Casein Kinase II Is Required for Cell Cycle Progression during G₁ and G₂/M in Saccharomyces cerevisiae*

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The catalytic subunit of Saccharomyces cerevisiae casein kinase II (Sc CKII) is encoded by the CKA1 and CKA2 genes, which together are essential for viability. Five independent temperature-sensitive alleles of the CKA2 gene were isolated and used to analyze the function of CKII during the cell cycle. Following a shift to the nonpermissive temperature, cka2* strains arrested within a single cell cycle and exhibited a dual arrest phenotype consisting of 50% unbudded and 50% large-budded cells. The unbudded half of the arrested population contained a single nucleus and a single focus of microtubule staining, consistent with arrest in G₁. Most of the large-budded fraction contained segregated chromatin and an extended spindle, indicative of arrest in anaphase, though a fraction contained an undivided nucleus with a short thick intranuclear spindle, indicative of arrest in G₂ and/or metaphase. Flow cytometry of pheromone-synchronized cells confirmed that CKII is required in G₁, at a point which must lie at or beyond Start but prior to DNA synthesis. Similar analysis of hydroxyurea-synchronized cells indicated that CKII is not required for completion of previously initiated DNA replication but confirmed that the enzyme is again required for cell cycle progression in G₂ and/or mitosis. These results establish a role for CKII in regulation and/or execution of the eukaryotic cell cycle.

Casein kinase II (CKII) is a serine/threonine protein kinase which is ubiquitous among eukaryotic organisms (for review, see Issinger, 1993; Pinna, 1990; Tuazon and Traugh, 1991). The enzyme is composed of a catalytic α and regulatory β subunit that combine to form a native α₂β₂ holoenzyme which is constitutively active in vitro. How (and indeed whether) the enzyme is regulated in vivo is unknown, though regulation via allosteric effectors (e.g. polyamines), covalent modification, cellular redistribution, and substrate-directed effects have all been proposed. CKII recognizes a Ser of Thr residue followed by a series of acidic residues and phosphorylates a broad and intriguing spectrum of both nuclear and cytoplasmic substrates.

Although the physiological role of CKII is not known, several lines of evidence suggest a role for the enzyme in cell prolifer-
inactivated within a time window with respect to the length of the cell cycle. Analysis of these temperature-sensitive strains reveals that CKII is required for cell cycle progression at two points in the cell cycle, one in G1 and in the other in G2 and/or mitosis. Furthermore, the data indicate that CKII activity is not required for ongoing DNA replication during S phase.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Sigma or J. T. Baker Chemical Co., media from Difco Laboratories, and restriction enzymes and other molecular biological reagents from Promega, New England Biolabs, or Life Technologies, Inc. Escherichia coli (Escherichia coli) was grown at 25°C for 6 h (log phase), and then treated either with n-factor at 2.5 μg/ml for 4.5 h or with hydroxyurea (added directly to the culture as a powder at 0.1 μg/ml for 5 h). Synchronized cultures were released by filtering through a 2.5-cm HA 0.45-μm filter (Millipore) mounted in a Swinnex 25 filter apparatus, followed by washing and resuspending the cells at the original density in fresh YPD at 25°C or the appropriate nonpermissive temperature.

Fluorescence Microscopy—For visualizing nuclear and spindle morphology, a sample of culture containing approximately 8 × 10^6 cells was fixed by the addition of 37% formaldehyde to a final concentration of 3.7% followed by incubation at 23°C for 3–4 h. Fixed cells were collected by centrifugation, washed twice with sterile water, and resuspended in 1 ml of Buffer A (0.1 M K-HPO₄, pH 7.0, 1.2 μM sorbitol). Fifty μl of Zymolyase 60,000 (ICN) at 4 units/ml in Buffer A was added, and the cells were incubated at 37°C for 1 h. Cells were washed twice in Buffer A and resuspended in 0.2 ml of Buffer A. Ten μl of this suspension was spotted into a polystyrene-coated well (polystyrene 300K, 0.1% in H₂O) of an eight-well Teflon-coated slide (Carlson Scientific Inc.) and incubated for 10 min. The secondary antibody was aspirated and the cells washed as described above. The slide was incubated at 25°C for 1–2 h in a humidified chamber. The primary antibody was aspirated and the cells washed as described above. Ten μl of secondary antibody (Amerham) was resuspended in 10 μl of secondary antibody (Amerham) and spotted onto the primary antibody. The slide was incubated at 25°C for 1–2 h in a humidified chamber. The secondary antibody was aspirated and the cells washed as described above. The wells were mounted in 90% glycerol, 0.02% p-phenylenediamine, 0.2 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Chemaid), and coverslips were sealed with nail polish. Cells were observed using a Zeiss IM 35 epifluorescence microscope equipped with a ×100 objective and either DAPI or fluorescein filters. Photographs were made with Kodak Technical Pan film.

