusion in yeast, the ability of calmodulin to bind to the mitotic apparatus in PTK cells is not dependent on the presence of Ca2+. Even a modified calmodulin unable to activate cyclic nucleotide phosphodiesterase can stabilize the kinetochore microtubules (Sweet et al., 1988). Thus, a possible Ca2+-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. Osheoff. 1988. Phosphorylation of DNA topoisomerase II in vivo and in total homogenates of *Drosophila* Kc cells. *J. Biol. Chem.* 263:12653–12660.

Baech, 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.

Baeting, C., J. Alderton, M. Poesien, H. Schulman, and R. A. Steinhardt. 1990. Multifunctional Ca2+/calmodulin-dependent protein kinase is necessary for nuclear envelope breakdown. *J. Cell Biol.* 111:1763–1773.

Banapathi, R., N. Kamath, A. Constantinitou, 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 duplication: homology of its product with Ca2+-binding proteins. *Proc. Natl. Acad. Sci. USA.* 83:5512–5516.

Baum, P., C. Yip, L. Goetsch, and B. Byers. 1988. A yeast gene essential 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 gene 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
Jacobs, C. W., A. E. M. Adams, P. J. Szaniszlo, and J. R. Pringle. 1988. Functional analysis of calcium-binding proteins in yeast. In Stimulus Response: The Role of Intracellular Calcium-binding proteins. V. L. Smith and J. R. Dedman, editors. CRC Press, Boca, FL. 237-249.

Davis, T. 1990. Genetic analysis of calcium-binding proteins in yeast. In Stimulus Response: The Role of Intracellular Calcium-binding proteins. V. L. Smith and J. R. Dedman, editors. CRC Press, Boca, FL. 117-577-606.

Davies, T. 1990. Genetic analysis of calcium-binding proteins in yeast. In Stimulus Response: The Role of Intracellular Calcium-binding proteins. V. L. Smith and J. R. Dedman, editors. CRC Press, Boca, FL. 117-577-606.

Sobue, K., Y. Muramoti, M. Fujita, and S. Kakiuchi. 1981. Purification of a calcium-binding protein from chicken gizzard that interacts with F-actin. Proc. Natl. Acad. Sci. USA. 78:5632-5635.

Schatzman, R. C., R. S. Turner, and J. F. Knoll. 1984. Phospholipid-sensitive Ca2+-dependent protein phosphorylation. In Calcium and Cell Function. Vol. V. W. 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. Pink, and J. B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 186 pp.

Sobue, K., Y. Muramoti, M. Fujita, and S. Kakiuchi. 1981. Purification of a calcium-binding protein from chicken gizzard that interacts with F-actin. Proc. Natl. Acad. Sci. USA. 78:5632-5635.

Schatzman, R. C., R. S. Turner, and J. F. Knoll. 1984. Phospholipid-sensitive Ca2+-dependent protein phosphorylation. In Calcium and Cell Function. Vol. V. W. Cheung, editor. Academic Press Limited, London. 33-66.

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Scherer, F., G. R. Pink, and J. B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 186 pp.

Sobue, K., Y. Muramoti, M. Fujita, and S. Kakiuchi. 1981. Purification of a calcium-binding protein from chicken gizzard that interacts with F-actin. Proc. Natl. Acad. Sci. USA. 78:5632-5635.

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Sobue, K., Y. Muramoti, M. Fujita, and S. Kakiuchi. 1981. Purification of a calcium-binding protein from chicken gizzard that interacts with F-actin. Proc. Natl. Acad. Sci. USA. 78:5632-5635.

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Scherer, F., G. R. Pink, and J. B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 186 pp.

Sobue, K., Y. Muramoti, M. Fujita, and S. Kakiuchi. 1981. Purification of a calcium-binding protein from chicken gizzard that interacts with F-actin. Proc. Natl. Acad. Sci. USA. 78:5632-5635.

Schatzman, R. C., R. S. Turner, and J. F. Knoll. 1984. Phospholipid-sensitive Ca2+-dependent protein phosphorylation. In Calcium and Cell Function. Vol. V. W. Cheung, editor. Academic Press Limited, London. 33-66.

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Scherer, F., G. R. Pink, and J. B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 186 pp.

Sobue, K., Y. Muramoti, M. Fujita, and S. Kakiuchi. 1981. Purification of a calcium-binding protein from chicken gizzard that interacts with F-actin. Proc. Natl. Acad. Sci. USA. 78:5632-5635.

Schatzman, R. C., R. S. Turner, and J. F. Knoll. 1984. Phospholipid-sensitive Ca2+-dependent protein phosphorylation. In Calcium and Cell Function. Vol. V. W. 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.