The EMBO Journal vol.12 no.4 pp.1723–1732, 1993

Characterization of the fission yeast *mcs2* cyclin and its associated protein kinase activity

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Communicated by T.Hunt

We have previously described the isolation of *mcs2-75*, a mutation obtained as an allele-specific suppressor of a dominant allele of *cdc2*. *mcs2* was cloned and determined to be an essential gene, the product of which shares homology with the cyclin family of proteins. In contrast to the behavior of some, but not all cyclins, the mcs2 protein is constant in its abundance and localization throughout the cell cycle. A kinase activity that co-precipitates with mcs2 can be detected when myelin basic protein (MBP) is provided as an exogenous substrate. This kinase activity is constant throughout the cell cycle. mcs2 does not appear to associate with the *cdc2* protein kinase or an antigenically related kinase. Finally, a protein kinase termed csk1 (cyclin suppressing kinase) was isolated as a high copy suppressor of an *mcs2* mutation. *csk1* is not essential, however, the level of kinase activity that co-precipitates with mcs2 is reduced ~3-fold in strains harboring a *csk1* null allele. Therefore, *csk1* may encode a protein kinase physically associated with mcs2 or alternatively may function as an upstream activator of the mcs2-associated kinase.

Key words: cdc2/cell cycle/cyclins/fission yeast/protein kinase

Introduction

The fission yeast *Schizosaccharomyces pombe* has proven to be a powerful genetic system for study of the eukaryotic cell cycle. The *cdc2* protein kinase, a universal regulator of the G_2/M transition in eukaryotic cells, was initially identified using genetic screens in fission yeast. The *cdc2* gene product is required at two points in the fission yeast cell cycle; mutations that abolish *cdc2* function result in a cell cycle arrest either in G_1, prior to S phase or in G_2, prior to mitosis (Nurse *et al.*, 1976; Nurse and Bissett, 1981). Dominant alleles of *cdc2* also exist (*cdc2w* or the *cdc2* 'wee alleles'), and these mutations shorten G_2, which results in small cells, termed 'wee' cells (Nurse and Thuriaux, 1980). The *cdc2w* alleles bypass *cdc2* regulatory pathways and suggest that *cdc2* kinase activity is rate limiting for entry into mitosis (Russell and Nurse, 1986, 1987).

cdc2 protein kinase activity oscillates dramatically throughout the cell cycle, reaching maximal activity at mitosis, although the abundance of the *cdc2* polypeptide remains relatively constant (Booher *et al.*, 1989). The activity of the *cdc2* kinase is regulated by phosphorylation of the *cdc2* protein (Gould and Nurse, 1989; Ducommum *et al.*, 1991; Gould *et al.*, 1991) and by its interaction with regulatory subunits that have been identified genetically as suppressors of conditional lethal alleles of *cdc2* (Hayles *et al.*, 1986; Booher and Beach, 1987; Hindley *et al.*, 1987).

An allele of one such gene, *cdc13*, was isolated as a suppressor of a *cdc2* mutation defective exclusively in the G_2 function of *cdc2* (Booher and Beach, 1987). Subsequently, allele-specific interactions were detected between *cdc13* and *cdc2w* alleles, which suggests the possibility of a physical association of the gene products. The *cdc13* and *cdc2* gene products have been demonstrated to form a complex, the kinase activity of which is required for entry into mitosis (Booher *et al.*, 1989). The *cdc13* polypeptide is homologous to members of the cyclin family of cell cycle regulatory proteins (Solomon *et al.*, 1988; Goebl and Byers, 1988). Cyclins are proteins first identified in marine invertebrates and have since been shown to function as regulatory subunits of cdc2-like protein kinases (reviewed in Draetta, 1990; Pines and Hunter, 1991b). The abundance of most cyclin polypeptides, as well as their associated kinase activities, oscillate throughout the cell cycle.

Mitotic catastrophe is a conditional lethal phenotype characterized by premature entry into mitosis, resulting in aberrant cellular and nuclear divisions leading to cell death. One way in which this phenotype can be induced is by combining a particular dominant allele of *cdc2* (*cdc2-3w*) with a loss of function allele of the *wee1* mitotic inhibitor (*wee1-50*; Russell and Nurse, 1987). Mutations that suppress the lethality of a *cdc2-3w wee1-50* double mutant strain might identify new mitotic inducers, in that a defect in a mitotic inducer might delay entry into mitosis, thus balancing the affects of mutations causing premature entry into mitosis. Alternatively, mutations in *cdc2* substrates might be predicted to abrogate the effects of the mutant *cdc2-3w* protein kinase. We have previously described the isolation and characterization of extragenic suppressors of mitotic catastrophe (mitotic catastrophe suppressors; Molz *et al.*, 1989). Two of the *mcs* mutations (*mcs2-75* and *mcs6-13*) display allele specific interactions with *cdc2w* mutations. Such interactions are strikingly similar to those detected between *cdc13* and *cdc2w* alleles, which suggests the possibility of a physical association of *mcs2* or *mcs6* and *cdc2* gene products.

We have cloned *mcs2* and determined that it encodes a new cyclin that is essential for progression through the fission yeast cell cycle. We can detect a protein kinase activity that co-precipitates with mcs2 and we have determined that this activity is not due to an association of mcs2 with the *cdc2* protein kinase or an antigenically related kinase. Neither the mcs2 polypeptide nor its associated kinase activity oscillate throughout the cell cycle. Finally, we have obtained a gene encoding a putative protein kinase (*csk1*: cyclin suppressing kinase) as a high copy suppressor of an *mcs2* mutant strain. *csk1* is not an essential gene, however, the level of the *mcs2* associated kinase activity is reduced 3-fold in strains which carry a null allele of *csk1*.

Results

Isolation and nucleotide sequences of the mcs2 gene

The allele of *mcs2* initially isolated (*mcs2-75*) does not confer any cell cycle or growth defect when present in an otherwise wild-type strain and could not be used to clone *mcs2* directly (Molz *et al.*, 1989). We have, however, previously demonstrated that the *mcs2-75* mutation is synthetically lethal when introduced into a *cdc2-3w cdc25-22* double mutant strain. A *cdc2-3w cdc25-22* double mutant strain is viable, whereas an *mcs2-75 cdc2-3w cdc25-22* triple mutant strain displays a temperature-sensitive cell cycle arrest (Molz *et al.*, 1989). Introduction of *mcs2* into this triple mutant strain should suppress the cell cycle arrest phenotype and allow cell growth. To isolate *mcs2*, a fission yeast genomic library was introduced into an *mcs2-75 cdc2-3w cdc25-22* triple mutant strain and two genes were recovered that efficiently rescued the lethal phenotype. Integration mapping demonstrated that one of these genes was *mcs2*. The other was found to be a high copy suppressor of the *mcs2-75* mutation (see below).

We determined the nucleotide sequence of a genomic DNA fragment carrying *mcs2* and found an open reading frame (ORF) interrupted by two introns (Figure 1A). The precise location of these introns was confirmed by comparing the genomic DNA sequence with the sequence of an *mcs2* cDNA. *mcs2* encodes a predicted polypeptide of 322 amino acids and is homologous to cyclins within a 190 amino acid region conserved among cyclins termed the 'cyclin box' (Figure 1B). The *mcs2* protein shares 22% identity (40% identity and conservative changes) with its closest relative, human cyclin C (Lew *et al.*, 1991).

mcs2 null allele

We constructed a null allele of *mcs2* using gene replacement to investigate further the role of *mcs2* in the *S.pombe* cell cycle. The entire coding region of *mcs2* was removed by replacing a *Sty*I fragment (Figure 1A) with the *S.pombe ura4* gene (Grimm *et al.*, 1988). This construct was used to replace one copy of *mcs2*$^+$ with *ura4*$^+$ in a diploid strain. This diploid was sporulated and we analysed 43 tetrads in which all four spores germinated. In each tetrad, two of the segregants divided to form microcolonies of elongated cells, which could not be propagated (Figure 2A). The two viable segregants from each tetrad were always Ura$^-$ and by inference, the inviable segregants carry the replacement of *mcs2* with *ura4*. We wished to verify that the lethal phenotype of a strain carrying the *mcs2* deletion was due to loss of *mcs2* and not due to altered expression of an

