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Conservation of Function and Regulation within the Cdc28/*cdc2* Protein Kinase Family: Characterization of the Human Cdc2Hs Protein Kinase in *Saccharomyces cerevisiae*

CURT WITTENBERG AND STEVEN I. REED*

Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

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Whereas the Cdc28 protein kinase of the budding yeast *Saccharomyces cerevisiae* plays an essential role in cell cycle progression during the G1 interval, a function in the progression from the G2 interval into M phase has been inferred for its homologs, including the Cdc2Hs protein kinase of humans. To better understand these apparently disparate roles, we constructed a yeast strain in which the resident *CDC28* gene was replaced by its human homolog, *CDC2Hs*. This transgenic yeast strain was able to perform the G1 functions attributed to the Cdc28 protein kinase, including the ability to grow and divide normally, to respond to environmental signals that induce G1 arrest, and to regulate the Cdc2Hs protein kinase appropriately in response to these signals.

Some of the regulatory elements of cell cycle control have recently been shown to be highly conserved throughout the eucaryotic phylogeny. The Cdc28 protein kinase (22, 29), established by genetic analysis to be essential for cell cycle progression in the budding yeast *Saccharomyces cerevisiae* (11, 28), has been found to have close structural homologs in numerous eucaryotic species (1, 7–9, 13, 18–20). However, while the critical functions of the Cdc28 protein kinase have been shown to be limited to the G1 interval of the cell cycle in *S. cerevisiae* (11, 12, 24, 28, 33), a function in the G2/M transition (6–9, 18) has been inferred for the Cdc28 homologs of higher eucaryotes. To help resolve this apparent paradox, we constructed a yeast strain in which the resident *CDC28* gene was replaced by its human homolog (20), *CDC2Hs*. We found that this transgenic strain was able to execute the G1 functions that have been attributed to the Cdc28 protein kinase.

Using fragment-mediated gene replacement (30) and standard molecular biological techniques (23), the resident *CDC28* gene in an *S. cerevisiae* strain was replaced with a chimeric gene containing the protein-coding region of *CDC2Hs* flanked by the noncoding 5′ and 3′ regions of *CDC28* (Fig. 1A). The starting yeast strain for this construction was SR679-1, which contained the chromosomal configuration shown in Fig. 1A and described previously (34). In this strain, a small segment 5′ to the *CDC28* polypeptide-coding region (designated Y) has been replaced by a segment containing the *TRP1* gene and the opposing *GAL10* and *GAL1* promoters and upstream regulatory elements (15). Thus, SR679-1, which contains a chromosomal *trp1* marker, is prototrophic for tryptophan and expresses the essential *CDC28* gene only when galactose is present and glucose is absent. SR679-1 was transformed with a DNA fragment containing normal chromosomal sequences flanking the *CDC28* polypeptide-coding region on the 5′ (X and Y) and 3′ (Z) ends but containing the *CDC2Hs* polypeptide-coding region instead of the *CDC28* coding region. The chimeric donor DNA was derived from a plasmid constructed by using the *CDC2Hs* cDNA (20). Transformants were selected on the basis of ability to form colonies on medium containing glucose. The parental strain, unable to transcribe the *GAL1*::*CDC28* gene under these conditions, can grow for only a limited number of generations. Since gene convertants of the desired configuration should have eliminated the *TRP1* marker, transformants capable of growth on glucose were screened for tryptophan auxotrophy. Glucose-resistant tryptophan auxotrophs were obtained at the frequency expected for one-step gene replacement in several independent transformations. To construct a congenic wild-type strain, the same procedure was followed except that the donor DNA for the transformation was derived from a plasmid containing the normal genomic *CDC28* and flanking sequences.

A Southern blot comparing genomic DNA from the resulting transgenic strain with that of the parental strain is shown in Fig. 1B. A probe internal to the *CDC28* coding region hybridized only with the parental DNA (lane 3), whereas a probe corresponding to the *CDC2Hs* coding region hybridized only with DNA from the transgenic strain (lane 2), demonstrating that replacement of the *CDC28* coding region had occurred. This was confirmed by the inability to detect the Cdc28 polypeptide by immunoblotting of protein extracts from the transgenic strain (data not shown). The transgenic strain will therefore be referred to as [*HS*], and the congenic wild-type strain will be referred to as [*SC*].

