Human cDNAs encoding homologs of the small p34^{Cdc28/Cdc2}-associated protein of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

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The Cks1 protein is a component of the Cdc28 protein kinase in the budding yeast *Saccharomyces cerevisiae*. This paper reports the cloning of two homologs of the *S. cerevisiae* *CKS1* gene from human cells. These homologs, *CKShs1* and *CKShs2*, both encode proteins of 79 amino acids that share considerable homology at the amino acid level with the products of *CKS1* from *S. cerevisiae* and *suc1*⁺ from the fission yeast *Schizosaccharomyces pombe*. Both human homologs are capable of rescuing a null mutation of the *S. cerevisiae* *CKS1* gene when expressed from the *S. cerevisiae* *GAL1* promoter. *S. pombe* *suc1*⁺ expressed from the *GAL1* promoter is also capable of rescuing a *S. cerevisiae* *cks1* null mutation. Ckshs1 or Ckshs2 protein linked to Sepharose beads can bind the Cdc28/Cdc2 protein kinase from both *S. cerevisiae* and human cells. The *CKShs1* and *CKShs2* mRNAs are expressed in different patterns through the cell cycle in HeLa cells, which may reflect specialized roles for the encoded proteins.

[Key Words: Cell cycle; *suc1*⁺; CKS1; p13; HeLa cells]

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Considerable progress has been made recently in understanding the molecular mechanisms that operate in eukaryotic cells to control entry into the cell cycle (G₁- to S-phase transition) and entry into mitosis (G₂- to M-phase transition). Of particular importance is the finding that many of the components involved in these control points are conserved in a wide range of eukaryotes from yeasts to humans (for review, see Cross et al. 1989; Hunt 1989; Murray and Kirschner 1989a; O'Farrell et al. 1989; Pardee 1989; Smith 1989). One of these conserved proteins, the Cdc28/Cdc2 protein kinase, plays a central role in cell-division control in eukaryotic cells. The Cdc28/Cdc2 protein kinase was discovered first in the budding yeast *Saccharomyces cerevisiae* (Hartwell et al. 1974; Reed 1980; Lorincz and Reed 1984; Reed et al. 1985) and the fission yeast *Schizosaccharomyces pombe* (Nurse and Bissett 1981; Beach et al. 1982; Hindley and Phear 1984; Simanis and Nurse 1986) and was subsequently identified in humans (Draetta et al. 1987; Lee and Nurse 1987), *Xenopus* (Dunphy et al. 1988; Gautier et al. 1988), starfish (Labbe et al. 1988), mouse (Lee et al. 1988; Cisek and Corden 1989), chicken (Krek and Nigg 1989), and plant cells (John et al. 1989). In *S. pombe* the Cdc2 protein kinase has been shown to play a central role in both G₁ and G₂ control points (Nurse and Bissett 1981). In *S. cerevisiae* the G₁ role of the Cdc28 protein

kinase has been studied extensively (Reed 1980; Mendenhall et al. 1987, 1988; Wittenberg and Reed 1988), and a G₂ role has also been demonstrated recently (Reed and Wittenberg 1990). In higher eukaryotes an essential role for the Cdc2/Cdc28 kinase activity has as yet only been demonstrated at the G₂ to M phase of the cell cycle (Riabowol et al. 1989). However, in the absence of genetic analysis, a G₁ role in somatic cells cannot be excluded.

Recently, a connection has been established between the Cdc2/Cdc28 protein kinase and maturation promoting factor (MPF), an activity initially described in mature *Xenopus* oocytes which, when injected into immature oocytes, induces them to undergo meiotic maturation (Masui and Markert 1971; Reynhout and Smith 1974). MPF has been purified from *Xenopus* and starfish oocytes and has been shown to contain, as an essential component, the Cdc2/Cdc28 protein kinase (Gautier et al. 1988; Lohka et al. 1988; Labbe et al. 1989a). It also appears that an activity known as growth-associated histone H1 kinase, which is present during mitosis in a number of organisms (Bradbury et al. 1974; Chambers et al. 1983), contains the Cdc2/Cdc28 protein and is likely to be identical to MPF (Arion et al. 1988; Labbe et al. 1988, 1989a; Brizuela et al. 1989; Draetta et al. 1989; Langan et al. 1989).

It has been demonstrated in several systems that the active form of Cdc2/Cdc28 protein kinase exists as a

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multimeric complex with other proteins (Brizuela et al. 1987, 1989; Draetta et al. 1987, 1989; Arion et al. 1988; Draetta and Beach 1988; Gautier et al. 1988, 1990; Wittenberg and Reed 1988; Labbe et al. 1989b; Meijer et al. 1989; Pondaven et al. 1990). One of the proteins that associates with Cdc2/Cdc28 has been identified as cyclin (Booher et al. 1989; Draetta et al. 1989; Labbe et al. 1989b; Meijer et al. 1989; Pines and Hunter 1989; Gautier et al. 1990). Cyclins were first described in oocytes of marine invertebrates as proteins that change dramatically in abundance throughout the cell cycle, reaching maximal levels just before the onset of mitosis (Rosenthal et al. 1980; Evans et al. 1983; Pines and Hunt 1987; Standart et al. 1987; Westendorf et al. 1989). Cyclins have subsequently been identified in *Drosophila* (Lehner and O'Farrell 1989; Whitfield et al. 1989), *Xenopus* (Minshull et al. 1989), humans (Pines and Hunter 1989), and the fission yeast *S. pombe* (Booher and Beach 1988; Goebel and Byers 1988; Hagan et al. 1988; Solomon et al. 1988). These cyclins are presumed activators of the Cdc28/Cdc2 protein kinase in the G₂-to-M-phase transition (Swenson et al. 1986; Booher and Beach 1987, 1988; Booher et al. 1989; Lehner and O'Farrell 1989; Minshull et al. 1989; Murray and Kirschner 1989b; Murray et al. 1989). Recently, a second class of cyclins, the G₁-specific cyclins, involved in activation of Cdc28/Cdc2 kinase in the G₁-to-S-phase transition, have been identified in the budding yeast *S. cerevisiae* (Cross 1988; Nash et al. 1988; Hadwiger et al. 1989b; Richardson et al. 1989; Wittenberg et al. 1990).

Another protein that interacts with Cdc2/Cdc28 is the product of the *S. pombe* *suc1*⁺ gene (p13) or the *S. cerevisiae* *CKS1* gene (p18). The *S. pombe* *suc1*⁺ gene was isolated as a high-copy suppressor of temperature-sensitive mutations in *cdc2* (Hayles et al. 1986b). *CKS1* was isolated from *S. cerevisiae* by a similar procedure (Hadwiger et al. 1989a) and encodes a protein highly related at the amino acid level to the *S. pombe* *suc1*⁺ gene product. In *S. pombe* the *suc1*⁺ gene product has been shown by genetic and biochemical approaches to interact with Cdc2 (Hayles et al. 1986a,b; Brizuela et al. 1987; Hindley et al. 1987; Moreno et al. 1989). Likewise, in *S. cerevisiae* the Cks1 protein has been demonstrated to interact with Cdc28 (Hadwiger et al. 1989a). A homolog of Cks1/Suc1 has been reported to exist in human cells (Draetta et al. 1987; Draetta and Beach 1988; Bailly et al. 1989; Riabowol et al. 1989).