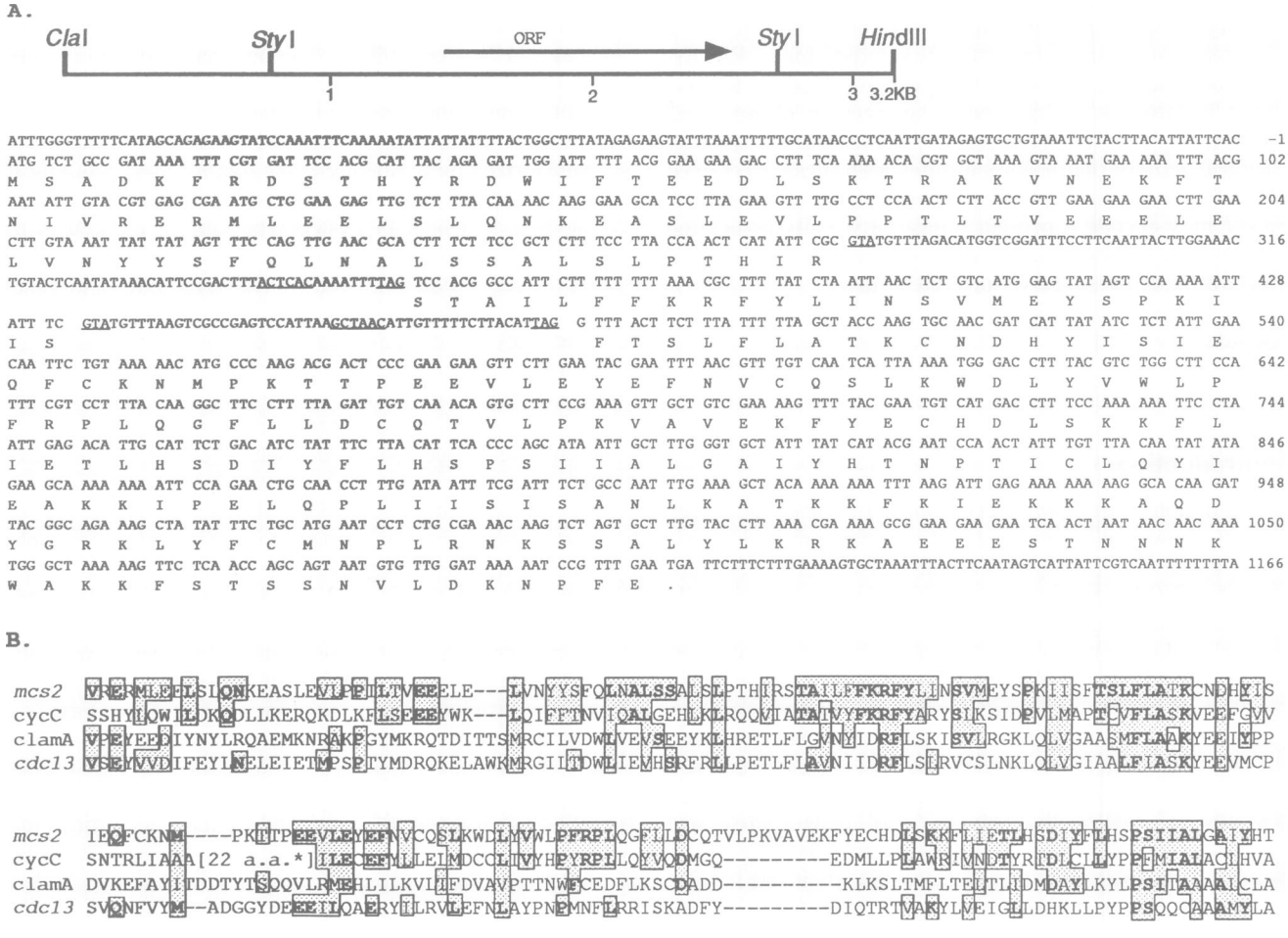

Fig. 1. *mcs2* encodes a 322 amino acid polypeptide that is homologous to cyclins. (**A**) Restriction map of a 3.2 kb *Hin*dIII−*Cla*I genomic fragment that carries *mcs2*. Nucleotide and predicted amino acid sequence of *mcs2* is shown below the restriction map. This gene contains two introns and the splicing consensus sequences are underlined. (**B**) Comparison of the deduced amino acid sequence of *mcs2* with human cyclin C (Lew *et al.*, 1991), clam cyclin A (Swenson *et al.*, 1986) and the *S.pombe cdc13* (Booher and Beach, 1988) proteins. Amino acids conserved between mcs2 and these cyclins are shaded and identical residues are also in bold. The following groups of amino acids are considered to be conserved: A, V, L, I, M; D, E; K, R; N, Q; F, Y; S, T. *, 22 amino acids are omitted from the human cyclin C sequence.

adjacent gene. An *mcs2* cDNA was cloned into an expression vector that carries the strong alcohol dehydrogenous (ADH) promoter. Expression of the *mcs2* product from the ADH promoter fully rescues the lethality of a strain carrying the *mcs2* gene replacement. Therefore, the lethal phenotype of this strain results from loss of the *mcs2* gene product (data not shown).

We determined whether the cells carrying the *mcs2* null allele arrested at a discrete point in the cell cycle by examining the phenotype of arrested cells germinated from spores carrying the *mcs2* deletion. A diploid strain heterozygous for the *mcs2* null allele was sporulated and the spores were purified on a glucose gradient. Only those spores carrying the *mcs2* null allele will be uracil phototrophs and germinate in liquid minimal media lacking uracil. Spores obtained from wild-type and mutant strains were inoculated into minimal media and allowed to germinate and divide. Every 2 h, an aliquot of cells was fixed with glutaraldehyde and then stained with diamidinophenylindole (DAPI) to visualize nuclear structure. Spores carrying the *mcs2* null allele germinated and divided 2−3 times before arresting (Figure 2C), in contrast to spores from a wild-type strain, which germinated and divided until reaching stationary phase (Figure 2B). The *mcs2*$^-$-arrested cells frequently contained division septa. The septation index was 14% in an exponentially growing culture of germinated wild-type spores, while 60% of terminally arrested cells that carry the *mcs2* deletion contained division septa. Also, the nuclei of the arrested cells were more compact than those of a wild-type strain, possibly indicating the presence of condensed chromatin. Although *mcs2*$^-$-arrested cells did not arrest at a uniform point in the cell cycle, the cells did, however, display characteristics of cells arrested in mitosis.

Detection of the mcs2 protein

In order to determine whether mcs2 displays any of the biochemical characteristics of cyclin proteins, we generated specific reagents for detection of mcs2. Full-length mcs2 purified from a bacterial expression system was used to immunize rabbits, for the purpose of raising a polyclonal anti-serum. This affinity-purified anti-serum was used to probe an immunoblot of *S.pombe* lysates and it recognized a single band migrating at the approximate molecular weight of *mcs2* (37.5 kDa), which exactly co-migrated with the bacterially-produced mcs2 protein (Figure 3A). This band was only slightly enhanced when lysates were prepared from strains that contain a multicopy plasmid that carries *mcs2* expressed from its own promoter. This band was strongly enhanced when lysates were prepared from a strain in which *mcs2* was expressed from the *ADH* promoter (Figure 3A). Also, this band was absent when lysates were prepared from cells germinated from spores harboring an *mcs2* null allele, as described above (Figure 3B). Therefore, this antibody appeared to specifically recognize mcs2 when used to probe an immunoblot of *S.pombe* lysates. This antibody was used to localize the *mcs2* protein (see below).