Since transformant cells containing the gene replacement described above were able to form colonies, it is clear that the protein kinase encoded by *CDC2Hs* is capable of fulfilling the G1 requirement for the Cdc28 protein kinase, allowing cells to proliferate. In fact, the growth rate of [*HS*] cells as determined by cell counting was only marginally slower than that of [*SC*] cells (Fig. 2A). Microscopic observation of the cells indicated that they were morphologically normal but significantly larger than the congenic wild-type cells (Fig. 2C). By using a model ZM electronic cell counter (Coulter Electronics, Inc.), it was determined that the [*HS*] cells were approximately 80% larger than the congenic wild-type cells (Fig. 2D). Furthermore, it was observed that whereas wild-type cells adapted normally to a nutritional downshift, decreasing in size by a factor of 2 (21), the [*HS*] cells did not adjust in size (Fig. 2D). The larger size of the [*HS*] cells and their inability to adjust size in response to a nutritional downshift may represent an intrinsic defect in the

* Corresponding author.

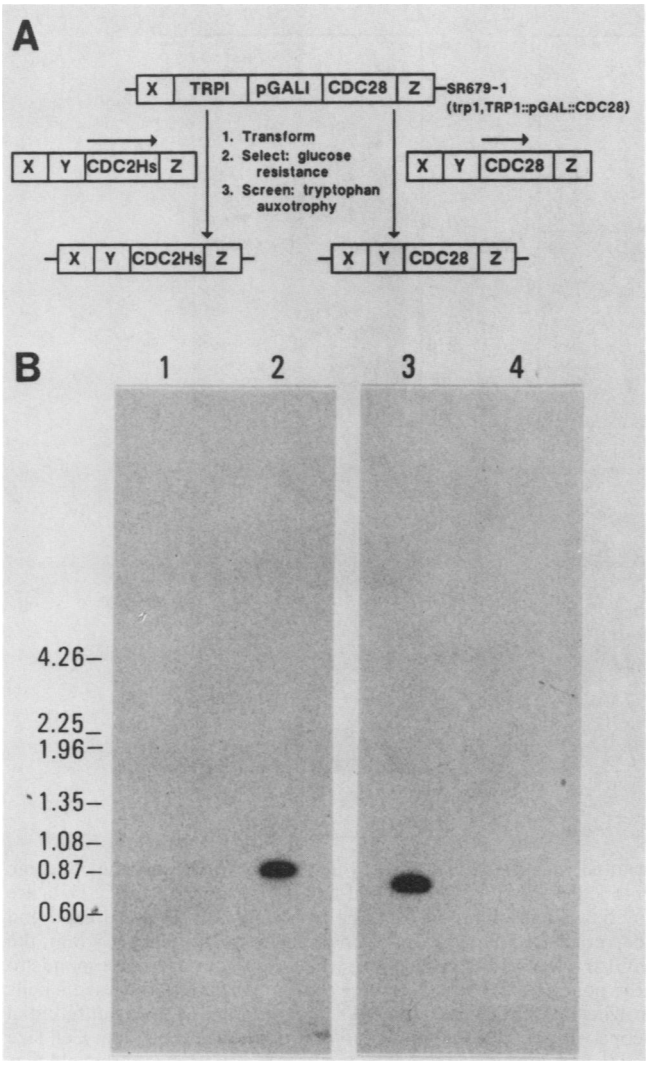

FIG. 1. Construction of a transgenic yeast strain. (A) Substitution of the polypeptide-coding region of *CDC28* by the *CDC2Hs* polypeptide-coding region, using DNA fragment-mediated gene replacement. See text for details. (B) Yeast genomic DNA was prepared from strain SR679-1 and a putative transgenic strain containing *CDC2Hs* in place of *CDC28*. DNA from strain SR679-1 was restricted with *Bam*HI and *Hin*dIII (lanes 1 and 3); DNA from the putative transgenic strain was restricted with *Bam*HI (lanes 2 and 4). The blot corresponding to lanes 3 and 4 was probed with a labeled restriction fragment internal to the *CDC28* polypeptide-coding region; the parallel blot, corresponding to lanes 1 and 2, was probed with labeled DNA consisting of the *CDC2Hs* polypeptide-coding region. The *CDC28* probe (horizontal arrow in panel A) reacted with a fragment of approximately 800 base pairs only in the parental DNA (lane 3), as expected, whereas the *CDC2Hs* probe (horizontal arrow in panel A) reacted only with a 950-base-pair fragment in the putative transgenic DNA (lane 2), confirming that the gene replacement had occurred. Molecular size markers (in kilobase pairs; shown on the left) were bacteriophage lambda DNA cleaved with *Hin*dIII and phage X174 DNA cleaved with *Hae*III.