The precise role of Cks1/Suc1 in the control of the cell cycle is not clear. There has been an accumulation of conflicting data based on work with fission yeast, budding yeast and vertebrates (Hayles et al. 1986a; Hindley et al. 1987; Dunphy et al. 1988; Dunphy and Newport 1989; Hadwiger et al. 1989a; Moreno et al. 1989; Riabowol et al. 1989; S.I. Reed, unpubl.). Whether the apparent discrepancies concerning the role(s) of *CKS1/suc1* result from fundamental differences or simply reflect differences in experimental design, will require additional study in these organisms and in other systems. Therefore, to investigate further the role of the Cks1/Suc1 homologs in the cell cycle, we chose to use a dif-

ferent system, that of somatic vertebrate cells. As a first step, we cloned two cDNAs encoding Cks1/Suc1 homologs from human cells. These cDNAs are capable of rescuing a null mutation in the endogenous *S. cerevisiae* *CKS1* gene. We show that both human Cks1/Suc1 homologs can bind the Cdc2/Cdc28 protein kinase complex in vitro and present data on the expression patterns of the corresponding mRNAs through the cell cycle in HeLa cells.

Results

Cloning CKS1 homologs from human cells

CKS1 from *S. cerevisiae* and *suc1*⁺ from *S. pombe* are 67% identical at the amino acid level (Hadwiger et al. 1989a). By comparison of the amino acid sequences of these proteins, two degenerate oligonucleotides were designed that could be used in the polymerase chain reaction (PCR) (C. Lee et al. 1988; Saiki et al. 1988) to amplify a region of 198 bp from the *S. cerevisiae* *CKS1* gene and from an intronless version of the *S. pombe* *suc1*⁺ gene (obtained from J. Hayles and P. Nurse). The 5' oligonucleotide was derived from the peptide sequence DD(NE)YEYRHVM and was 8000-fold degenerate, whereas the 3' oligonucleotide was derived from the peptide sequence PEPHILLFK and was 33,000-fold degenerate (see Fig. 2, below). Synthetic restriction sites were placed at the 5' ends of these degenerate oligonucleotides so that the PCR products could be cloned easily, and the DNA sequence could be determined.

The degenerate oligonucleotides were used in the PCR to amplify DNA from a HeLa cell cDNA library and from *S. cerevisiae* and *S. pombe* genomic libraries as controls. Bands of the predicted molecular weight were obtained from the *S. cerevisiae* and *S. pombe* genomic DNA libraries. Two bands in the range of 150–200 bp were obtained from the human cDNA library. These PCR products were cloned, and DNA sequence analysis revealed three distinct clones. The first two contained an open reading frame (ORF) showing extensive amino acid sequence homology to the *S. cerevisiae* *CKS1* and *S. pombe* *suc1*⁺ genes. These clones were named *CKShs1* and *CKShs2* (CKS being an acronym for Cdc28/Cdc2 kinase subunit). The third did not contain an ORF that extended from the amino- to carboxy-terminal primers and showed no homology to the yeast *CKS1* and *suc1*⁺ genes; therefore, it was not pursued further.

The PCR clones of the human *CKS1* homologs were used to probe HeLa cell cDNA libraries to obtain full-length cDNA clones (see Experimental procedures). DNA sequence analysis of these clones revealed that *CKShs1* and *CKShs2* are very similar (Fig. 1). The largest *CKShs2* clones contained a 612-bp insert with an ORF that is predicted to encode a 79-amino-acid protein and has a 3'-untranslated region of 280 bp before the poly(A) segment. The largest *CKShs1* contained a 717-bp insert with an ORF that potentially encodes a protein of 79 amino acids and has a 3'-untranslated region of 470 bp. The *CKShs1* clones obtained did not contain a poly(A)

Figure 1. (A) DNA sequence of *CKShs1* and (B) *CKShs2* cDNA clones. The cDNA clones are diagrammatically represented in bar figures. Plasmid regions are represented by open boxes. The ORFs are indicated by shaded boxed regions. For cloning the cDNA, poly(G/C) or poly(A/T) regions immediately preceding and following the two *EcoRI* sites, respectively (A) and immediately preceding the *PstI* site (B) were used. Relevant restriction sites are indicated: (B) *BamHI*; (Bg) *BglII*; (E) *EcoRI*; (H) *HindIII*; (P) *PstI*; (S) *SacI*. The DNA sequences of the cDNAs and the predicted amino acid sequence are shown below with relevant restriction sites. Arrows indicate direction of transcription.

tail (see Experimental procedures); thus, the 3'-untranslated region may be longer than 470 bp. The predicted start sites for both human CKS cDNAs have the conserved A at -3, but only *CKShs2* has the conserved G at +4 (Kozak 1981).

Figure 2 shows an alignment of the two human Cks1 homologs with *S. cerevisiae* Cks1 and *S. pombe* Suc1. The predicted Ckshs1 and Ckshs2 proteins (79 amino acids) are considerably smaller than their homologs from

S. cerevisiae (150 amino acids) and *S. pombe* (113 amino acids). Compared with the yeast homologs, the human Cks1 homologs contain truncations at both the amino and carboxyl termini and also have a region of 9 amino acids deleted in the middle of the protein. Ckshs1 and Ckshs2 are highly related proteins containing 64 of 79 identical amino acids (81% identity). Ckshs1 shares 53% identity with both Cks1 and Suc1, and Ckshs2 shares 57% identity with each of the yeast homologs.

Human CKS1 homologs rescue an S. cerevisiae CKS1 null mutation

Although the human Cks1 homologs share a high level of homology with the yeast Cks1 and Suc1 proteins, they are predicted to encode considerably smaller proteins. For this reason it was important to determine whether *CKShs1* and *CKShs2* are functional homologs of the yeast genes, by testing whether they are able to replace the *S. cerevisiae* *CKS1* gene to allow normal growth of cells. To allow expression of the human cDNAs in *S. cerevisiae*, *CKShs1* and *CKShs2* were placed under the control of the *S. cerevisiae* *GAL1* promoter in the plasmid YCpG2 (*CEN1*, *LEU2*, *URA3*, *pGAL1*; see Experimental procedures). The *GAL1* promoter is strongly induced by galactose and repressed by glucose (Johnston and Davis 1984). Thus, expression of the human *CKS1* homologs could be controlled by growing cells in galactose or glucose media. As controls, an intronless version of the *S. pombe* *suc1*⁺ gene and the *S. cerevisiae* *CKS1* gene were placed under control of the *GAL1* promoter in YCpG2 (see Experimental procedures).