The affinity-purified antibody described above did not specifically precipitate mcs2 from *S.pombe* lysates. We therefore utilized an epitope addition system for immunoprecipitation of mcs2 (Field *et al.*, 1988; Tyers *et al.*, 1992). *mcs2* was tagged at the carboxyl-terminus with three copies of the influenza virus hemagglutinin epitope (HA1 epitope) or three HA1 epitopes adjacent to a factor X_a protease cleavage site. A monoclonal antibody is commercially available that recognizes the HA1 epitope. The tagged genes were then used to replace precisely the genomic copy of *mcs2*, resulting in a strain identical to a wild-type strain, except for the presence of the HA1 epitopes. An anti-HA1 antibody was used to probe an immunoblot of *S.pombe* lysates and it recognized a band of the predicted molecular weight of each particular fusion protein (Figure 3C). These bands were absent when lysates were prepared from strains in which *mcs2* was not tagged (Figure 3C, lanes 1 and 2) and were enhanced when lysates were prepared from strains

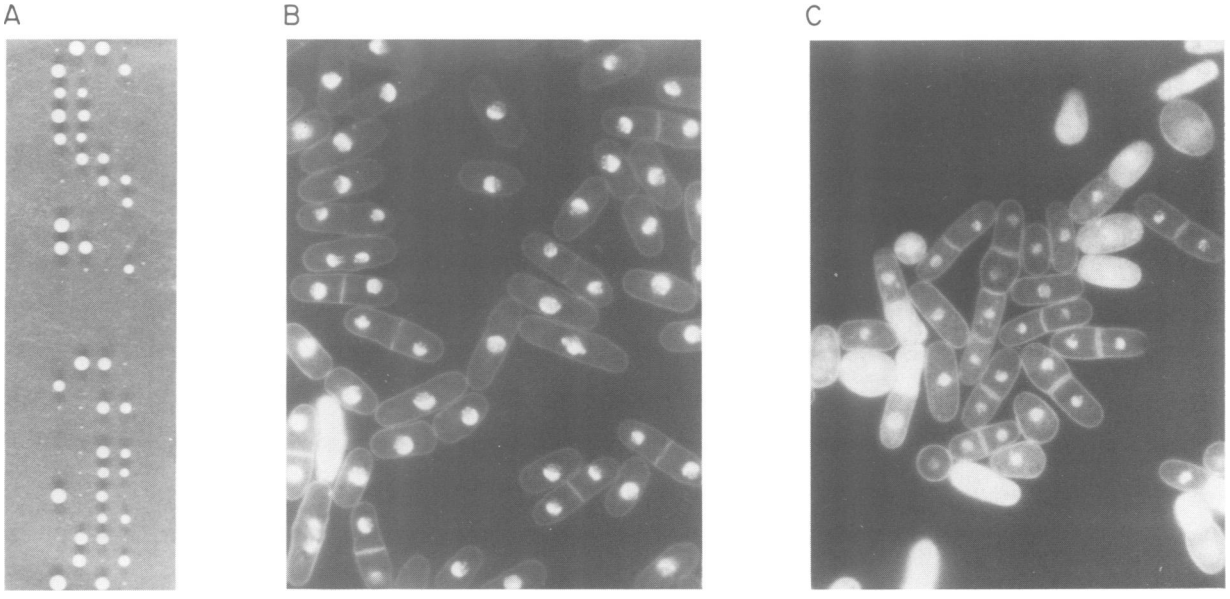

Fig. 2. *mcs2* gene replacement. Photograph of tetrads dissected from a diploid heterozygous for *mcs2* replacement (**A**). The two spores from each tetrad that carry *mcs2* replacement germinated and formed microcolonies of inviable cells. We have examined in more detail the phenotype of arrested cells carrying *mcs2* replacement. Wild-type spores (**B**) and spores carrying the *mcs2* null allele (**C**) were germinated in liquid media, fixed with glutaraldehyde and stained with DAPI.

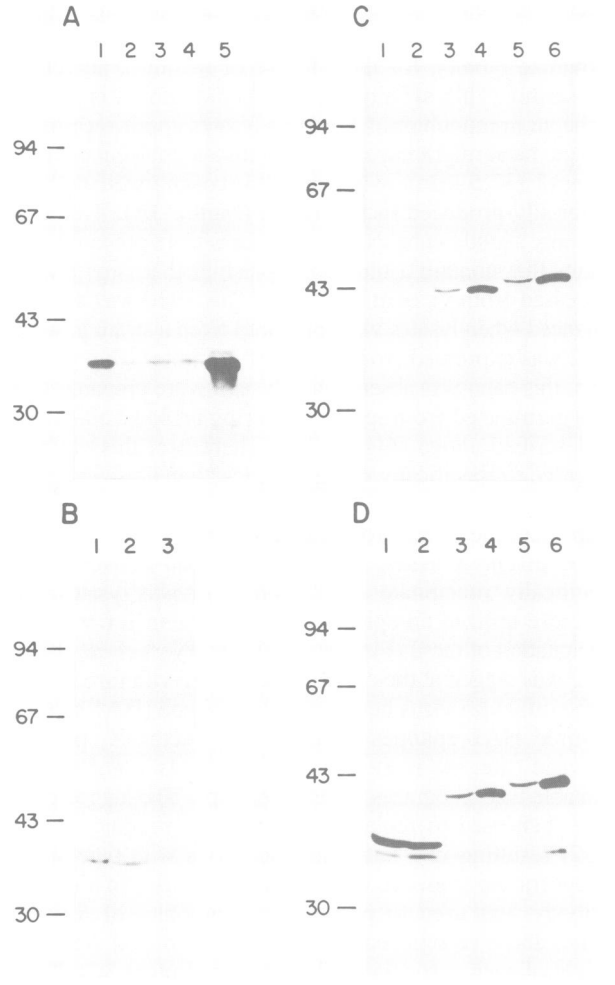

Fig. 3. Detection of *mcs2*. (**A**) Immunoblot of *S.pombe* lysates (100 μg) probed with an anti-mcs2 polyclonal serum. Approximately 2 ng of bacterially-produced mcs2 protein was loaded in lane 1. Lysates were prepared from strains carrying a replicating plasmid (pDB248x, lane 2), a pDB248x derivative carrying *mcs2* expressed from its own promoter (pmcs2b, lane 3), a replicating plasmid carrying the *ADH* promoter (pART3, lane 4) and a pART3 derivative carrying *mcs2* expressed from the *ADH* promoter (pmcs2-ART3, lane 5). Due to the strong expression from the *ADH* promoter, only 10 μg of an *S.pombe* lysate was loaded on lane 5. (**B**) Immunoblot of *S.pombe* lysates (100 μg) probed with an anti-mcs2 polyclonal serum. Lane 1 contains bacterially-produced mcs2. Lysates were prepared from cells germinated from spores obtained by sporulating a wild-type diploid (lane 2) or a diploid carrying the *mcs2* gene replacement (lane 3). (**C**) Immunoblot of *S.pombe* lysates (100 μg) probed with anti-HA1 monoclonal antibodies. Lysates were prepared from wild-type strains and strains in which the wild-type copy of *mcs2* had been precisely replaced with tagged *mcs2* genes. Lysates were prepared from a wild-type strain (lane 1) and from the same strain transformed with the replicating vector pART3 (lane 2), which carries the strong *ADH* promoter. Lysates were also prepared from a strain in which the genomic copy of *mcs2* was tagged with three HA1 epitopes and subsequently transformed with pART3 (lane 3) or a pART3 derivative in which the HA1-tagged *mcs2* gene was expressed from the *ADH* promoter (lane 4). Finally, lysates were prepared from a strain in which the genomic copy of *mcs2* was tagged with three HA1 epitopes adjacent to a factor X_a protease cleavage site and subsequently transformed with pART3 (lane 5), or with a pART3 derivative in which the HA1-X_a-tagged *mcs2* gene was expressed from the *ADH* promoter. Due to the strong expression from the *ADH* promoter, only 5 μg of an *S.pombe* lysate was loaded in lanes 4 and 6. (**D**) An immunoblot identical to that described in (C) was probed with an anti-mcs2 polyclonal serum.

that express the tagged genes from the strong ADH promoter (Figure 3C, lanes 4 and 6). Also, the wild-type and HA1-tagged *mcs2* proteins were recognized by the anti-mcs2 polyclonal antibody (Figure 3D). Therefore, the anti-HA1 monoclonal serum specifically recognized mcs2 when used to probe an immunoblot of *S.pombe* lysates.