Flow Cytometry—Cells were prepared for flow cytometry essentially as described by Hutter and Eipel (1979). Approximately 5 × 10^6 cells were analyzed. The cells were fixed in 70% ethanol in PBS, and coverslips were sealed with nail polish. Cells were observed using a Zeiss IM 35 epifluorescence microscope equipped with a ×100 objective and either DAPI or fluorescein filters. Photographs were made with Kodak Technical Pan film.
Cell Cycle Requirement for CKII

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Nucleotide substitutions and amino acid replacements in cka2 alleles

| Allele  | Nucleotide substitution(s) | Amino acid replacement(s) | Corresponding residue in cAMPdPK |
|---------|-----------------------------|---------------------------|---------------------------------|
| cka2-7  | GGC → ACG                   | A190T                     | A188                            |
|         | ACG → ATG                   |                           | T363M                           |
| cka2-8  | GAA → AAA                   | E51K                      | E44                             |
|         | GGC → GAC                   | G102D                     | F100                            |
| cka2-11 | GAC → AAC                   | D225N                     | D220                            |
|         | GAG → AAG                   | E299K                     | None                            |
| cka2-12 | GGC → GTG                   | A190V                     | A188                            |
|         | CAC → TAC                   | H294Y                     | None                            |
| cka2-13 | GAC → AAC                   | D225N                     | D220                            |

Substitutions are shown in the context of the affected codon. All substitutions are explainable on the basis of a C to T transition (on either the coding or noncoding strand), as expected for hydroxylamine mutagenesis.

All residues in the Cka2 polypeptide are numbered as in Padmanabha et al. (1990), i.e., relative to the initiating methionine as residue 1.

Residues in cAMPdPK are numbered as in Fig. 1 of Hanks et al. (1988).

E299K and H294Y lie within an insertion between subdomains X and XI (Hanks and Quinn, 1991). A comparable insertion is not present in cAMPdPK.

RESULTS

Construction of Temperature-sensitive Strains—Strains temperature sensitive for CKII activity were constructed by plasmid shuffling (Boeke et al., 1987). Because of the functional redundancy of the CKA1 and 2 genes (Padmanabha et al., 1990), either could have been selected for mutagenesis, and the CKA2 gene was chosen arbitrarily. A LEU2 CEN/ARS plasmid bearing the wild-type CKA2 gene was mutagenized in vitro with hydroxylamine and then transformed into RPG41-1a, a cka1 cka2 strain rescued by the wild-type CKA2 gene on a URA3 CEN/ARS plasmid (Padmanabha et al., 1990). Two-thousand independent transformants were plated in duplicate on minimal medium containing 5-FOA (to select against the URA3-marked plasmid) and incubated at both 25 and 37°C. Seven independent transformants that exhibited temperature-sensitive growth were identified and colony-purified. The LEU2-marked plasmids from five of these transformants were recovered in E. coli. Linkage between the mutagenized plasmid and the temperature-sensitive phenotype was confirmed by retransformation of RPG41-1a followed by plating on 5-FOA. None of the five strains was temperature sensitive prior to evolution of the CKA2 gene, confirming that all five alleles are recessive.

The nucleotide substitution(s) and corresponding amino acid replacement(s) present in each temperature-sensitive cka2 allele were determined by sequencing the protein coding region. As expected for hydroxylamine, which deaminates cytosines, all mutations could be explained by C → T transitions (see Table I). One allele (cka2-13) contained a single substitution while the remaining four (cka2-7, -8, -11, and -12) contained two, and every substitution resulted in an amino acid replacement. The temperature sensitivity of the single mutant (cka2-13) is presumably explained by the sole amino acid replacement in this mutant (D225N). The affected residue, which corresponds to D220 of cAMPdPK (Table I), is invariant in the protein kinase family (Hanks and Quinn, 1991) and is postulated to function in stabilizing the catalytic loop (Knighton et al., 1991). The D225N replacement also occurs as one of the two mutations present in the cka2-11 allele. The A190T and A190V replacements of cka2-7 and cka2-12, respectively, affect a residue which is moderately variable among protein kinases generally but is invariant among known CKIIIs. Replacements in the other two alleles do not affect strongly conserved residues either in protein kinases generally or in CKII (Hanks and Quinn, 1991).