Cdc2Hs protein kinase with respect to its ability to respond to *S. cerevisiae* size control signals and, therefore, a defect in coordination between cellular growth and division in the [*HS*] cells. Alternatively, the larger size may be a trivial ramification of an inefficiency of the heterologous protein kinase. To analyze the cell cycle kinetics of congenic [*HS*] and [*SC*] cells, the distribution of cellular DNA content in growing populations was analyzed by flow cytometry, using a Becton Dickinson FACS IV analyzer after staining of the DNA with propidium iodide (14). Within the limits of sensitivity of the assay, the ratios of G1 to G2 and M cells were identical for the two populations (Fig. 2B), indicating that the cell cycle in [*HS*] cells was unperturbed.

Previous studies have suggested that the Cdc28 protein kinase is involved in mediating the G1 arrest response of cells to both nutrient limitation and mating pheromones (24, 33). We therefore sought to determine whether transgenic *CDC2Hs*-substituted yeast cells could respond to these stimuli in an appropriate manner by arresting in the G1 interval of the cell cycle. [*HS*] and [*SC*] cells in liquid culture were transferred to medium lacking a source of nitrogen. Both cell number and budding index were monitored by microscopic examination. In *S. cerevisiae*, budding index is an indication of cell cycle synchrony or arrest, where a budding index of 0 usually represents a homogeneous G1 population. Both cultures increased in cell number by nearly a factor of 2, whereas the budding index decreased to 0 (Fig. 3A). This is consistent with G1 arrest in the first cell cycle in response to nitrogen starvation. Similarly, [*HS*] and [*SC*] cultures were subjected to nutrient depletion by allowing growth in rich medium over the course of several days. Normally, attainment of stationary phase is accompanied by G1 arrest. The [*HS*] and [*SC*] cultures arrested equally well in G1 as judged by the decrease of the budding index to 0 (Fig. 3B). The difference in cell number achieved by the two strains upon entry into stationary phase is most likely a reflection of the difference in cell size.

To assess the response to mating pheromone (3), an α-factor halo test (16) was performed (Fig. 3C). For this experiment, the original [*HS*] strain was crossed to a yeast strain containing a *bar1* mutation (31). This mutation, which eliminates the function of a protease that cleaves the α-factor peptide, renders cells approximately 10-fold more sensitive to this pheromone, facilitating assays. Halo assays were performed in duplicate on each of two [*HS*] *bar1* and two [*SC*] *bar1* segregants from the cross described above. Drops containing 200 ng of the mating pheromone α-factor were centered upon newly plated lawns of approximately 5×10^4 [*HS*] or [*SC*] cells of the **a** mating type, and the resulting zone of growth inhibition was measured after incubation for 2 days at 23°C. The results of this assay indicated that there was no detectable difference in mating pheromone sensitivities of these strains. All assays gave a halo diameter of 2.1 ± 0.1 cm. Microscopic examination of cells within the zone of growth inhibition indicated that they were arrested in the G1 interval of the cell cycle. Furthermore, liquid cultures of [*HS*] and [*SC*] cells treated with mating pheromone were observed to synchronize in the G1 interval with identical kinetics (data not shown).

Using an in vitro assay, we have shown that the Cdc28 protein kinase is regulated in response to conditions which lead to arrest in G1 (24, 33). Specifically, the Cdc28 protein kinase is inactivated by mating pheromone and nutritional starvation, as judged by its ability to phosphorylate either an endogenous substrate or the exogenous substrate histone H1. Since genetic data indicate that inactivation of the Cdc28 protein kinase leads to G1 arrest (11, 28), these observations are consistent with a model in which external signals regulate cell division by controlling the Cdc28 protein kinase. We sought to determine whether the Cdc2Hs protein kinase was similarly regulated in *S. cerevisiae*. In vitro assays of the Cdc2Hs and Cdc28 protein kinases were performed in par-