mcs2 encodes a nuclear protein

Several mitotic cyclins from humans and yeast are localized to the nucleus in a cell cycle-dependent manner (Alfa *et al.*, 1991; Booher *et al.*, 1989; Girard *et al.*, 1991; Pagano *et al.*, 1991; Pines and Hunter, 1991a). We wished to determine whether mcs2 is also localized to a subcellular structure in a cell cycle-dependent manner. Cultures of wild-type fission yeast were grown to mid-log phase, fixed with methanol and stained with preimmune or anti-mcs2 serum. Staining with anti-mcs2 serum revealed nuclear fluorescence (Figure 4C and D), which was absent in cells treated with the preimmune serum (Figure 4A and B). The nuclear fluorescence was also absent in growing cells harboring the *mcs2* deletion (Figure 4G and H), but present in wild-type cells (Figure 4E and F). Therefore we believe the anti-mcs2 antibodies specifically recognized the *mcs2* gene product. The mcs2 protein was localized to the nucleus in every cell, regardless of its position in the cell cycle. Also, mcs2 was localized to a broad nuclear region encompassing more than the DNA region of the nucleus visualized by DAPI staining (see arrows, Figure 4C and D).

mcs2 protein does oscillate in abundance during the cell cycle

The protein level of many previously characterized cyclins has been shown to oscillate throughout the cell cycle. In order to determine whether the levels of mcs2 also oscillate throughout the cell cycle, we obtained a synchronous culture of cells by a temperature block−release experiment using a *cdc25-22* mutant strain. The *cdc25* gene product is required for entry into mitosis and at the restrictive temperature cells of a *cdc25-22* mutant strain arrest late in G_2. After release to the permissive temperature, the culture was allowed to progress through two cell cycles and aliquots of cells were taken every 20 min for the preparation of lysates to be used for immunoblotting. The percentage of cells that contain a division septum was used as an indication of synchronous progression of the culture through two cell cycles (Figure 7A). The level of mcs2 was constant throughout the cell cycle (Figure 7B), which is consistent with experiments where the localization and intensity of anti-mcs2 immunofluorescence was shown to be constant throughout the cell cycle. In order to insure that addition of the HA1 epitopes did not affect the stability of mcs2, a synchronized culture was prepared using a *cdc25-22* strain carrying an untagged *mcs2*. An immunoblot of lysates prepared from aliquots of the synchronized culture was probed with the anti-mcs2 polyclonal serum described above and again, the level of mcs2 was constant throughout the cell cycle (data not shown).

mcs2 has an associated kinase activity that is not cdc2

Since the mcs2 is homologous to cyclins, which are regulatory subunits of cdc2-like protein kinases, it was of

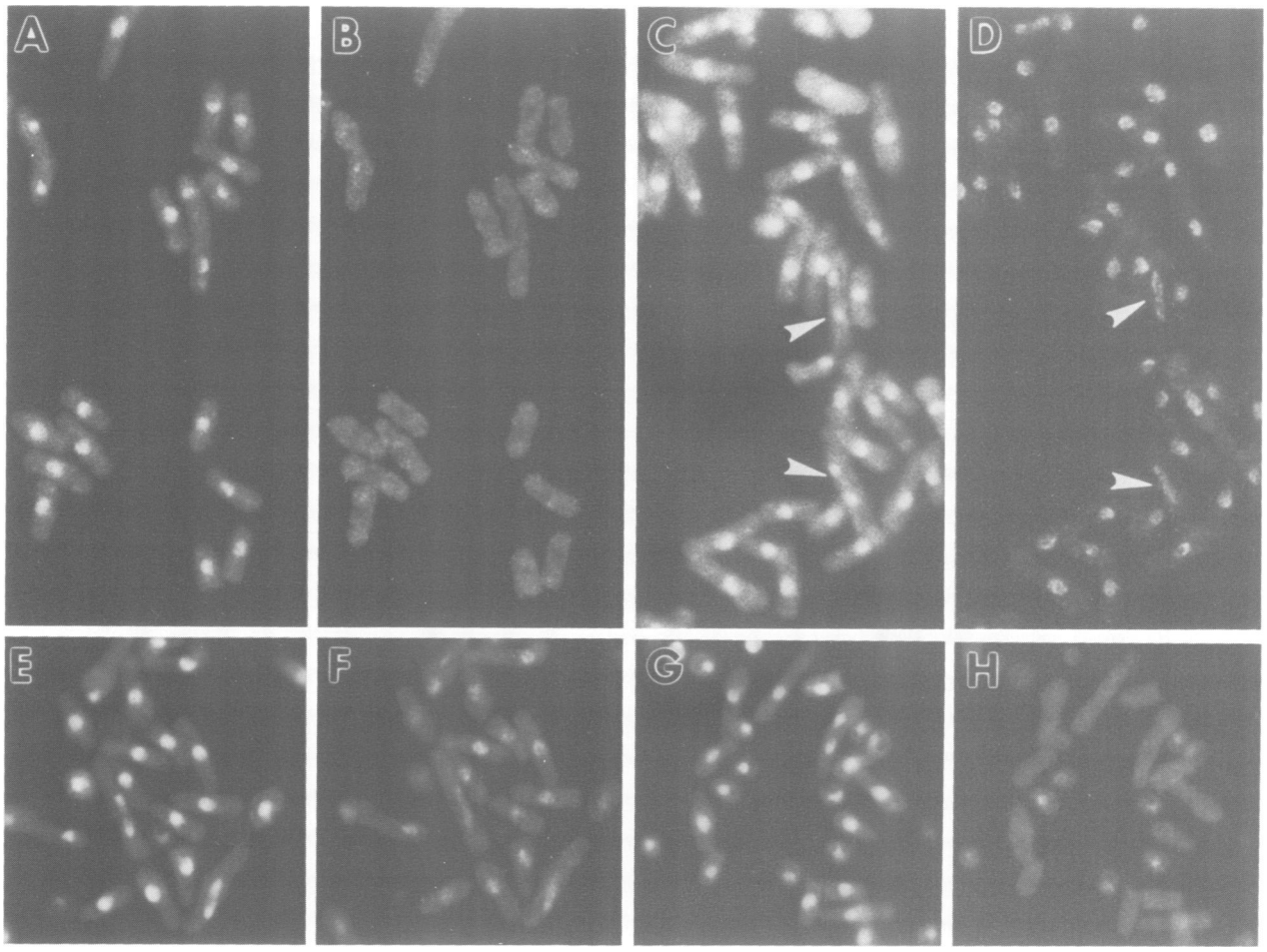

Fig. 4. Nuclear localization of mcs2. Asynchronously growing cells were stained with preimmune serum (**B**) or anti-mcs2 polyclonal serum (**D**). The same cells were stained with DAPI (**A** and **C**). Wild-type spores (**F**) and spores carrying the *mcs2* gene replacement (**H**) were germinated and stained with anti-mcs2 serum. The same cells were stained with DAPI (**E** and **G**). The ungerminated spores in (H) carry an intact *mcs2* gene and therefore displayed nuclear fluorescence.

interest to determine whether a protein kinase activity co-precipitates with the HA1-tagged mcs2. We failed to detect a kinase activity co-precipitating with mcs2 when histone H1 was provided as an exogenous substrate, although histone H1 is a good substrate for the cdc2 kinase. We did, however, detect a kinase activity that co-precipitates with mcs2 when myelin basic protein (MBP) was provided as an exogenous substrate (Figure 5A). This MBP kinase activity was not present in immunoprecipitations using lysates prepared from an untagged control strain and was competed by pre-incubating the anti-HA1 serum with HA1 peptide (Figure 6). Therefore, we believe that the kinase activity detected *in vitro* co-precipitated with mcs2 and was not precipitated by virtue of a non-specific interaction with the protein A−sepharose or the anti-HA1 antibody. The mcs2-associated kinase activity was optimal at a pH of 7.5 and 2.5 mM Mn^{2+} (data not shown). These assay conditions were different from those used for the cdc2 protein kinase (Booher *et al.*, 1989), which preferred Mg^{2+} as a divalent cation. cdc2 kinase precipitated using p13−Sepharose also phosphorylated MBP, although to a lesser extent than histone H1 (Figure 5B).