The maximum permissive and minimum restrictive temperatures of the five mutant strains are shown in Table III. The lower temperature transition of YDH11 relative to YDH13 implies that the E299K replacement in the cka2-11 allele is also a destabilizing mutation, at least in the context of the D225N mutation.

All five strains exhibited some phenotypic defect at permissive temperature. As shown in Table III, all five strains exhibited some increase in flocculation. This effect was severe in those strains showing the greatest temperature sensitivity, most notably YDH12, which grew essentially as a pellet at 25°C in liquid medium. Strains displaying the greatest temperature sensitivity also exhibited a modest slow growth phenotype at permissive temperature, as assessed by colony size on plates (data not shown). Because flocculation and slow growth are both characteristic of CKII depletion (Padmanabha et al., 1990), these results imply a reduction in CKII activity in these mutants at permissive temperature. Based on the above results, YDH8 was selected for the bulk of the studies described below because it has the lowest transition temperature consistent with a near normal growth rate (Fig. 1A) and a tractable level of flocculation (Table III) at permissive temperature.

Terminal Phenotype at the Restrictive Temperature—When shifted from 25 to 37°C during log phase growth, YDH8 exhibited arrest within one cell doubling, consistent with arrest during the first cell cycle following the shift (Fig. 1B). In contrast, the wild-type control strain YDH6 exhibited only a transient heat shock-induced depression in growth rate and then grew to saturation normally. As anticipated for cells depleted of CKII activity (Padmanabha et al., 1990), YDH8 exhibited complete flocculation within 1–2 h following the shift to restrictive temperature (data not shown). Similar behavior was observed with the other four temperature-sensitive alleles.

Budding profiles for the experiment shown in Fig. 1B are...
TABLE III

Properties of cka2– strains

| Strain   | Allele | Maximum permissive temperature\(a\) | Minimum restrictive temperature\(b\) | Flocculation at 25 °C | % |
|----------|--------|--------------------------------------|--------------------------------------|-----------------------|---|
| YPH250\(a\) | CKA2   | 39                                   | 41                                   | 8 ± 4                 |   |
| YDH6\(a\)   | CKA2   | 38,5                                 | 40,5                                 | 37 ± 4                |   |
| YDH7       | cka2–7 | 35                                   | 36                                   | 58 ± 5                |   |
| YDH8       | cka2–8 | 33                                   | 35                                   | 77 ± 14               |   |
| YDH11      | cka2–11| 31                                   | 34                                   | 87 ± 5                |   |
| YDH12      | cka2–12| 29                                   | 33                                   | 97 ± 1                |   |
| YDH13      | cka2–13| 33                                   | 35                                   | 90 ± 1                |   |

\(a\) Maximum permissive temperature is the highest temperature which allows essentially normal colony formation on YPD plates.

\(b\) Minimum restrictive temperature is the lowest temperature which prevents macroscopically detectable growth on YPD plates.

\(c\) Percent flocculation at 25 °C was assayed as described under "Experimental Procedures." The mean and standard error for three independent cultures of each strain is shown.

Data for the parental strain YPH250 and the isogenic control strain YDH6 are shown for comparison. The slight phenotype of YDH6 relative to YPH250 presumably reflects the absence of a functional CKA1 gene in YDH6 and/or occasional loss of the CEN plasmid carrying the rescuing CKA2 gene.

Fig. 1. Growth curves of asynchronously growing CKA2 and cka2–8 strains at 25 and 37 °C. Strains were inoculated into YPD, grown for 6 h at 25 °C, and then either maintained at 25 °C (A) or shifted to 37 °C (B). Cell density was determined as described under "Experimental Procedures." ○, YDH6 (CKA2); ●, YDH8 (cka2–8).

Presented in Table IV. Both YDH6 and YDH8 exhibited a transient, heat shock-induced increase in the proportion of unbudded cells during the first 1–2 h following the shift to restrictive temperature. YDH8 cells reached a stable state (terminal phenotype) by 4–6 h after the shift. The arrested population consisted of approximately equal numbers of unbudded and large-budded cells, with small-to-medium-budded cells being present at very low levels. In contrast, YDH6 gradually returned to the roughly equal mixture of unbudded and small-to-medium-budded cells characteristic of log phase growth. Flow cytometry of YDH8 cells 4–6 h after the shift indicated a mixture of cells containing 1 and 2 n DNA complement (data not shown). These results imply the existence of at least two distinct arrest points in the mutant. Because YDH8 cells do not arrest with a single terminal morphology, cka2–8 does not qualify as a classical cell division cycle mutation (Hartwell et al., 1973).