These plasmids were used to transform a *S. cerevisiae* haploid strain containing a null mutation in *CKS1* made by insertion of *LEU2* near the 5' end of the gene (*cks1* :: *LEU2*) but carrying a plasmid copy of the *CKS1* gene (*CEN1*, *TRP1*, *CKS1*). Transformants of this strain with the YCpG2[*CKS1* homolog] plasmids were selected on plates lacking uracil and containing glucose (noninducing conditions). To test whether expression of the human *CKS1* homologs under *GAL1* promoter control were able to replace the *S. cerevisiae* *CKS1* gene, the cells were cured of the plasmid containing the wild-type *CKS1* gene while expression of the human genes from the *GAL1* promoter was allowed, as follows. Cells were grown for several generations on plates containing tryptophan (to allow loss of the plasmid carrying the wild-type *CKS1* gene and the *TRP1* marker) and galactose (to allow expression of the *CKS1* homolog from the *GAL1* promoter). Loss of the plasmid containing the wild-type *CKS1* gene was monitored by testing colonies for tryptophan auxotrophy.

By using this approach on the control plasmids, it was found that the plasmid containing the wild-type *CKS1* gene could be lost from cells containing the plasmids YCpG2[*CKS1*] or YCpG2[*suc1*⁺] but not from cells containing a plasmid with the *CKS1* gene in the reverse orientation (YCpG2[*CKS1*rev]). The ability of the expression of *suc1*⁺ from the *GAL1* promoter to allow loss of the plasmid containing the wild-type *CKS1* gene indi-

Figure 2. Alignment of the *CKShs1* and *CKShs2* amino acid sequence with *S. cerevisiae* Cks1 and *S. pombe* Suc1. Identical amino acids between Ckshs1 or Ckshs2 and the yeast Cks1 homologs are boxed. Amino acids that are conserved between all four homologs are indicated above the Cks1 sequence. The regions used to generate degenerate primers for PCR are underscored.

cates that *S. pombe* *suc1*⁺ is indeed a functional homolog of *S. cerevisiae* *CKS1*. As shown in Figure 3A–C, cells containing YCpG2[*CKS1*] or YCpG2[*suc1*⁺] grown in galactose medium are slightly larger than wild-type *CKS1*⁺ cells (which may be due to the overexpression of the *CKS1* homologs from the *GAL1* promoter, as will be discussed below). However, after several generations of growth in glucose medium, these cells become grossly enlarged and abnormally shaped and stop dividing (as in Fig. 3F).

When cells containing YCpG2[*CKShs1*] or YCpG2-[*CKShs2*] and the wild-type *CKS1* plasmid were grown in galactose medium containing tryptophan, Trp⁻ colonies (cured of the *GEN1-CKS1-TRP1* plasmid) were obtained at a frequency similar to that with YCpG2[*CKS1*] (20–30% after 20 generations of nonselective growth). This result indicates that *CKShs1* and *CKShs2* can replace the *S. cerevisiae* *CKS1* gene to allow cell growth and thus verifies that the human CKS gene products are indeed functional homologs of *S. cerevisiae* *CKS1*.

In galactose medium, cells containing YCpG2-[*CKShs1*] or YCpG2[*CKShs2*] exhibit an enlarged morphology (Fig. 3D,E). The cells actually appear slightly larger than the equivalent cells expressing the yeast *CKS1* homologs from the *GAL1* promoter (Fig. 3B,C). This may be due to the inability of the human *CKS* homologs to fully rescue the *S. cerevisiae* *CKS1* null mutation or to the overexpression of the heterologous proteins resulting in a delay in the cell cycle. The relative abilities of the human and yeast *CKS1* homologs to support growth of the *cks1*⁻ cells were analyzed by determining their growth rates in galactose medium and after shifting the cells to glucose medium (where transcription of the *CKS1* homologs from the *GAL1* promoter is repressed). In galactose medium, cells expressing the human *CKS1* homologs showed similar growth rates to those expressing the yeast homologs (approximately one doubling every 5–6 hr). This suggests that the human *CKS1* homologs and the *S. pombe* *suc1*⁺ gene expressed from the *GAL1* promoter are equally capable of allowing cell-cycle progression as the *S. cerevisiae* *CKS1* gene expressed from the *GAL1* promoter. When cells carrying YCpG2[*CKShs1*] or YCpG2[*CKShs2*] are shifted to glucose medium (Fig. 4), they are capable of undergoing ~6 doublings before arresting as large aberrantly shaped cells, many of which showed a G₁-arrest morphology (large unbudded cells; Fig. 3F). Large budded cells were

also present (Fig. 3F), and experiments are in progress to determine whether *CKS1* plays a role in the G₂- to M-phase transition of *S. cerevisiae*. Cells carrying YCpG2[*CKS1*] or YCpG2[*suc1*⁺] shifted to glucose medium allow ~8 and 13 rounds of division, respectively (data not shown). Thus, Cks1 and its three homologs are extremely stable proteins that can persist in functional form long after their synthesis has been terminated. The

Figure 3. Photomicrographs of *S. cerevisiae cks1⁻* cells rescued by *CKShs1* or *CKShs2* expressed from the *GAL1* promoter. (A) *CKS1⁺* cells carrying YCpG2 in galactose medium; (B) *cks1⁻* cells carrying YCpG2[*CKS1*] in galactose medium; (C) *cks1⁻* cells carrying YCpG2[*suc1⁺*] in galactose medium; (D) *cks1⁻* cells carrying YCpG2[*CKShs1*] in galactose medium; (E) *cks1⁻* cells carrying YCpG2[*CKShs2*] in galactose medium; (F) *cks1⁻* cells carrying YCpG2[*CKShs1*] 72 hr after shifting to glucose medium.

Figure 4. Kinetics of growth arrest of *S. cerevisiae cks*⁻ cells after repressing transcription of *CKShs1* or *CKShs2* from the *GAL1* promoter. Cell numbers were determined with time from cultures grown in galactose minimal media without uracil or after the addition of glucose. The data are represented as the number of divisions (the natural log of the increase in cell number) as a function of time. (▲) *cks*⁻ YCpG2[*CKShs1*] in galactose; (○) *cks*⁻ YCpG2[*CKShs2*] in galactose; (●) *cks*⁻ YCpG2[*CKShs1*] in glucose; (■) *cks*⁻ YCpG2[*CKShs2*] in glucose.

fact that the human *CKS1* homologs do not allow as many divisions as the yeast *CKS1* homologs may be due to differential levels of expression or to differential protein stability.