These data suggest that the cdc2 protein kinase did not contribute to the MBP kinase activity that co-precipitates with mcs2. We used *cdc2* mutants to completely eliminate this possibility. *cdc2* kinase precipitated from *cdc2* mutant strains (*cdc2-33*, *cdc2-56* and *cdc2-L7*) displayed reduced kinase activity towards histone H1 and MBP. In contrast, the level of the MBP kinase activity coprecipitating with mcs2 was not reduced in any of these *cdc2* mutant strains (data not shown). Furthermore, we failed to find any evidence for a physical association between mcs2 and cdc2. We failed to detect cdc2 in anti-mcs2 immunoprecipitations from lysates that were prepared from wild-type strains or strains carrying dominant alleles of *cdc2* (data not shown). Also, mcs2 was never detected in cdc2 precipitations (data not shown).

Since we have shown that the level of mcs2 is constant throughout the cell cycle, it was of interest to determine if the mcs2-associated kinase activity is cell cycle regulated. Cells were synchronized by a temperature block−release of a *cdc25-22* mutant strain and harvested as described above. mcs2 was immunoprecipitated from lysates and the immunoprecipitations were assayed for MBP kinase activity. As shown in Figure 7C and E, the mcs2-associated kinase activity was not cell cycle regulated (Figure 7C, E). In contrast, cdc2 protein kinase activity did oscillate in a cell cycle-dependent manner, as previously described (Figure 7C and D; Booher *et al.*, 1989).

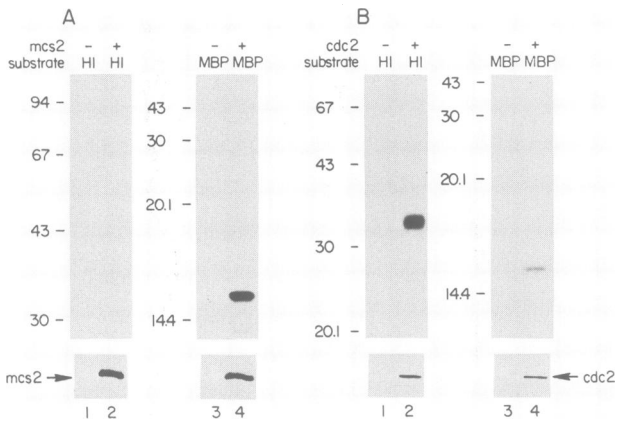

Fig. 5. An MBP kinase activity coimmunoprecipitates with mcs2. **(A)** Cell lysates were prepared from strains carrying untagged (lanes 1 and 3) or HA1-tagged (lanes 2 and 4) mcs2. Cell lysates were immunoprecipitated with an anti-HA1 serum and half of the immunoprecipitations were then immunoblotted with the same antibody for quantification of the precipitated mcs2 protein (see arrows). The remaining half of the precipitations was assayed for kinase activity using histone H1 (lanes 1 and 2) or MBP (lanes 3 and 4) as exogenous substrates. Phosphorylated histone H1 and MBP proteins were resolved using SDS–PAGE and then exposed to film for the same amount of time for comparison of relative substrate specificities. **(B)** cdc2 protein was precipitated from lysates using p13–Sepharose; protein A–Sepharose was used for control precipitations. The immunoprecipitations were split in half for use in either kinase assays or immunoblotting with an anti-cdc2 serum (G8), exactly as described in **(A)**.

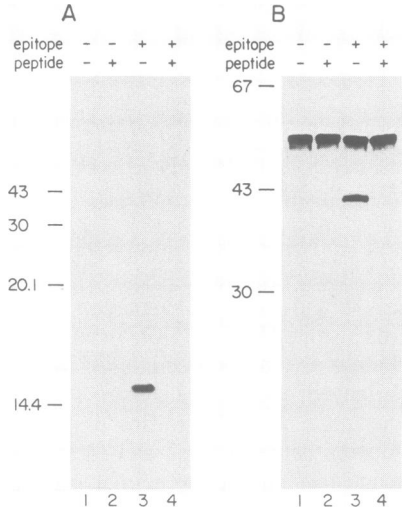

Fig. 6. The kinase activity co-immunoprecipitating with mcs2 is specific. Cell lysates prepared from strains carrying untagged (lanes 1 and 2) and HA1-tagged mcs2 (lanes 3 and 4) were immunoprecipitated with either anti-HA1 serum (lanes 1 and 3) or anti-HA1 serum that had been preincubated with the HA1 peptide (lanes 2 and 4). Half of each immunoprecipitation was assayed for MBP kinase activity **(A)**, while the remaining half was immunoblotted with anti-HA1 serum for quantification of mcs2 **(B)**.

Isolation of a protein kinase as high copy suppressor of the mcs2-75 mutation

We have described above the isolation of a gene that efficiently suppressed the cell cycle arrest phenotype of an *mcs2-75 cdc2-3w cdc25-22* triple mutant strain when introduced into this strain on a high copy plasmid. This gene,

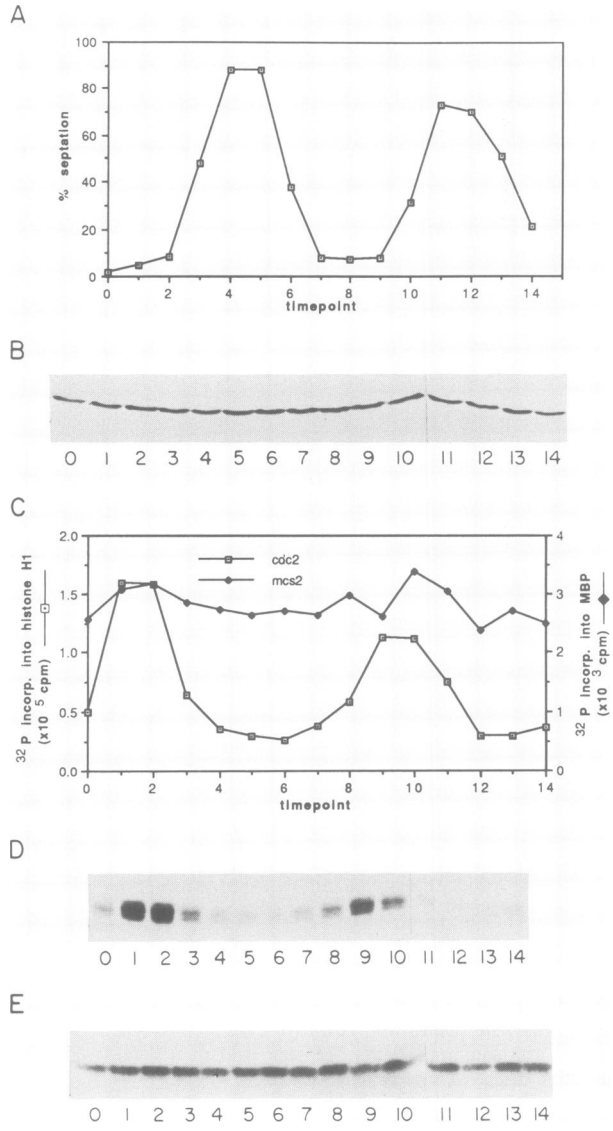

Fig. 7. The mcs2 polypeptide and its associated kinase activity are constant throughout the cell cycle. Cells were synchronized by a temperature block release of a *cdc25-22* mutant and an aliquot was taken every 20 min for the preparation of lysates. **(A)** Percentage of cells with a septa, which were visualized by calcifluor staining. **(B)** Visualization of mcs2 by probing an immunoblot of the lysates described above (100 μg) with anti-HA1 serum. **(C)** Quantification of c.p.m. incorporated into MBP and histone H1 in **(D)** and **(E)**. **(D)** cdc2 precipitations using p13–Sepharose were assayed for histone H1 kinase activity. **(E)** mcs2 immunoprecipitations were assayed for MBP kinase activity.

Table I. *csk1* is a high copy suppressor of *mcs2-75*

	+vector	+pmcs2	+pcsk1
mcs2-75	+	+	+
cdc2-3w	wee	wee	wee
mcs2-75 cdc2-3w	cdc^{-L}	wee	wee
cdc2-3w cdc25-22	+	+	+
mcs2-75 cdc2-3w cdc25-22	cdc	+	+

+, these cells are wild-type in cell length at division.
$^{-L}$, poorly growing colonies of cells that ranged from wild-type to highly elongated in length at cell division and accumulated multiple nuclei and division septa.