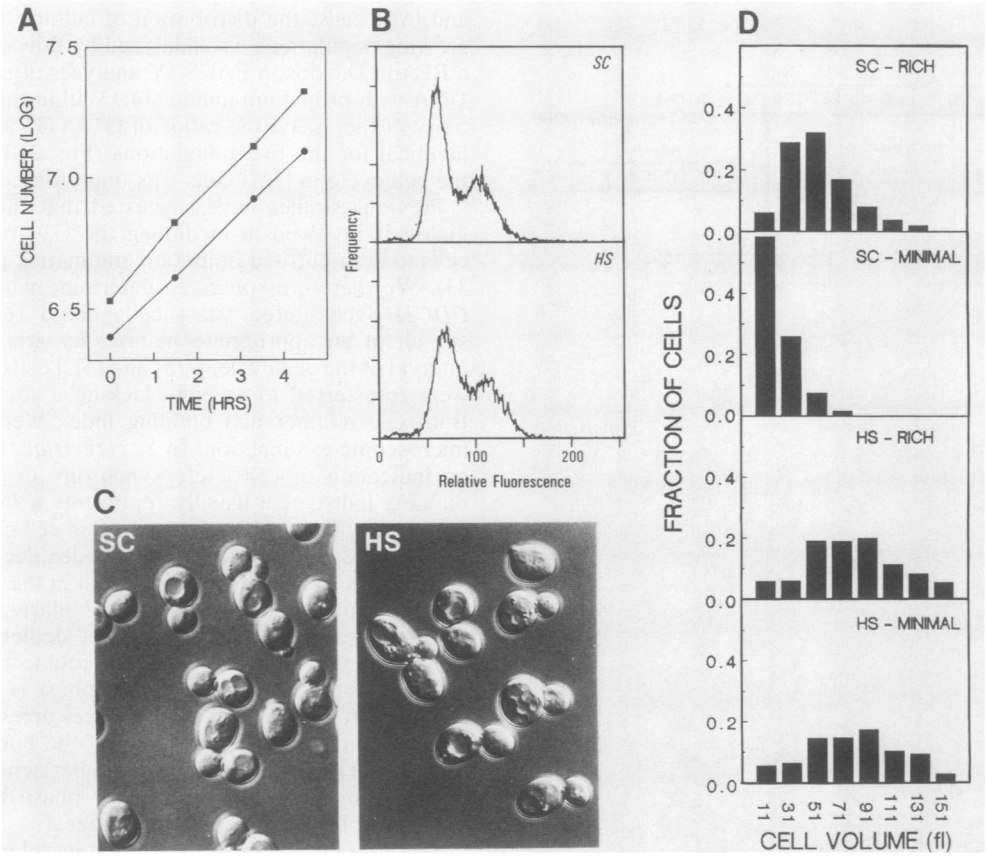

FIG. 2. Growth and size characteristics of a transgenic *CDC2Hs*-substituted yeast strain. (A) Cell cycle time. [*HS*] (■) and the congenic wild-type strain [*SC*] (●) were grown in the rich medium, yeast extract-peptone-dextrose (YEPD), to mid-logarithmic phase at 30°C. Data are presented as semilog plots of cell number versus time. (B) Cell cycle kinetics. Logarithmic-phase [*HS*] and [*SC*] cultures were fixed and stained with propidium iodide; DNA fluorescence was analyzed and plotted versus frequency within each population. In each histogram, the left peak corresponds to cells with a 1n content of DNA, representing the G1 fraction of the population, while the right peak corresponds to those with a 2n content of DNA, representing the G2-plus-M fraction of the population. The saddle between the two peaks represents cells in S phase. (C) Cell morphology. Logarithmic-phase [*HS*] and [*SC*] cells growing in YEPD were mounted and photographed, using differential interference contrast (Nomarski) optics on a Zeiss axiophot photomicroscope with an ×100 oil immersion objective. (D) Cell size. Cell size distributions were obtained by analyzing sonicated Formalin-fixed cells with an electronic cell counter; the lower particle size threshold was kept constant while the upper threshold was progressively raised to develop the histogram intervals. Cultures analyzed were [*HS*] and [*SC*] grown in rich medium (YEPD) and minimal medium (yeast nitrogen base, essential amino acids, 2% raffinose).