Human *CKS1* cDNAs encode smaller proteins than yeast *CKS1* genes

It has been reported by Draetta et al. (1987) that HeLa cells contain a protein that cross-reacts with a polyclonal antibody directed against *S. pombe* Suc1. These investigators report evidence that this potential homolog, present in HeLa cell extracts, migrates on an SDS-polyacrylamide gel at the same molecular mass as the *S. pombe* Suc1 protein (13 kD). The *CKS1* homologs that we cloned from HeLa cells are expected to encode proteins of only 8.7 kD and thus would be expected to migrate on SDS-polyacrylamide gels at a smaller molecular mass compared with the *S. pombe* Suc1 protein. To investigate the relative molecular masses of the Cks1 homologs, *CKShs1*, *CKShs2*, *S. cerevisiae* *CKS1* and *S. pombe* *suc1*⁺ were cloned into the bacterial expression vector pRK171 (Rosenberg et al. 1987; see Experimental procedures). The bacterially produced proteins were precipitated with 80% ammonium sulfate, purified through a Sephacryl S-200HR sizing column, run on a 20% SDS-polyacrylamide gel, and stained with Coomassie Blue (Fig. 5). As shown in Figure 5, the Ckshs1 and Ckshs2 proteins migrate at ~9 kD (lanes 1 and 2), the *S. pombe* Suc1 protein migrates at 13 kD (lane 4), and the *S. cerevisiae* Cks1 protein migrates at 18 kD (lane 3). Thus, it is clearly evident that the human Cks proteins migrate with a greater mobility than the yeast Cks proteins, as predicted from their amino acid sequences. The discrepancy between the molecular masses of the proteins en-

coded by the human *CKS1* homologs determined here and that reported by Draetta et al. (1987) will be addressed in the Discussion.

mRNA abundance of the two human *CKS1* homologs varies through the cell cycle in HeLa cells

The presence of two *CKS1* homologs in human cells suggested that they may be used differently in the cell cycle. Thus, it was of interest to determine whether the mRNAs corresponding to the two *CKShs* homologs were expressed differentially through the cell cycle. HeLa cells were fractionated on the basis of cell size by centrifugal elutriation (see Experimental procedures). The first fractions contain the smallest cells in the G₁ phase of the cell cycle, and the last fractions are enriched for cells in G₂ and M phases. To confirm that good separation of cells had been obtained, the fractions were analyzed for nuclear DNA content by staining with propidium iodide, followed by flow cytometry (Fig. 6A). RNA was prepared and analyzed on a single Northern blot by first using a probe specific for *CKShs1* and stripping and reprobing for *CKShs2* mRNA (see Experimental procedures). mRNAs of ~0.7 kb were detected for both *CKShs1* and *CKShs2* (Fig. 6B), consistent with the size of the cDNA clones. The intensity of the mRNA signal in each lane was quantitated against an internal standard (human β-actin mRNA) by laser densitometric analysis (Fig. 6C). As shown in Figure 6C, *CKShs1* mRNA abundance varies differently from that of *CKShs2* through the cell cycle. *CKShs1* mRNA is low in G₁ cells and increases by fourfold in fractions rich in G₂- to M-phase cells, whereas *CKShs2* mRNA is barely detectable in fractions rich in G₁ cells and increases by sevenfold in G₂- to M-phase cells. Similar results were obtained with cells synchronized by using a double thymidine block/release rather than by elutriation (Rao and Johnson 1970; data not shown). There is some indication from

Figure 5. Comparison of the molecular weights of *E. coli*-produced Cks1 homologs. Purified bacterially produced proteins were run on a 20% SDS-polyacrylamide gel and stained with Coomassie Blue. (Lane 1) Ckshs1; (lane 2) Ckshs2; (lane 3) Cks1; (lane 4) Suc1.

Figure 6. Transcription of *CKShs1* and *CKShs2* through the cell cycle. HeLa cells in a suspension culture were separated on the basis of size with an elutriator rotor. Aliquots from each sample of cells were either stained with propidium iodide and analyzed for DNA content by flow cytometry, or RNA was prepared for Northern blot analysis. (A) A graphic representation of the fraction of cells in each sample with a 4n DNA content (in G₂ or M phase). (B) Autoradiographs of Northern blots of RNA prepared from the elutriated samples, with radiolabeled probes specific for *CKShs1*, *CKShs2* or β -actin mRNA. The Northern blot was first hybridized with the *CKShs1* probe and stripped and rehybridized with the *CKShs2* and β -actin probes. (C) A graphic representation of the relative level of *CKShs1* and *CKShs2* mRNA normalized to β -actin mRNA.

these data that expression of *CKShs1* mRNA is biphasic, with a small peak at the G₁ to S boundary and a larger peak near the end of the cell cycle. The peak of S-phase cells in the profile (fractions 6 and 7 in Fig. 6A), based on flow cytometric analysis, separates the two maxima of *CKShs1* mRNA. Further experiments are needed to determine whether observed variations in mRNA abundance are due to changes in the synthesis or degradation of mRNA and whether this variation translates into differences in the abundance of the Cks proteins through

the cell cycle. Such changes in Cks proteins levels would be predicated on the proteins being unstable, which is apparently not so when they are expressed in yeast. Their stability in human cells remains to be determined. Note that the estimate of mRNA variation is a minimal one due to the impurity of the elutriated fractions rich in S-phase and G₂- to M-phase cells. Only fractions 1–4 were homogeneous, containing exclusively G₁ cells. In addition, we have used β -actin mRNA as an internal standard, assuming that it would be constant in level through the cell cycle. However, it is apparent that this mRNA increases slightly in abundance as a function of cell-cycle progression (Fig. 6B). Both the fluctuations in *CKShs1* and *CKShs2* mRNAs, as well as in actin mRNA, were reproducible in several different experiments.

Cks1 and *Cks2* bind to the Cdc2/Cdc28 protein kinase from *S. cerevisiae* and HeLa cells

A property of the *S. pombe* Suc1 protein and the *S. cerevisiae* Cks1 protein is that they bind to Cdc2/Cdc28 (Brizuela et al. 1987; Draetta et al. 1987; Draetta and Beach 1988; Dunphy et al. 1988; Hadwiger et al. 1989b). Both Suc1/Cks1 antibodies and Suc1–Sepharose beads have been used to adsorb Cdc2/Cdc28 protein kinase activity. To confirm that the cloned *CKShs1* and *CKShs2* cDNAs encode proteins that are able to associate with Cdc2/Cdc28, the proteins produced in *Escherichia coli* were coupled to Sepharose beads (see Experimental procedures). The Cks–Sepharose beads were added to cell extracts from exponentially growing HeLa cells. After incubation, the Cks–Sepharose beads were washed and assayed for Cdc2hs protein kinase activity using histone H1 as the substrate. As shown in Figure 7A (lanes 1 and 3), both Cks1– and Cks2–Sepharose beads bind a protein kinase activity that is active against histone H1. This protein kinase activity was specifically associated with the Cks proteins because the ability to phosphorylate histone H1 could be blocked by prior addition of free Cks protein to the cell extract (Fig. 7A, lanes 2, 4, 6, 7). These results suggest that the human Cks1 homologs specifically associate with the Cdc2 protein kinase in human cells.