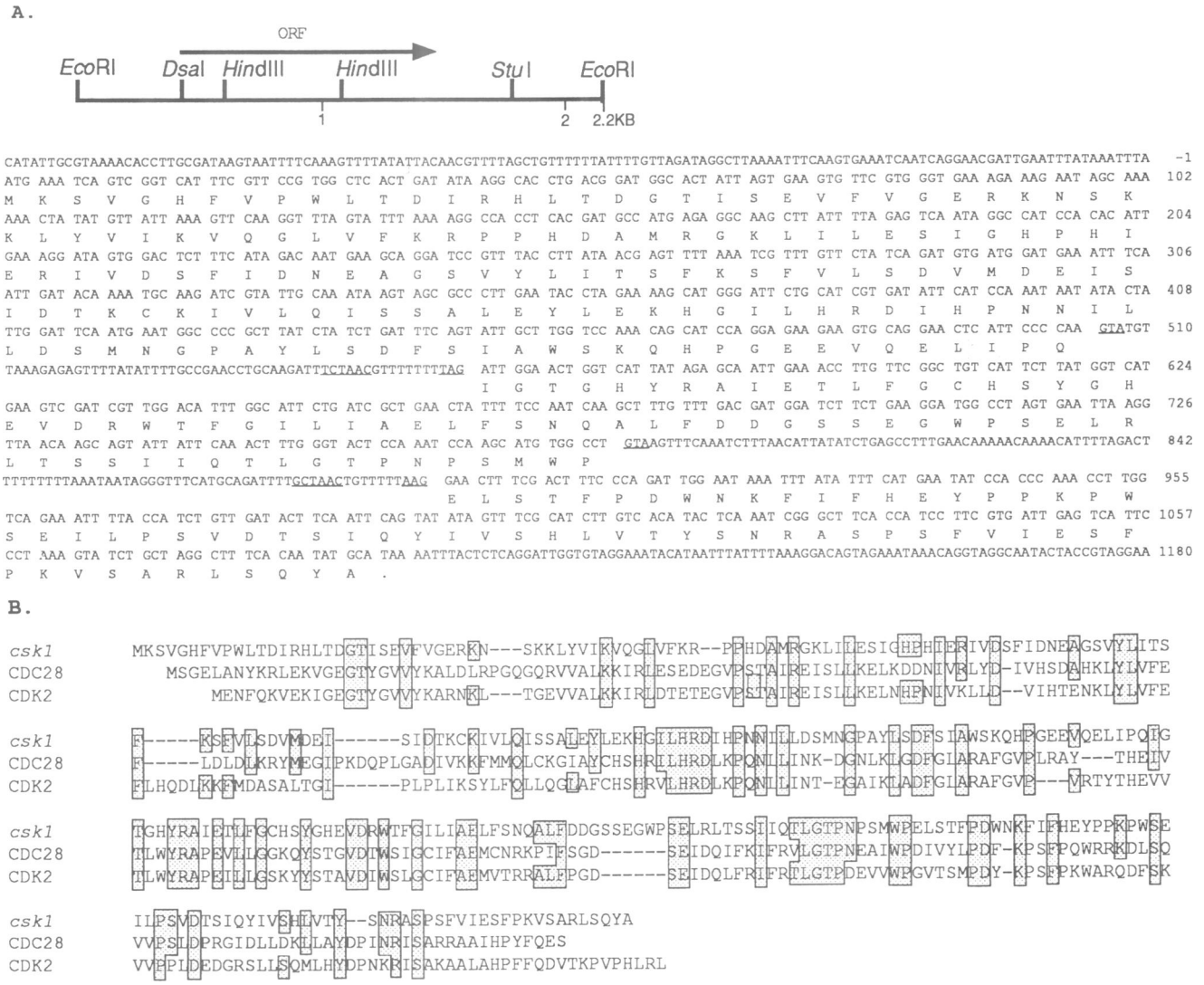

Fig. 8. *csk1* encodes a 306 amino acid polypeptide that is homologous to protein kinases. (**A**) Restriction map of a 2.2 kb genomic *Eco*R fragment carrying *csk1*. The nucleotide and predicted amino acid sequence of *csk1* is shown below the restriction map. *csk1* contains two introns and consensus splicing sequences are underlined. (**B**) Comparison of the deduced amino acid sequence of csk1 with *S.cerevisiae* CDC28 (Beach *et al.*, 1982) and human CDK2 (Tsai *et al.*, 1991) gene. Identical amino acids between csk1 and these kinases are shaded. Sequence similarity of the mcs2 polypeptide with cyclins and the csk1 polypeptide with protein kinases was obtained by searching data bases with the program FASTA (Pearson and Lipman, 1988).

designated *csk1* for reasons described below, might rescue the lethal phenotype of this strain by abrogating the affects of any of the three mutations present in this strain. We have determined that overexpression of *csk1* appears to rescue the defect conferred by the *mcs2-75* mutation. The *mcs2-75* mutation displays no cell cycle defect when present in an otherwise wild-type strain, but it confers a cell cycle defect in combination with the *cdc2-3w* mutation (Molz *et al.*, 1989). A *cdc2-3w* mutant strain displays a wee phenotype, while an *mcs2-75 cdc2-3w* double mutant strain is elongated and accumulates multiple division septa and nuclei. Also, a *cdc2-3w cdc25-22* double mutant strain displays a wild-type phenotype, while an *mcs2-75 cdc2-3w cdc25-22* triple mutant strain displays a temperature-sensitive cell cycle arrest. Introduction of *csk1* into *mcs2-75 cdc2-3w* or *mcs2-75 cdc2-3w cdc25-22* mutant strains reversed the affect of the *mcs2-75* mutation and restored the phenotypes to wee and wild-type, respectively (Table I). Therefore, it appears that *csk1* is an efficient suppressor of the *mcs2-75* mutation; *csk1* does not, however, rescue the lethal phenotype of a null allele of *mcs2*.

A genomic DNA fragment carrying *csk1* was sequenced and determined to contain a 306 amino acid ORF interrupted by two introns (Figure 8A). The precise location of these introns was confirmed by comparison of the genomic DNA sequence with the sequence of a *csk1* cDNA. The *csk1* polypeptide is homologous to protein kinases (Figure 8B) and contains the 11 kinase subdomains defined by Hanks *et al.* (1988). The csk1 protein is slightly more homologous to members of the cdc2 family of kinases than to other protein kinases, sharing ~30–33% identity with cdc2-like kinases across the catalytic domain and ~20–25% identity with other kinases.

We determined whether *csk1* is essential for progression through the *S.pombe* cell cycle. A 1.3 kb *Dsa*I–*Stu*I restriction fragment (Figure 8A) carrying the entire *csk1* coding region was replaced with the *sup3-5* tRNA suppressor, which rescues an *ade6-704* mutation in *S.pombe* (Chikashige *et al.*,

1989). This construct was used to replace one copy of the *csk1*$^+$ gene with the *sup3-5* tRNA suppressor in a diploid strain. The diploid was sporulated and we analysed 30 tetrads in which all four spores germinated and formed colonies of viable cells. In each tetrad, two segregants were Ade$^+$ and two were Ade$^-$, indicating that *csk1* is not an essential gene.

csk1 is required for full activity of the mcs2-associated kinase

csk1 was isolated as a high copy suppressor of the *mcs2-75* mutation. We therefore investigated whether the level of mcs2-associated kinase activity was altered in a strain carrying a *csk1* null allele. mcs2 was immunoprecipitated from a wild-type strain and strain carrying the *csk1* null allele. The immunoprecipitates were divided in half for quantification of mcs2 and for kinase assays. Strains carrying a *csk1* null allele contained approximately equal amounts of the mcs2 protein compared with a wild-type strain (Figure 9B), however, the kinase activity co-precipitating with mcs2 was reduced ~3-fold in a *csk1*$^-$ strain (Figure 9A and C). In contrast, levels of the mcs2-associated kinase activity were unchanged in strains carrying *cdc2-3w, cdc2-1w, wee1-50* and *cdc25-22* mutations (data not shown). This result suggests at least two possibilities: either *csk1* encodes a kinase physically associated with the mcs2, or *csk1* encodes an upstream activator of the kinase that physically associates with mcs2. We are currently unable to distinguish between these two possibilities. These data suggest that the kinase that co-precipitates with the tagged mcs2 protein is not simply precipitating by virtue of a non-specific association with the HA1 epitope. The *csk1* deletion strain contains wild-type levels of the tagged mcs2 protein, yet the kinase activity co-precipitating with mcs2 is significantly reduced. It is possible but unlikely that *csk1* is affecting the level or activity of a kinase associating with the HA1 epitope. *csk1* was obtained as a specific and efficient suppressor of an *mcs2* mutation, which suggests that it affects *mcs2* gene function.