allel on extracts from [*HS*] and [*SC*] cells, respectively (Fig. 4). The immune complexes containing the protein kinase were prepared by using an antiserum directed against a peptide homologous to amino acids 11 to 27 of the predicted product of the *CKS1* gene (10). This polypeptide has been shown to be associated with the Cdc28 protein kinase in wild-type cells. Protein kinase activity directed against both the endogenous substrate p40 and exogenously added histone H1 could be extracted from growing exponential cultures of [*SC*] (Fig. 4, lanes 1 and 7) and [*HS*] (lanes 2 and 8) cells. Both activities were lost or substantially reduced when [*SC*] and [*HS*] cells were treated with mating pheromone (lanes 5 and 6) or underwent starvation as a result of growth to stationary phase (lanes 9 and 10). Thus, the Cdc2Hs protein kinase, when expressed in yeast cells, responds to natural regulatory signals in a fashion parallel to that of the endogenous Cdc28 protein kinase. In contrast to extracts from [*SC*] cells (lane 3), protein kinase assays of extracts of [*HS*] cells performed after immunoprecipitation in the presence of an excess of the peptide antigen contained approximately 10% of the activity observed without the addition of peptide (lane 4). Although this result may reflect a low level of nonspecific adsorption of activity in these immune complexes, it does not alter our conclusions.

We have shown that *S. cerevisiae* cells, normally dependent on the activity of the Cdc28 protein kinase for proliferation, can utilize the homologous protein kinase from human cells to execute all essential functions. This is not surprising in light of the demonstration that the *CDC2Hs* gene can rescue mutations in the homologous gene, $cdc2^+$, in the fission yeast *Schizosaccharomyces pombe* (20). Furthermore, we have demonstrated that transgenic yeast cells containing only the human protein kinase execute division control properly in response to environmental signals, a function attributed to the Cdc28 protein kinase. Consistent with this observation, the human protein kinase is regulated by these environmental signals in a manner identical to that of the Cdc28 protein kinase. The ability of Cdc2Hs to form tight associations with p40, the endogenous substrate of Cdc28 (24, 29), and the 18-kilodalton product of the *CKS1* gene (10) suggests a high degree of conservation of structure and function between the yeast and human homologs.

The extent to which the role of these protein kinases is conserved between organisms is presently in question.

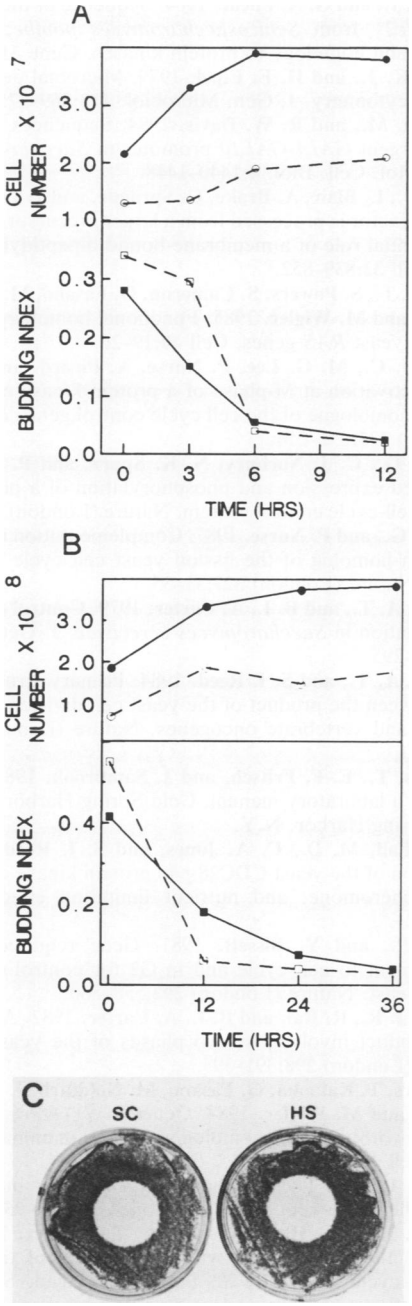

FIG. 3. Cell cycle arrest of transgenic *CDC2Hs*-substituted cells. (A) Nitrogen starvation. Logarithmic-phase cultures of [*HS*] (open symbols) and [*SC*] (solid symbols) cells grown in minimal raffinose medium (yeast nitrogen base, essential amino acids, 2% raffinose) were shifted to nitrogen starvation medium (minimal raffinose medium with sodium sulfate replacing ammonium sulfate) by centrifugation, washed once in nitrogen starvation medium, and resuspended in an equal volume of nitrogen starvation medium. Equal portions were removed from each culture at regular intervals for determination of cell number and budding index (the fraction of budded cells in the population), a measurement of arrest in G1, by microscopic examination. (B) Nutrient depletion. [*HS*] (open symbols) and [*SC*] (solid symbols) cultures were allowed to undergo nutrient depletion in rich medium (YEPD). Equal portions were removed and analyzed as described above, beginning in late logarithmic phase. (C) Mating pheromone. Sensitivity of [*HS*] and [*SC*] cells to mating pheromone arrest was compared by using an α-factor halo assay.