The ability of Cks1 and Cks2 to interact with Cdc2/Cdc28 was also investigated in *S. cerevisiae* cells where mutations in *CDC28* could be used to confirm that the human Cks1 homologs were indeed interacting specifically with the Cdc28 protein kinase. As shown in Figure 7B (lanes 3 and 5) Cks–Sepharose beads incubated with a protein extract from exponentially growing *S. cerevisiae* *CDC28*⁺ cells bind a kinase activity that is active against exogenously added histone H1 and an endogenous 40-kD protein, p40 (Reed et al. 1985; Mendenhall et al. 1987). Under the same assay conditions, both exogenously added histone H1 and the endogenous substrate p40 are phosphorylated when Cdc28 antisera or Cks1 antisera are used to precipitate Cdc28 protein kinase from exponentially growing *S. cerevisiae* cell ex-

Figure 7. Protein kinase assays with Cks1s-Sepharose beads. (A) Protein kinase assays of HeLa cell protein extracts. (Lane 1) Cks1s-Sepharose beads; (lane 2) Cks1s-Sepharose beads with the addition of excess free Cks1s protein; (lane 3) Cks2s-Sepharose beads; (lane 4) Cks2s-Sepharose beads with the addition of excess free Cks2s protein; (lane 5) mock beads; (lane 6) Cks1s-Sepharose beads with the addition of excess free Cks2s protein; (lane 7) Cks2s-Sepharose beads with the addition of excess free Cks1s protein. (B) Protein kinase assays of *S. cerevisiae* CDC28+ or cdc28-4 (cdc28 temperature-sensitive allele) protein extracts. (Lane 1) immunoprecipitation from CDC28+ cell extracts with Cdc28 amino-terminal antiserum; (lane 2) immunoprecipitation from cdc28-4 cell extracts with Cdc28 amino-terminal antisera; (lane 3) Cks1s beads with CDC28+ cell extracts; (lane 4) Cks1s beads with cdc28-4 cell extracts; (lane 5) Cks2s beads with CDC28+ cell extracts; (lane 6) Cks2s beads with cdc28-4 cell extracts; (lane 7) mock beads with CDC28+ cell extracts; (lane 8) mock beads with cdc28-4 cell extracts. The band above the histone H1 band (indicated) is the endogenous substrate p40 (Reed et al. 1985; Mendenhall et al. 1987). Samples were prepared as described in Experimental procedures.

tracts (Fig. 7B, lane 1; Hadwiger et al. 1989a). When the Cks1s-Sepharose beads were incubated with extracts from a strain carrying a temperature-sensitive allele of CDC28, cdc28-4 (from which no Cdc28 activity is detected in the in vitro assay; Reed et al. 1985; Mendenhall et al. 1987), no significant activity was observed against histone H1 and no p40 phosphorylation was detectable. This result indicates that the human Cks proteins are indeed able to bind to the Cdc28 protein kinase complex in vitro in an active form.

Discussion

By using a cloning strategy based on the technique of PCR, we identified two homologs of the *S. cerevisiae* CKS1 gene from human cells. The degenerate primers used for PCR were derived from the amino acid sequences of two CKS1 homologs from the distantly related yeasts *S. cerevisiae* and *S. pombe*. This strategy has also been used successfully to clone CKS1 homologs from *Xenopus* and *Drosophila* (H.E. Richardson, P. Russell, and S.I. Reed, unpubl.). In addition, this approach

has been successful for obtaining homologs of the *S. pombe* mitotic inducer cdc25+ from human cells (Sadhu et al. 1990).

The human CKS1 homologs, CKShs1 and CKShs2, represent the first members of the Cks1 protein family from higher eukaryotes. Although CKShs1 and CKShs2 cDNAs encode proteins that have ~55% identity to the yeast CKS1 homologs, they show several distinct differences, the most striking being the deletion of 9 amino acids in the middle of the protein relative to the yeast proteins. Despite this deletion and their considerably smaller size (a molecular mass of 9 kD compared with 18 kD for *S. cerevisiae* Cks1 and 13 kD for *S. pombe* Suc1), they are both able to rescue a null mutation in the *S. cerevisiae* CKS1 gene. Cks1 has a region of polyglutamine at the carboxyl terminus that is not present in either the *S. pombe* or human Cks1 homologs. The observation that both *S. pombe* suc1+ and human CKShs1 and CKShs2 are able to replace the *S. cerevisiae* CKS1 gene to allow normal mitotic growth of *S. cerevisiae* cells suggests that the polyglutamine region does not perform any essential function.

The fact that the human CKShs1 and CKShs2 cDNAs encode considerably smaller proteins than the yeast CKS1 homologs was surprising in light of the results of Draetta et al. (1987). Using anti-p13^{suc1} antisera, Draetta et al. (1987) detected a protein from human cells that comigrated with the *S. pombe* Suc1 protein at ~13 kD. This discrepancy prompted a comparison of the molecular masses of the four Cks1 homologs by SDS-PAGE (Fig. 5). The proteins encoded by CKShs1 and CKShs2 cDNAs migrate at ~9 kD on an SDS-polyacrylamide gel (Fig. 5). There are several possible explanations for this apparent discrepancy: (1) Different gel systems may have been used; (2) Cks1s protein may be modified in vivo, thereby modifying its mobility; (3) additional homologs of 13 kD may exist in human cells that were not detected by the PCR approach; and (4) the species detected by Draetta et al. (1987) was not a true Cks1/Suc1 homolog. Antibodies against the human Cks1 homologs are presently being prepared to distinguish between the various possibilities.