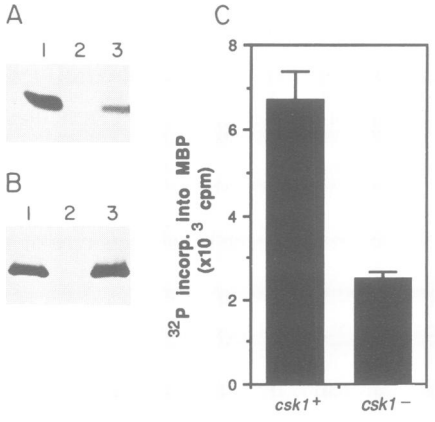

Fig. 9. The kinase activity co-precipitating with the mcs2 protein is reduced in strains carrying the *csk1* gene replacement. Cell lysates were prepared from a wild-type strain carrying a HA1-tagged *mcs2* (lane 1), an untagged strain carrying the *csk1* gene replacement (lane 2) and a tagged strain carrying the *csk1* gene replacement (lane 3). Cell lysates were precipitated with anti-HA1 serum and half of the immunoprecipitations were assayed for MBP kinase activity (**A**). The remaining half was immunoblotted with the anti-HA1 antibody for quantification of mcs2 (**B**). Quantification of MBP kinase assays from a wild-type strain and a strain carrying the *csk1* gene replacement (**C**). The background c.p.m. incorporated from anti-HA1 precipitations from an untagged strain was substracted. The results represent an average of the values obtained from four immunoprecipitations from the same lysate and one representative set of precipitations is shown in (A) and (B).

Discussion

We have previously described the isolation of *mcs2-75*, a mutation obtained as an allele-specific suppressor of the *cdc2-3w* mutation. In this paper, we describe the cloning of *mcs2* and characterization of its gene product. The *mcs2* gene is essential; spores carrying a null allele of *mcs2* germinate and undergo several divisions to form micro-colonies of inviable cells. These cells arrest with a heterogeneous phenotype, although many display characteristics of mitotic cells. These cells frequently contain division septa and their nuclei appear to be more compact in comparison with those of wild-type strain, possibly indicating the presence of condensed chromatin.

The *mcs2* gene product shares limited homology with cyclins. The mcs2 protein is 22% identical (40% identity and conservative changes) to its closest relative, human cyclin C in the cyclin box, a region which is conserved among cyclins. Cyclin C homologs have been cloned from humans and *Drosophila* by complementation of a strain of *Saccharomyces cerevisiae* deficient in G_1 cyclin function (Leopold and O'Farrell, 1991; Lew *et al.*, 1991). These genes share 72% identity and therefore we believe that mcs2 is not an *S.pombe* cyclin C homolog, but instead defines a new class of cyclin.

Cyclins comprise a diverse family of cell cycle regulatory proteins that often display characteristic biochemical properties (reviewed in Draetta, 1990; Pines and Hunter, 1991b). Many cyclins are unstable proteins that oscillate in their abundance or subcellular localization throughout the cell cycle. We have determined that mcs2 is localized to the nucleus throughout the cell cycle. Cyclins have also been determined to be regulatory subunits of cdc2 or cdc2-like protein kinases. We have detected an *in vitro* kinase activity that co-precipitates with mcs2 when MBP is provided as an exogenous substrate. The level of this kinase activity is constant throughout the cell cycle, a characteristic that is not unique to the mcs2 cyclin. Neither the abundance of the polypeptide nor the levels of kinase activity associated with the CLN3 'G_1' cyclin from *S.cerevisiae* is cell cycle-regulated (M.Tyers and B.Futcher, personal communication). It is possible that the activity of the mcs2-associated kinase is constant throughout the cell cycle, but that other factors, such as the accessibility of its substrates, are cell cycle regulated. Alternatively, the activity of mcs2-associated kinase might be required at a discrete point in the cell cycle, even though the kinase activity itself is not cell cycle-regulated.

The allele-specific interactions between *mcs2* and *cdc2* mutations suggest a physical association of the gene products. We failed, however, to find evidence for a stable complex between cdc2 and mcs2 either in wild-type strains or in strains carrying dominant *cdc2* mutations. Therefore, mcs2 and cdc2 physically associate in a transient manner that we presently cannot detect or the allele-specific interactions do not reflect a physical association of the proteins.

The mcs2 cyclin appears to associate with a protein kinase that is not the cdc2 kinase. Furthermore, the mcs2-associated kinase differs from presently characterized cdc2-like kinases,

in that the mcs2-associated kinase does not phosphorylate histone H1 and prefers Mn^{2+} (instead of Mg^{2+}) in the kinase assay buffer. Indeed, recent evidence suggests that cyclin dependent-kinases may comprise a diverse family of cell cycle regulatory kinases. For example, a human D-type cyclin, which appears to function in G_1, has been shown to associate with a protein kinase (cdk4, cyclin-dependent kinase) that is only 44% identical to the human cdc2 and lacks some of the structural motifs conserved among cdc2 homologs (Matsushime *et al.*, 1992). Like the mcs2-associated kinase, the cdk4−cyclin D complex does not appear to phosphorylate histone H1 *in vitro*. The cdk4−cyclin D complex will, however, phosphorylate a different exogenous substrate, the product of the retinoblastoma gene. In fission yeast, three cyclins have been identified and to date only the product of the *cdc13* gene, a B-type cyclin, has been demonstrated to physically associate with the cdc2 kinase. It is presently unknown whether the other two cyclins, cig1 and puc1, physically associate with the cdc2 kinase or other presently unidentified protein kinases (Bueno *et al.*, 1991; Forsburg and Nurse, 1991).

Finally, we have cloned and sequenced *csk1*, an efficient suppressor of the *mcs2-75* mutation. *csk1* encodes a protein kinase that is not essential for progression through the *S.pombe* cell cycle. In a strain carrying a deletion of *csk1*, the level of the kinase activity that co-precipitates with mcs2 is reduced 3-fold, despite the fact that this strain contains approximately wild-type levels of mcs2. This fact suggests that *csk1* encodes either a kinase that physically associates with mcs2 or an upstream activator of the kinase(s) that physically associates with mcs2. If *csk1* does encode a kinase that physically associates with mcs2, redundant kinases must exist to account for the remaining activity that can be co-precipitated with mcs2 from a strain carrying a null allele of *csk1*. An identification of the gene(s) encoding the mcs2-associated kinase will be essential for further understanding of the role of *mcs2* in the *S.pombe* cell cycle.

Materials and methods

S.pombe strains and genetic techniques

Cultures of *S.pombe* were grown to mid-log ($0.5-2.0 \times 10^7$ cells/ml) in complex media (YEA: 3% glucose, 0.5% yeast extract, 75 μg/ml adenine) or the modified minimal media EMM2 (Nurse, 1975). *S.pombe* genetic nomenclature (Kohli, 1987) and techniques (Gutz *et al.*, 1974) were performed as previously described. *S.pombe* mutations *mcs2-75* (Molz *et al.*, 1989), *cdc25-22* and *cdc2-56* (Nurse *et al.*, 1976), *cdc2-1w*, *wee1-50* and *cdc2-L7* (Nurse and Thuriaux, 1980) and *cdc2-3w* (Fantes, 1981) have been previously described.