To determine whether the dual arrest phenotype was specific to YDH8, the budding profile of two other temperature-sensitive strains was examined at the restrictive temperature. Strains YDH11 and 13, whose cka2 alleles bear replacement(s) distinct from those of cka2–8 (Table II), also arrested as a 50:50 mixture of unbudded and large-budded cells (data not shown). This outcome indicated that the dual arrest phenotype is not an allele-specific response.

To better define the points of arrest, YDH8 cells incubated at the restrictive temperature for 5 h were double-stained with the DNA-binding dye DAPI (to visualize nuclear morphology) and an antitubulin monoclonal antibody (to visualize the tubulin cytoskeleton, including the spindle). Stained cells were analyzed by immunofluorescence microscopy (Fig. 2B). The unbudded half of the arrested population uniformly displayed a single mass of DAPI-stainable material, indicative of an undivided nucleus. The majority of these cells contained an array of cytoplasmic microtubules radiating from a single focus, a morphology typical of cells arrested in G1. The large-budded half of the arrested population was heterogeneous. The majority of these cells (approximately two-thirds) contained two lobes of DAPI-stainable material and an elongated spindle. Cells in which the two lobes remained joined through the bud aperture and cells in which the two nuclei appeared to be fully separated were both observed, at approximately equal frequency. Both phenotypes are indicative of arrest in anaphase (Surana et al., 1993). The remaining third of budded cells contained a single, round nucleus traversed by a short, thick intranuclear spindle (not shown). The latter phenotype is characteristic of arrest in G2 or metaphase (Irniger et al., 1995; Surana et al., 1991). At the level of resolution achieved by immunocytochemistry, the morphology of the spindle did not appear to be abnormal in any of these arrested cells. The mutant grown at 25 °C (Fig. 2A) and the wild-type strain grown at either 25 or 37 °C (not shown) exhibited the expected array of nuclear and cytoskeletal morphologies typical of logarithmically growing cells.

The results obtained with asynchronous cultures suggested that CKII is required for cell cycle progression in G1, as well as in G2 and/or M. In order to probe these arrest points independently, we analyzed cell cycle progression of synchronized mutant and wild-type cultures. Cells were synchronized in G1 by two different protocols, nutrient limitation and exposure to mating pheromone, and at the G1/S boundary by treatment with hydroxyurea.

CKII Is Required for Cell Cycle Progression in G1—S. cerevisiae respond to nutrient limitation by arresting cell division (and growth) early in the G1 phase of the cell cycle. In order to synchronize cells at this point in the cycle, YDH6 and YDH8 were grown to late stationary phase in YPD at 25 °C (90–95% unbudded cells). The synchronized cells were then released into fresh medium prewarmed to 37 °C (Fig. 3). In contrast to the control, which grew to saturation normally at this temperature, YDH8 exhibited no increase in cell number, clearly indicating arrest in the first cell cycle following release (Fig. 3A). Moreover, analysis of the budding profile indicated a marked defect in G1 progression (Fig. 3B). Relative to the control, which budded synchronously in the first few hours after release, YDH8 cells budded with slower kinetics, and at least 80% of the initially unbudded population failed to bud at all. The small percentage of cells which did escape G1 arrest subsequently arrested as large-budded cells (not shown).
The failure to obtain a quantitative G1 arrest could be explained by the finite time required to inactivate the cka2-8 allele (or to dephosphorylate the relevant substrates) or by residual activity of the enzyme at the restrictive temperature. Preincubation of YDH8 at 37°C for either 1 or 2 h prior to release had little if any effect on the proportion of cells able to pass the G1 block. In contrast, preincubation for 1 h at 38.5°C released had little if any effect on the proportion of cells able to clear the G1 block (75–80% budded cells, the vast majority large-budded).}

**Fig. 2.** Nuclear morphology and microtubule cytoskeleton of YDH8 (cka2-8) at 25 and 37°C. YDH8 was inoculated into YPD as in Fig. 1, grown for 6 h at 25°C, and then either maintained at 25°C for an additional 5 h (A) or shifted to 37°C for 5 h (B). Nuclear morphology was visualized with DAPI, and the microtubule cytoskeleton was visualized by staining with a monoclonal anti-tubulin antibody, as described under "Experimental Procedures." The identical field of cells is shown in the left and right panels.

The failure to obtain a quantitative G1 arrest could be explained by the finite time required to inactivate the cka2-8 allele (or to dephosphorylate the relevant substrates) or by residual activity of the enzyme at the restrictive temperature. Preincubation of YDH8 at 37°C for either 1 or 2 h prior to release had little if any effect on the proportion of cells able to pass the G1 block. In contrast, preincubation for 1 h at 38.5°C (the maximum permissive temperature of the control strain; Table III) and subsequent release at that temperature resulted in a complete G1 block (data not shown). This result suggests some leakiness of the cka2-8 allele at 37°C.