Role of Cks1 homologs

In this study we initiated an investigation of the role of the human Cks1 homologs in the cell cycle by cloning the cDNAs and demonstrating that they can replace the *S. cerevisiae* CKS1 gene in allowing normal mitotic growth. The rescue of the *S. cerevisiae* CKS1 null mutation by the human CKShs1 and CKShs2 cDNAs indicates that they can replace all functions of the endogenous gene. We have shown that this is also true for the *S. pombe* suc1+ gene. In *S. cerevisiae*, the CKS1 function has been demonstrated to be required for the G₁- to S-phase transition (Hadwiger et al. 1989a; S.I. Reed, unpubl.). A role for the *S. cerevisiae* Cks1 protein in the G₂ to M phase has not been demonstrated, yet it is known that Cks1 associates with Cdc28 protein kinase at this stage of the cell cycle (C. Wittenberg and S.I. Reed, un-

publ.). Cdc28 has recently been demonstrated to have a G_2 role (Reed and Wittenberg 1990); however, this role was not apparent from the terminal phenotype of *cdc28* temperature-sensitive alleles (Hartwell et al. 1974; Reed 1980) and could only be demonstrated by inhibitor shift experiments where Cdc28 kinase activity was allowed to decay in cells arrested in S or G_2 phase (Wittenberg et al. 1990). The data suggest that in *S. cerevisiae*, the requirement for Cdc28 is greater in the G_1 - to S-phase transition than in the G_2 - to M-phase transition. Thus, when Cdc28 activity is made limiting, there is a tendency for cells to arrest at G_1 . It is possible that the G_1 arrest conferred by *cks1* mutations is a manifestation of this apparent differential requirement of Cdc28 activity in G_1 relative to G_2 . Further analysis will be required to confirm this. The biphasic pattern of expression of *CKShs1* mRNA relative to the human cell cycle is consistent with the possibility of both a G_1 and G_2 /M function in higher cells.

In *S. pombe* cells, the role of the *suc1*⁺ gene in the cell cycle has been investigated by analysis of germinating *suc1*⁻ spores (Moreno et al. 1989). It was found that *suc1*⁻ cells arrested with long mitotic spindles, no cytoplasmic microtubules, and condensed chromosomes, consistent with an anaphase or telophase arrest. It was also observed that Cdc2 protein kinase activity was threefold higher in *suc1*⁻ cells compared with *suc1*⁺ germinating spores. These results were interpreted as evidence that Suc1 is required in anaphase/telophase for the reduction in activity of the Cdc2 protein kinase, which is necessary for cells to exit from mitosis (Murray et al. 1989). However, these results may also be interpreted to indicate that Suc1 is required for the function of Cdc2 protein kinase in mitosis, as explained below. Because only 5% of the Suc1 protein present in the cell is complexed with Cdc2 (Brizuela et al. 1987), there may be enough of the protein present in germinating spores (even though it was undetectable by immunoblot analysis; Moreno et al. 1989) to allow the Cdc2 protein kinase to function in the G_1 - to S-phase transition and to initiate mitosis, but the level of Suc1 may have decreased below a threshold level required for the completion of some early mitotic events. The higher level of Cdc2 protein kinase activity observed in the *suc1*⁻ germinating spores may be explained by the fact that these cells are arresting in mitosis and thus are synchronized at a point where kinase activity is high, whereas the *suc1*⁺ cells continue cycling. The fact that Cdc2 protein kinase activity remains high in *suc1*⁻ germinating spores suggests that the loss of Suc1 is not required for the maintenance of Cdc2 protein kinase activity but may be involved in targeting the Cdc2 protein kinase to substrates or some other critical function. Thus, in *suc1*⁻ cells the appropriate substrates may not be phosphorylated in mitosis, resulting in mitotic arrest even with an excess of active kinase.

Several studies involving the *S. pombe* Suc1 protein have suggested that the Cks1 homologs are inhibitors of mitotic induction. The overexpression of Suc1 in *S. pombe* results in a delay in entry into mitosis (Hayles et

al. 1986b; Hindley et al. 1987). In the work described in this paper, *S. cerevisiae* *CKS1*, *S. pombe* *suc1*⁺ and human *CKShs1* and *CKShs2* cDNAs were overexpressed in the *S. cerevisiae* *cks1*⁻ null mutant. All four strains gave rise to slightly larger and more elongated cells than a *CKS1*⁺ strain (Fig. 3), and preliminary analysis suggests that this is attributable to a delay at the G_2 - to M-phase transition. Similarly, experiments in the *Xenopus* system have led to the proposal that Cks1 homologs act as an inhibitor of Cdc2 activation at the G_2 - to M-phase transition by preventing dephosphorylation at tyrosine residues on the Cdc2 protein (Dunphy et al. 1988; Dunphy and Newport 1989). However, in that study the heterologous *S. pombe* Suc1 protein was added to extracts from *Xenopus* embryos, and the results obtained may be due to inappropriate interactions occurring between heterologous proteins. Likewise, the same argument can be used to explain the inhibitory effect on cell division observed when *S. pombe* Suc1 is injected into rat fibroblasts in the experiments of Riabowol et al. (1989). Alternatively, it is possible that the concentrations of Suc1 used in these experiments were much higher than the normal cellular levels of the endogenous proteins; therefore, their results may represent a non-physiological consequence of a stoichiometric imbalance in interacting proteins. This explanation is consistent with the inhibition of cell-cycle progression observed when the Cks1 homologs are overexpressed in yeast (Hayles et al. 1986b; Hindley et al. 1987; H.E. Richardson and S.I. Reed, unpubl.).

Studies on the Cdc2 protein kinase (after purification of MPF activity or histone H1 kinase activity) from *Xenopus* oocytes, marine invertebrate oocytes, and human cells have not addressed whether Cks1 homologs are normally components of the active kinase complex (Arion et al. 1988; Gautier et al. 1988, 1990; Labbe et al. 1988, 1989a,b; Lohka et al. 1988; Brizuela et al. 1989; Draetta et al. 1989; Langan et al. 1989; Meijer et al. 1989; Pondaven et al. 1990). In the analysis of the components of purified MPF or histone H1 activity, these workers used gel systems that would not have resolved the small Cks1 homologs. Furthermore, in some cases, p13^{Suc1}-Sephacrose beads were used to purify the activity, and Suc1 may therefore have displaced the endogenous protein. In this work we have demonstrated that Cks1s-Sephacrose beads can bind the active form of the Cdc2 protein kinase, as assayed by the ability to phosphorylate histone H1. The preparation of antisera against Cks1s and Cks2s proteins will enable us to investigate whether these proteins are normally components of MPF and histone H1 kinase in human cells and whether their relative abundance varies through the cell cycle, as is suggested by their transcription patterns.

In conclusion, we have demonstrated that human cells contain proteins that are structurally and functionally conserved with the small subunit (Cks1/Suc1⁺) of the Cdc28/Cdc2⁺ protein kinase from yeast. This presents another example of the strong conservation of cell-cycle control mechanisms and components in eukaryotic organisms.

Experimental procedures

Yeast strains, genetic procedures, media, and growth conditions

All yeast strains used in this study were derivatives of BF26415D *ura3⁻*, α *ade1 his2 leu2-3,112 trp1-1^a ura3 Δ ns* (Reed et al. 1985; Richardson et al. 1989). The *cdc28* temperature-sensitive strain, (*cdc284*), used to prepare protein extracts, was D4, α *ade1 his2 leu2-3, 112 trp1-1^a cdc28-4* (Reed et al. 1985).

Standard genetic procedures for yeast were used (Sherman et al. 1982). Yeast transformations were carried out by the alkali cation method (Ito et al. 1983). Genomic sequence replacements were performed as described by Rothstein (1983).