Cloning and sequencing of mcs2 and csk1

Standard procedures for a molecular genetic analysis of *S.pombe* were followed (Moreno *et al.*, 1991). *mcs2* and *csk1* were cloned from a gene bank of fission yeast DNA carried in the *S.pombe/Escherichia coli* shuttle vector pDB248× (Beach *et al.*, 1982) by complementation of the temperature-sensitive defect in the strain SP755 (*h*$^{-s}$ *mcs2-75 cdc2-3w cdc25-22 leu1-32*). The plasmids recovered were designated pmcs2a and pmcs2b, respectively. Standard procedures for cloning were performed according to Sambrook *et al.* (1989). A 3.2 kb *Hin*dIII fragment from pmcs2b and a 2.2 kb *Eco*RI fragment from pmcs2b were determined to complement SP755 (see below).

We constructed plasmids used for integration mapping. The complementing fragments from pmcs2a and pmcs2b were cloned into unique *Hin*dIII or *Eco*RI sites in the integrating vector puc19-SU4 (Booher and Beach, 1988). The *Hin*dIII fragment isolated from pmcs2b was also cloned in both orientations into the *Hin*dIII site of puc119 and the *Eco*RI fragment isolated from pmcs2a was cloned in both orientations into puc118. Unidirectional deletions were made in these plasmids using the method of Henikoff (1987) and sequenced by the method of Sanger *et al.* (1977) using Sequenase (United States Biochemical Corp.). The *csk1* gene did not produce single stranded phage from the non-coding strand. Therefore, the non-coding strand of *csk1* was sequenced using a plasmid template and oligonucleotides spanning the 2.2 kb *Eco*RI fragment.

Analysis of the DNA sequence revealed that both genes contained putative introns. An *mcs2* cDNA was synthesized from total cellular RNA and amplified by PCR as described in DeVoti *et al.* (1991). A *csk1* cDNA was recovered from an *S.pombe* cDNA library by hybridization with a probe made from *csk1*. This library was a generous gift from L.Guarente and has been previously described (Olesen *et al.*, 1991). Strains carrying *mcs2* or *csk1* gene replacements were constructed using a one step gene replacement procedure (Rothstein, 1983). Construction of plasmids in which either the *mcs2* or *csk1* coding regions were replaced with a selectable marker was described in Results. Southern blotting was used to identify transformants harboring simple gene replacements.

Construction of overproducing plasmids

Wild-type and epitope-tagged *mcs2* and *csk1* gene products were overexpressed using the constitutive *ADH* promoter in the replicating vector, pART3 (McLeod and Beach, 1987). An *Nde*I−*Bam*HI fragment carrying the *mcs2* cDNA was cloned into the *Nde*I and *Bam*HI sites in pART3, resulting in a plasmid designated pmcs2-ART3. An *Nde*I−*Sac*I fragment carrying the *csk*I cDNA was cloned into the *Nde*I and *Sac*I sites in pART3, resulting in the plasmid designated pcsk1-ART3.

We constructed plasmids which overproduced the epitope-tagged *mcs2* gene product in the pART3 expression vector. A 378 base pair *Bsa*BI−*Nae*I fragment carrying the carboxyl-terminus of the tagged *mcs2* gene was ligated into the *Bsa*BI and *Sma*I sites of pmcs2-ART3.

Bacterial expression and protein purification for immunization of rabbits

The *mcs2* gene product was expressed in *E.coli* using a T7 promoter expression system (Studier *et al.*, 1990). An *Nde*I site was created at the initiating methionine of the *mcs2* using oligonucleotide-directed mutagenesis. An *Nde*I−*Hin*dIII fragment carrying *mcs2* was cloned into the *Nde*I−*Hin*dIII sites of pAR3038 (described in McLeod and Beach, 1986). This plasmid was used to produce mcs2 in the *E.coli* strain BL21(DE3)LysS as previously described by Studier *et al.* (1990). The soluble and insoluble fractions were prepared as previously described by Booher *et al.* (1989) and mcs2 was found to be insoluble. The pellet of insoluble material containing mcs2 was resuspended in a solution of 8.0 M urea, 25 mM Tris pH 8.0, 1 mM DTT and 0.05% TritonX-100, incubated overnight at 37°C, and then centrifuged at 100 000 *g*. mcs2 was the major protein present in the pellet. The pellet was washed three times in a solution of 25 mM Tris pH 8.0, 2 mM EDTA, and 1 mM DTT. The purified mcs2 protein (100−200 μg) was mixed with complete Freund's adjuvant and injected subcutaneously into two New Zealand white rabbits. The resulting antisera was affinity purified as previously described by Jessus and Beach (1992).

Epitope addition

mcs2 was tagged at the carboxyl-terminus with a triple tandem HA1 epitope or a triple tandem HA1 epitope adjacent to a factor X_a protease cleavage site. The plasmids pGTEP1 and pGTFX_a contain these epitopes on *Not*I cassettes (Tyers *et al.*, 1992) and therefore site-directed oligonucleotide mutagenesis was used to introduce a *Not*I site at the carboxyl-terminus of *mcs2*. Linear DNA fragments carrying the tagged genes were transformed into the diploid SP954 (*h*^{90}/*h*$^{+N}$ *mcs2/mcs2::ura4 ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-216/ade6-210*) and 5′fluoroorotic acid was used to select diploids that had replaced the *mcs2::ura4* locus with the tagged *mcs2* genes. Southern blotting was used to identify transformants harboring simple gene replacements. A monoclonal antibody that recognizes the HA1 epitope (12CA5) and the HA1 peptide was purchased from the Berkeley Antibody Company.

Preparation of S.pombe extracts and immunochemical assays

Standard immunochemical procedures were performed as described in standard manuals (e.g. Harlow and Lane, 1988). Lysates were prepared using cold buffer H, without detergents, as previously described by Booher *et al.* (1989). mcs2 was immunoprecipitated by incubating 1.0 mg of protein with 1.0 μl of the anti-HA1 monoclonal antibody. Immunoprecipitations were diluted to a 500 μl volume in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholic acid, 0.1% SDS) and incubated for 2.0 h at 4°C. Immune complexes were then isolated, as previously described by Booher *et al.* (1989). cdc2 was precipitated from lysates using p13−Sepharose (Dunphy *et al.*, 1988), G8 serum or anti-PSTAIRE serum. For immunoblotting, proteins were resolved using SDS−

PAGE, transferred to nitrocellulose using a semi-dry transfer apparatus (Millipore) and then processed using an enhanced chemiluminescence kit (Amersham).

Kinase assays

Immunoprecipitates were prepared as described above and cdc2 kinase assays were performed as described previously. Following three washes in RIPA buffer, immunoprecipitates were washed three times in mcs2 kinase assay buffer (50 mM HEPES pH 7.5, 2.5 mM $MnCl_2$, 1 mM DTT) and resuspended in 35 μl of mcs2 kinase assay buffer. ATP and substrates were then added in a small volume to final concentrations of 100 μM ATP, 1.25 μC/μl [α-^{32}P]ATP (NEN) and 0.125 μg/μl myelin basic protein. Kinase assays were incubated for 4 min at room temperature, stopped by the addition of 20 μl of Laemmli sample buffer, boiled for 10 min and phosphorylated substrates were resolved using SDS–PAGE (Laemmli, 1970).

Immunofluorescence microscopy

Immunofluorescence microscopy was performed in a similar manner as described in Hagan and Hyams (1988) except that the cell wall was removed by incubating cells for 0.5 h at 37°C in 5.0 ml of a solution of 1.2 M sorbitol, 50 mM Na_2HPO_4, 40 mM sodium citrate, 40 mM EDTA, 0.025 mg/ml novozyme and 0.75 μg/ml zymolase. Cells were incubated with anti-*mcs2* polyclonal serum, diluted 1/10.

Acknowledgements

We wish to thank Harriet Feilotter for providing p13–Sepharose, Nick Tonks for providing myelin basic protein and the Futcher lab for providing the epitope addition reagents. We are especially grateful to Jim Bischoff and Catherine Jessus for insightful discussions, as well as Robert Derby for technical assistance with immunofluorescence. We thank Maureen Caliguiri, Jim Bischoff, Gregg Hannon and Karen Lundgren for critical reading of the manuscript and Tim Connolly, Susan Allan and Nancy Kaplan for encouragement. We are grateful to Jim Duffy and Phil Renna for art and photography services. This work was supported by NIH grant GM34607 to D.B. as an investigator of the Howard Hughes Medical Institute.

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Received on November 9, 1992; revised on December 31, 1992