Wild-type cells exposed to the mating pheromone α-factor arrest cell division, but not growth, at a later stage in G1 than cells in stationary phase. To determine whether the point of G1 arrest in response to CKII depletion lies before or after the point of α-factor arrest, YDH6 and YDH8 were grown to log phase, synchronized in G1 with α-factor and then released at 25, 37, or 38.5°C. As shown in Fig. 4A, YDH8 responded to and recovered from α-factor with kinetics identical to those of the wild-type at 25°C. At 37°C (without preincubation) YDH8 exhibited only a slight G1 defect (data not shown). The strain budded with near normal efficiency, albeit approximately 1 h slower than wild-type, and did not arrest until the G2 and/or M block (75–80% budded cells, the vast majority large-budded).

**Fig. 3.** Growth curves and budding profiles of CKA2 and cka2-8 strains following release from stationary phase arrest. Cultures were grown in YPD at 25°C until stationary phase (90–95% unbudded cells) and then inoculated at 8 × 10⁶ cells/ml in YPD prewarmed to 37°C. Cell density (A) and the percentage of budded cells (B) were determined as described under "Experimental Procedures." ○, YDH6 (CKA2); •, YDH8 (cka2-8).

To confirm that arrest was indeed in G1, the DNA content of mating pheromone-synchronized mutant and wild-type cells was analyzed by flow cytometry (Fig. 5). At the time of α-factor release, all cells were arrested with a 1N DNA content. After release, the mutant at 38.5°C remained arrested with a 1N complement of DNA for up to 11 h, while both the mutant at 25°C and wild-type at 38.5°C proceeded through S and into G2/M, eventually losing synchrony after multiple cell divisions. The G1 peak of the arrested mutant exhibited a rightward drift...
upon prolonged incubation at 38.5°C. Such drifts have been noted before and are due to increased autofluorescence from cell enlargement (Reed and Wittenberg, 1990). This was consistent with the observed increase in the average size of the arrested cells throughout the course of the experiment. These experiments with pheromone-synchronized cells confirm that CKII is required for cell cycle progression in G1, at a point which must lie between the point of α-factor arrest and the onset of S phase.

In order to determine whether arrest at the G1 block is reversible, G1-arrested YDH6 cells were shifted back to 25°C after 4 h at 38.5°C. Approximately 3–4 h after being returned to 25°C these cells simultaneously initiated bud formation (Fig. 4C) and entered S phase (Fig. 5). By 5 h of recovery approximately 80% of the cells were budded and contained a 2N DNA complement. However, few if any cells appeared to be competent to divide at this point, and nearly all of these recovered cells formed an aberrant, elongated bud. Cells with the latter morphology have been observed previously following gradual depletion of CKII activity in a null background (Padminaba et al., 1990) and are also prominent in cka2Δ strains incubated at a semipermissive temperature. We speculate that this phenotype is associated with intermediate levels of CKII activity during G2/M, such that some CKII-dependent functions are completed but not others.

CKII Is Required for Cell Cycle Progression in G2/M—In order to probe the requirement for CKII at later points in the cell cycle, YDH6 and YDH8 were synchronized after the CKII G1 block using hydroxyurea, an inhibitor of ribonucleotide reductase which arrests cells in early S phase with the large-budded morphology. Cells were released from hydroxyurea arrest at 25 or 38°C (the latter after a 1-h preincubation). As shown in Fig. 6A, both strains exhibited a normal response to and recovery from hydroxyurea at the permissive temperature. At 38°C, the control strain YDH6 recovered and grew to saturation, though with the slower kinetics noted earlier (Fig. 6B). In contrast, YDH8 exhibited no increase in cell number at 38°C. This result implies the existence of at least one additional arrest point in the mutant. As with the first arrest point in G1, arrest at this second block also occurs in the first cycle following release from the synchronization block.