The strain used to test for rescue of the *S. cerevisiae cks1* null allele, *cks1 :: LEU2 [CEN1 TRP1 CKS1]*, was constructed as follows. The *cks1 :: LEU2* allele was prepared in vitro by insertion of the *LEU2* gene as a *HindIII*–*SmaI* fragment (from a pUC19 clone in the *SalI* site) into the unique *HindIII* site present in *CKS1*, which was cloned as a 0.72-kb *XhoI*–*EcoRI* fragment into a pBluescript plasmid (see Hadwiger et al. 1989a). The endogenous *CKS1* gene was replaced by the *cks1 :: LEU2* allele by transformation of BF264-15D *ura3⁻* diploid yeast cells with pBluescript[*cks1 :: LEU2*] DNA digested with *XhoI* and *StuI* (see Hadwiger et al. 1989a) and selection for *Leu⁺* colonies. This strain, heterozygous for *cks1 :: LEU2*, was transformed with the *CEN1 :: TRP1 :: CKS1* plasmid, (pSE271[*CKS1*]), and *Trp⁺* colonies were selected. The haploid strain carrying the *cks1 :: LEU2* null allele and the pSE271[*CKS1*] plasmid were obtained by sporulation and dissection of the diploid and screening for *Leu⁺* and *Trp⁺* segregants.

Yeast cultures were grown in YEP (1% yeast extract, 2% bacto-peptone, 0.005% adenine, 0.005% uracil) supplemented with glucose (2%) or galactose (2%) as the carbon source, except when selection for plasmids was required; cells were then grown in minimal media supplemented with amino acids and adenine but lacking uracil.

HeLa cells were grown in suspension culture in Joklik's modified Eagle medium containing 10% (vol/vol) equine serum and 10 mM HEPES (pH 7.4).

Cloning the CKShs cDNA

The degenerate oligonucleotides used in the PCR technique for cloning human homologs of *CKS1* had the following sequences: 5' primer, 5'-AGAGCTCGAGAYGAYRANTAYGARTAYM-GNCAYGTNATG-3' corresponding to the amino acid sequence DD(NE)YEYRHVM; 3' primer, 5'-ATCTCGAGATC-GATTRAANARNARNATRTGNGGYTCNGG-3', corresponding to the amino acid sequence PEPHILLFK (Y = C + T, R = A + G, M = A + C, N = A + G + C + T). The *XhoI* and *ClaI* restriction sites are underlined. The PCR incubations were for 40 cycles of 94°C for 1 min, 45°C for 2 min and 72°C for 3 min in a DNA Thermal Cycler (Perkin-Elmer Cetus). The reactions consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.025 units of *Taq* DNA polymerase (Perkin-Elmer Cetus), 10 μ g of each oligonucleotide set, and 5 μ g of DNA prepared from a HeLa cell cDNA library (kindly provided by Steve Gould, University of California, San Diego). The HeLa cell cDNA library was made in the plasmid pCD (Okayama and Berg 1983) by Steve Hanks (Salk Institute, La Jolla, California). The PCR products were digested with *XhoI* and *ClaI* restriction enzymes, gel-purified, and cloned into the *XhoI* and *ClaI* sites of pBluescript SK (Stratagene).

To obtain cDNA clones, the two *CKShs* PCR clones were used to make radiolabeled probes to screen a HeLa cell cDNA

library. Initially, the HeLa cell cDNA library made by Steve Hanks was used; however, only one clone (*CKShs2*) was obtained after screening 50,000 colonies. The DNA sequence of this clone was determined in both directions after constructing subclones from the internal *SacI*, *BglII*, and *PstI* sites (Fig. 1B) in pT7T319U (Pharmacia). A HeLa cell (D98/AH-2) cDNA library in the vector Lambda Zap II (Stratagene) was then screened with radiolabeled probes made from the *CKShs1* PCR clone and the *CKShs2* cDNA clone. Many positive clones were obtained (~100 of 200,000 plaques). Several of these were purified, and pBluescript SK phagemids were derived from the λ phage by in vivo excision following the manufacturer's protocol (Stratagene). Restriction analysis revealed that they represented both *CKShs2* and *CKShs1* cDNA clones. The largest *CKShs1* cDNA clone was sequenced completely from the pBluescript SK clone and from subclones obtained from the internal *HindIII* site (Fig. 1A) in pT7T319U (Pharmacia). Sequence analysis of the *CKShs* cDNA revealed that it lacked a poly(A) tail, which may have occurred during the in vivo excision process used to obtain the clone in a plasmid form.

Recombinant DNA manipulations

The *CEN1 :: TRP1 :: CKS1* plasmid, pSE271[*CKS1*], was constructed by insertion of a *BamHI*–*XhoI* fragment containing the *CKS1* gene, which was derived from an *EcoRI* clone of *CKS1* in the phagemid pT7T319U (Hadwiger et al. 1989a), into the unique *BamHI* and *SalI* sites of pSE271. The plasmid pSE271 was obtained from R.W. Davis.

Clones of *CKShs1*, *CKShs2*, *CKS1*, and *suc1⁺* were obtained in the yeast *GAL1* expression vector, YCpG2 (Richardson et al. 1989), by constructing *BamHI* restriction sites in vitro at the 5' and 3' ends of the protein-coding regions and cloning the *BamHI* fragments into YCpG2 at the unique *BamHI* site (located 3' of the *GAL1* promoter). The following pairs of primers were used to create *BamHI* restriction sites at the 5' and 3' ends of the protein-coding regions of *CKS1* homologs by PCR: *CKShs1* 5' end, 5'-GGGATCCGATCATGTGCGACAAAC-3', corresponding to bases 5–22 (Fig. 1A); *CKShs1* 3' end, 5'-GGGATCCAGTTACCAGCACATTC-3' corresponding to the reverse complement of bases 381–397 (Fig. 1A); *CKShs2* 5' end, 5'-GGGATCCGGATGGCCCACAAGCAG-3', corresponding to bases 94–110 (Fig. 1B); *CKShs2* 3' end, 5'-GGGATCCGATTTGACGATCCCCAG-3', corresponding to the reverse complement of bases 343–359 (Fig. 1B); *CKS1* 5' end, 5'-CCGGATCCAGTACTATGTACCATCA-3'; *CKS1* 3' end, 5'-CCGGATCCGAATTCAATTTTCAGTAAT-3' (see Hadwiger et al. 1989a); *suc1⁺* 5' end, CCGGATCCAATATGTC-GAAAAGTGGTG; *suc1⁺* 3' end, 5'-CCGGATCCGTTG-GAGTTGACAAACT-3' (see Hindley et al. 1987). *BamHI* restriction sites are underlined.