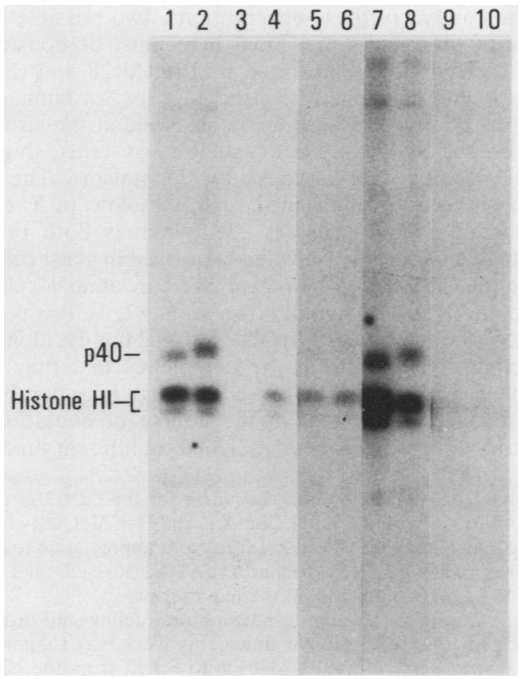

FIG. 4. Cdc28/Cdc2Hs protein kinase activity of a transgenic *CDC2Hs*-substituted yeast strain. Protein kinase assays were performed by using immunoprecipitates from extracts of actively growing [*SC*] (lanes 1, 3, and 7) or [*HS*] (lanes 2, 4, and 8) strains prepared either with the anti-$CKS1_{11-27}$ serum alone (lanes 1, 2, 7, and 8) or with serum that had been blocked by the addition of an excess of the $CKS1_{11-27}$ peptide (lanes 3 and 4) (10). [*SC*] and [*HS*] cultures were arrested by addition of the mating pheromone α-factor (lanes 5 and 6, respectively) or allowed to undergo nutrient depletion in rich medium (YEPD) (lanes 9 and 10, respectively) before the protein kinase assay. The assays in lanes 1 through 6 were performed by using [*SC*] and [*HS*] strains carrying the *bar1* mutation that are supersensitive to α-factor (see Fig. 3C); the assays in lanes 7 through 10 were performed by using the congenic [*HS*] and [*SC*] strains described in the legend to Fig. 1. Immunoprecipitation of protein kinase activity from *BAR1* strains was also blocked by the addition of an excess of the antigenic peptide (not shown). All autoradiography was performed for 5 h with a du Pont Cronex Lightning-Plus intensifying screen.

While the data presented here demonstrate the capacity of the Cdc2Hs protein kinase to perform essential G1 functions and to respond to G1 regulatory signals in yeast, a requirement for its activity during the human cell cycle has yet to be established. Available data suggest that the histone H1 kinase activity associated with the Cdc2Hs protein kinase is maximal during M phase in human cells (6). However, this does not preclude a G1 function for this kinase that is perhaps specific for different substrates. In fact, in the fission yeast *S. prombe*, a functional *cdc2* gene is required for cell cycle progression through both G1 and M phase (25). Although a similar requirement for the Cdc28 protein kinase for progression through M phase has been reported (26), we have been unable to confirm those results (M. D. Mendenhall and S. I. Reed, unpublished observations). However, the Cdc28 protein kinase is capable of performing both G1/S and G2/M functions of the $cdc2^+$ protein kinase of *S. pombe* (1). We must therefore conclude that although the Cdc28 protein kinase may play a role in regulating events occurring outside of the G1 interval in yeast, that role must not be essential for proper cell cycle progression.

In the context of these observations, two possible explanations of the results presented here must be considered. Either the functions performed by the Cdc28 and Cdc2Hs protein kinases are highly conserved between humans and yeasts or the proteins, although conserved at the structural level, interact with different regulatory systems, thus performing distinct roles in these two organisms. The latter hypothesis has precedent in the *ras* homologs of *S. cerevisiae* and of animal cells (4, 27). Whereas both proteins regulate adenylate cyclase when expressed in yeast cells (17, 32), neither does so when expressed in animal cells but instead interacts with an unknown target (2, 5). It is possible that the protein kinases of the Cdc28/Cdc2 family, along with interacting components of the complexes that they form, have been structurally conserved because they can be regulated efficiently but that in the course of evolution this regulatory capacity has been assigned to different functions.

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