In order to define the position of the second arrest point, cells were analyzed by flow cytometry (Fig. 7). Prior to release from hydroxyurea, the mutant at 25 and 38°C and the wild-type at 38°C all exhibited a DNA content between 1 and 2N, indicative of S phase arrest. Within the first 2 h after release, all three

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2 D. E. Hanna and C. V. C. Glover, unpublished observation.
cultures resumed DNA synthesis, completed S, and acquired a 2N DNA content. This result established that the mutant is able to complete previously initiated DNA synthesis at the restrictive temperature. At later time points, the two control cultures continued to cycle and ultimately lost synchrony, whereas the mutant at 38°C remained arrested with a 2N DNA complement for up to 9 h. The flow cytometry data thus positioned the second cell cycle block in G2 and/or M. The G2/M-arrested cells remained large-budded and, when examined for their nuclear and spindle morphologies, displayed the same characteristic range of large-budded phenotypes seen in asynchronous cultures at 37°C (data not shown). This suggested that the G2/M block defined using hydroxyurea synchronization is the same as that observed in the asynchronous cultures.

Viability at the Nonpermissive Temperature—The inability of YDH8 to resume normal cell cycle progression following release from CKII arrest at either the G1 or G2/M block implied that YDH8 becomes inviable at the nonpermissive temperature. Direct measurement of percent viability (Fig. 8) confirmed that the viability of YDH8 declines precipitously between 2 and 4 h following a shift to 38.5°C, a time frame which coincides with cessation of cell cycle progression. The fact that few if any cells in the population retained viability at the nonpermissive temperature confirmed that cells arrested at either CKII block are inviable. Essentially identical results were obtained following a shift to 37°C (data not shown).

RNA and Protein Synthesis—The rapid loss of viability of YDH8 at the nonpermissive temperature raised the possibility that cell cycle arrest might be secondary to a general depression of cell metabolism or macromolecular biosynthesis. To address this issue, we compared the rates of total RNA and protein synthesis in YDH6 and YDH8 at both 25 and 38°C. These rates were estimated by measuring uptake of [14C]uracil and [35S]methionine, respectively, into acid-precipitable material during a 10-min pulse. As shown in Fig. 9A, the wild-type and mutant strains displayed comparable rates of RNA synthesis during log phase growth at 25°C. Both strains also displayed a similar heat shock-induced depression in RNA synthesis following a shift to 38°C. While the latter effect was transient in the wild-type, the rate of total RNA synthesis in the mutant failed to recover and remained approximately 4-fold lower than that of the controls for at least 4 h. Whether
this reduction in the rate of RNA synthesis reflects a decrease in synthesis of rRNA, mRNA, or both is unknown. However, CKII phosphorylates a number of nucleolar proteins, including the mUBF transcription factor (Voit et al., 1992) and has been shown to activate rRNA transcription in isolated nuclei (Belen-guer et al., 1989).

The reduction in the rate of total RNA synthesis was not reflected in a comparable reduction in the rate of total protein synthesis (Fig. 9B). While uptake of [35S]methionine in the mutant was consistently lower than that of the wild-type, the magnitude of this effect (approximately 2-fold) was no greater at 38°C than at 25°C. By contrast, treatment with the protein synthesis inhibitor, cycloheximide, inhibited [35S]methionine incorporation more than 20-fold for up to 4 h (Fig. 9B). The arrest of growth in the mutant strain, therefore, does not appear to be due to an overall inhibition of protein synthesis, although an effect on the synthesis of specific messages limiting for cell cycle progression cannot be ruled out by these experiments. Also, because of the energetic expense of protein synthesis, cell cycle arrest is unlikely to be due to a general decline in metabolic activity.

**DISCUSSION**

Conditional CKII Alleles—We have used a plasmid shuffling technique to isolate five independent temperature-sensitive alleles of the cka2 gene. All of these alleles are recessive to wild-type and confer a phenotype similar to that of a null (including flocculation, loss of viability, and arrest as a mixture of budded and unbudded cells), suggesting that they are loss-of-function mutants. Consistent with this, extracts of YDH8 (grown at either 25 or 37°C) contain very low levels of CKII activity (Cardenas et al., 1993), and the phosphorylation of at least two well characterized CKII substrates, topoisomerase II (Cardenas et al., 1992) and eIF2α (Feng et al., 1994), is temperature-sensitive in vivo in YDH8. The rapidity with which cells arrest following a shift to the nonpermissive temperature suggests that these mutants are temperature-sensitive for activity per se rather than for enzyme synthesis or assembly (Hartwell et al., 1973). However, we have been unable to confirm this directly because of the low activity present in extracts of cells grown at permissive temperature.