For the production of Cks proteins in bacteria, clones of the *CKS1* homologs were obtained in the bacterial expression vector pRK171 (Rosenberg et al. 1987), as described below. A *NdeI* restriction site was constructed at the 5' end, and a *BamHI* restriction site was constructed at the 3' end of the protein-coding regions of the *CKS1* homologs by PCR, with the *CKShs* 3'-end *BamHI* primers described above and the following 5'-end *NdeI* primers: *CKShs1* 5'-end, 5'-CCCATGTGCG-CACAAACAAATT-3', corresponding to bases 10–27 (Fig. 1A); *CKShs2* 5'-end, 5'-CCCATATGGCCACAAGCAGATC-3', corresponding to bases 96–113 (Fig. 1B); *CKS1* 5'-end, 5'-GCCATATGTACCATCACTATCAC-3' (see Hadwiger et al. 1989a); *suc1⁺* 5'-end, 5'-GCCATATGTGCGAAAAGTGGTG-3' (see Hindley et al. 1987). *NdeI* restriction sites are underlined. The *NdeI*–*BamHI* fragments were cloned into the *NdeI* and

*Bam*HI sites of pRK171, resulting in the fusion of the protein-coding frames of *CKShs1* and *CKShs2* with a strong bacterial translation initiation signal (Rosenberg et al. 1987).

The PCR conditions used for the in vitro construction of restriction sites were as described above except that the hybridization temperature was adjusted to be 5°C below the average melting temperature of the two primers. The template DNA (from plasmid clones of the appropriate *CKS1* homologs) was added to the reactions at a final concentration of 10 pg/μl.

Photomicroscopy and cell counting

Yeast cells were fixed in 70% ethanol, and cell number was determined with a hemacytometer and a Nikon Optiphot microscope with phase-contrast optics at 400× magnification. Cells were photographed by use of differential interference contrast (Nomarski) optics with a 100× objective.

Cell synchrony and Northern analysis

For the fractionation of HeLa cells by centrifugal elutriation, a 3-litre suspension culture of HeLa cells was grown to a density of 5×10^5 cells/ml. Cells were loaded onto a Beckman JE5.0 elutriator rotor spinning at 2500 rpm at a rate of 125 ml/min, and the flow rate was adjusted to 75 ml/min and the centrifugal speed was adjusted to 1550 rpm. Aliquots (400 ml) were taken as the flow rate was increased at 10% increments. Samples were analyzed for DNA content after staining cells with propidium iodide, as described by Draetta and Beach (1988), with a Becton Dickinson FACS IV analyzer. RNA was prepared from the samples by use of guanidinium isothiocyanate (Chirgwin et al. 1979). RNA samples (10 μg of each) were electrophoresed on a 1% agarose-formaldehyde gel. RNA size markers (0.24- to 9.5-kb RNA ladder, BRL) were run on the gel and stained with ethidium bromide. RNA was electroblotted to a Biotrans (ICN) nylon membrane.

Radiolabeled probes were made by use of the random primer DNA labeling kit (Boehringer-Mannheim) according to the manufacturers' instructions. The *CKShs1* probe was prepared from the *Nde*I–*Bam*HI clone of *CKShs1* in pRK171, and the *CKShs2* probe was prepared from a 0.6-kb *Bam*HI–*Sac*I fragment containing the complete coding region obtained from the cDNA clone. The β-actin probe was prepared from a 2.3-kb *Bam*HI fragment encoding the cDNA (Ponte et al. 1984). Hybridizations and washes were performed as described by Reed et al. (1982). An Ultrascan XL laser densitometer (LKB) was used to scan the autoradiographs to quantitate the relative levels of the *CKShs* transcripts in each sample relative to β-actin mRNA.

Expression of proteins in bacteria and preparation of Ckshs–Sephacrose beads

Cks proteins were overproduced in bacteria by use of the T7 expression system, described by Rosenberg et al. (1987). Two grams of induced cells (B-lysS strain) carrying pRK171[*CKShs1*], pRK171[*CKShs2*], pRK171[*CKS1*], or pRK171[*suc1+*] plasmids, was lysed in 50 mM Tris-HCl (pH 8), 2 mM EDTA, and 10% glycerol, followed by sonication for 2 min. Polyethylene amine (pH 7.5) was added to 0.25% (vol/vol), and the lysate was centrifuged at 11,000g for 15 min. The supernatant was removed, ammonium sulfate was added to 80% (wt/vol), and proteins were precipitated at 9000g for 20 min. The protein pellet was resuspended in 50 mM Tris-HCl (pH 8), 100 mM NaCl, and 2 mM EDTA and run through a 1.6 × 65-cm Sephacryl S-200HR column at a flow rate of 11 ml/hr. Fractions containing the protein, identified by SDS-PAGE on a 15% gel, were pooled.

Ckshs protein, prepared as described above, was coupled to

cyanogen bromide–Sephacrose as described by the manufacturer (Pharmacia). The coupling density obtained for the Ckshs–Sephacrose beads was 0.14 mg/ml for both Ckshs1 and Ckshs2 protein.

Protein kinase assays and SDS-PAGE

Yeast cell protein extracts were prepared from exponentially growing *CDC28+* or *cdc28* temperature-sensitive mutant (*cdc28-4*) cells, as described by Wittenberg and Reed (1988). The HeLa cell protein extract was kindly supplied by Clare McGowan (Scripps Clinic, La Jolla, California), as a 140,000g supernatant at a concentration of 30 mg/ml.

For Cdc2/Cdc28 protein kinase assays with Ckshs–Sephacrose beads, 30 μl of Ckshs–Sephacrose beads was incubated with 15 μg of HeLa cell protein or 40 μl of yeast cell extract in lysis buffer [1% sodium deoxycholate, 1% Triton, 0.1% SDS, 50 mM Tris-HCl (pH 7.2), 1 mM sodium pyrophosphate] on ice for 1 hr. For competition experiments, Ckshs1 or Ckshs2 protein was added to the incubations at a final concentration of 0.05 mg/ml. After incubation, the Ckshs–Sephacrose beads were washed three times in RIPA buffer (lysis buffer containing 300 mM NaCl), once in lysis buffer without SDS or sodium pyrophosphate, and finally, two times with reaction buffer [20 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 0.8 mM EGTA]. Protein kinase assays were performed in reaction buffer with the addition of 80 μM [γ -³²P]ATP and 1 μg of histone H1 (Boehringer-Mannheim Biochemicals) for 30 min at 37°C. Reactions were stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer, and the labeled products were analyzed by electrophoresis on a 10% SDS-PAGE gel. Gels were dried and exposed to XAR-5 film (Kodak) at –70°C with a Cronex Lightning Plus intensifying screen.

Cdc28 antiserum precipitations of Cdc28 protein kinase activity were performed with antiserum directed against the amino terminus of Cdc28, as described by Wittenberg and Reed (1988).

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