We have used these temperature-sensitive alleles to define the requirement for CKII activity during the cell cycle in S. cerevisiae. As in any experiment employing a temperature-sensitive mutation, an important caveat is that the observed effects may be specific to heat-shocked cells. This concern is exacerbated in this case because of the higher temperatures required to obtain a tight arrest. While we cannot eliminate this caveat, we note that temperatures identical to those employed here have been effectively used to analyze cell cycle mutants in S. cerevisiae. For example, a temperature of 38°C was required to identify the G2/M function of Cdc28 (Reed and Wittenberg, 1990). Similarly, Tang and Reed (1993) used 38.5°C to obtain a tight G1 arrest of a cks1 allele. Requirement for CKII in G1—The results presented here demonstrate that CKII is required for cell cycle progression in G1. Studies with pheromone-synchronized cells indicate that CKII is required either at Start itself (defined as the point of α-factor arrest) or between Start and the initiation of DNA synthesis (the G1/S transition). We emphasize that the data do not exclude the possibility of additional arrest points in G1. Indeed, one apparent paradox in our results is that pheromone-synchronized cells do not arrest in G1 when released at 37°C, whereas approximately half of an asynchronous population arrests in G1 at this temperature. Although this result may simply reflect the shorter time available to inactivate the kinase and/or dephosphorylate the relevant substrates, it is possible instead that it implies an additional CKII-sensitive point in G1 (prior to Start) for which 37°C is sufficient for arrest. Consistent with this, cells arrested in G1 following release from stationary phase arrest at 37°C do not respond to α-factor (data not shown), indicating that they are not arrested at Start. Microinjection of antibodies against the β subunit of CKII has been shown to inhibit mammalian cell cycle progression at multiple points in G1, specifically the G0/G1 transition, early G1, and the G1/S transition (Pepperkok et al., 1994). A general requirement for CKII activity during G1 is consistent with data...
from mammalian systems indicating elevated levels of CKII activity in G2, particularly just prior to the G2/S transition (Carroll and Marshak, 1989; DeBenedette and Snow, 1991).

The targets of CKII which are essential for G1 progression remain to be identified. In S. cerevisiae, progression through Start is regulated by a series of complexes between the Cdc28 protein kinase and five different cyclins, Cin1, Cin2, Cin3, Clb5, and Clb6 (Schwob and Nasmyth, 1993). Russo et al. (1992) have shown that p34\textsuperscript{cdc2} is phosphorylated in vitro by purified CKII at Ser\textsuperscript{39} and that this site is phosphorylated in vivo during the G1 phase of the cell cycle. Whether Cdc28 is subject to the same modification is not known, but the relevant CKII phosphorylation site is conserved in Cdc28. Other proteins required for Start also represent potential targets. In an effort to identify genes which interact genetically with CKII, we have carried out a screen for multicopy suppressors of the cka2-13 allele.\textsuperscript{3} Among the genes identified in this screen is CDC37, a previously characterized gene required for Start (Reed, 1980). We have found that Cdc37 is a physiological substrate of CKII and that mutation of the CKII recognition site impairs CDC37 function in vivo.\textsuperscript{4} Although the biochemical function of Cdc37 is not known, failure to phosphorylate Cdc37 could explain the G1 arrest of cka2 mutants.

Our analysis of cells released from hydroxyurea arrest suggests that CKII activity is not required for completion of S phase, consistent with the low level of CKII activity detected during S phase in mammalian systems (Carroll and Marshak, 1989; DeBenedette and Snow, 1991). However, we emphasize that this conclusion rests upon the dual assumption that the enzyme is fully inactivated by a 1-h preincubation at 38 °C and that all relevant substrates become dephosphorylated. While the former appears likely in view of our studies of G2 arrest, there is no information concerning the latter. At least one well characterized substrate of CKII is involved in DNA replication (DNA ligase I), and the activity of this protein is increased in response to CKII phosphorylation (Prigent et al., 1992). We also emphasize that our results do not preclude a requirement for CKII at the G1/S transition, as hydroxyurea arrest occurs after this point in the cycle.

Requirement for CKII in G2/M—The post-S phase arrest of CKII mutants is heterogeneous, with two-thirds of the arrested population exhibiting an anaphase morphology and the remainder a morphology typical of arrest in G2 and/or metaphase. One interpretation of these data is that CKII is required at multiple points in G2/M. Alternatively, there may be a single requirement for CKII, but the resulting phenotype may be either leaky or intrinsically heterogeneous. We cannot at present distinguish among these possibilities. A post-S phase function for CKII is consistent with work in Xenopus laevis, where it has been demonstrated that microinjection of purified CKII into oocytes potentiates mitosis promoting factor-induced maturation (Mulner-Lorillon et al., 1988). Interestingly, the \( \beta \) subunit of Xenopus CKII is phosphorylated in vitro by mitotic p34\textsuperscript{cdc2}, and this phosphorylation results in increased CKII activity (Mulner-Lorillon et al., 1990). Both the \( \alpha \) and \( \beta \) subunits of mammalian CKII are also phosphorylated in vitro by p34\textsuperscript{cdc2}, and phosphorylation at these sites increases dramatically in human or chicken cells arrested in mitosis (Litchfield et al., 1991, 1992). Although no changes in activity were reported in the latter studies, collectively these results suggest that CKII may function downstream of mitotic CDK activity.

Although the targets of CKII relevant to arrest in G2/M remain to be identified, a promising candidate is the well-characterized CKII substrate, topoisomerase II. This enzyme is a major structural component of the metaphase chromosome scaffold and is essential both for chromosome condensation in metaphase and for sister chromatid segregation during anaphase (for review, see Cardenas and Gasser, 1993). S. cerevisiae topoisomerase II is an excellent substrate of CKII in vitro, and phosphorylation strongly stimulates enzyme activity (Cardenas et al., 1993). The protein is phosphorylated in vivo and becomes hyperphosphorylated during mitosis (Cardenas et al., 1992). The excellent correlation between in vivo and in vitro sites suggests that topoisomerase II is a physiological target of CKII in yeast. Consistent with this, phosphorylation of the protein is temperature sensitive in the YDH8 strain (Cardenas et al., 1992). Temperature-sensitive topoisomerase II (top2\textsuperscript{ts}) mutants arrest in mitosis with an elongated spindle and a nucleus which is stretched through the bud aperture (Holm et al., 1985), a phenotype similar to that of anaphase-arrested cka2\textsuperscript{ts} cells. Moreover, like cka2\textsuperscript{ts} mutants, top2\textsuperscript{ts} become inviable at the nonpermissive temperature, apparently during a failed attempt to segregate daughter chromosomes (Holm et al., 1985). Collectively, these data suggest that the anaphase arrest of cka2\textsuperscript{ts} strains may result from failure to phosphorylate and activate topoisomerase II. It appears unlikely that failure to activate topoisomerase II can account for those cka2\textsuperscript{ts} cells which arrest with a G2/M metaphase phenotype, suggesting that additional substrates must be involved.

Conclusions—The results described here indicate a requirement for CKII in both the G1 and G2/M phases of the cell cycle. An important unresolved issue is whether CKII is required only for the mechanics of cell cycle progression or whether it exerts a regulatory function in G1 and/or G2/M. Our results argue against the trivial possibility that CKII-mediated cell cycle arrest is the result of a general decline in metabolic activity or biosynthetic capacity, since CKII-arrested cells continue to grow after cell cycle progression is halted (Padmanabha et al., 1990) and retain a significant capacity for protein as well as DNA synthesis. Based on arguments outlined above, we speculate that CKII acts downstream of mitotic CDK activity, perhaps as part of the interacting network of protein kinases activated at mitosis (King et al., 1994). Because CKII arrest is partially reversible in G1 but irreversible in G2/M, an intriguing possibility is that a mechanism has evolved to monitor CKII activity in G1 (perhaps via Cdc37) in order to ensure that adequate levels of activity are available to phosphorylate the structural and enzymatic proteins (such as topoisomerase II) needed during mitosis. The failure of cka2\textsuperscript{ts} mutants to recover completely from G1 arrest may be an artifact associated with the necessity of resynthesizing CKII after the return to permissive temperature.

The properties of the CKII mutations described here differ in several respects from those reported recently for the orb5 allele of the S. pombe cka1\textsuperscript{a} gene (Snell and Nurse, 1994). Following a shift to the nonpermissive temperature, orb5 cells undergo several cell divisions and ultimately die as small spherical cells. No cell cycle defects were noted in this mutant, which was interpreted as a morphological mutant defective in reinitialization of polarized growth following cytokinesis (Snell and Nurse, 1994). At face value, the different behavior of temperature-sensitive CKII mutations in the two organisms suggests significant differences in the physiological role of CKII in S. pombe and S. cerevisiae. However, we have recently isolated two temperature-sensitive alleles of the Sc CKII \( \alpha \) subunit and find that these exhibit a behavior strikingly similar to that of the orb5 mutation, including the absence of first cycle arrest and adoption of a highly spherical

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\textsuperscript{3} R. O. McCann, D. E. Hanna, and C. V. C. Glover, unpublished observations.

\textsuperscript{4} R. O. McCann and C. V. C. Glover, unpublished observations.
morphology. This result implies some functional specialization of CKA1 and CKA2 in S. cerevisiae and argues that the function of CKII in the two yeasts may be similar. Additional studies in both organisms will be required to clarify and correlate the physiological role of CKII in the two species.

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