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A Role for the START Gene–specific Transcription Factor Complex in the Inactivation of Cyclin B and Cut2 Destruction

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Hyperactivation of Cdc2 in fission yeast causes cells to undergo a lethal premature mitosis called mitotic catastrophe. This phenotype is observed in *cdc2-3w wee1-50* cells at high temperature. Eleven of 17 mutants that suppress this phenotype define a single complementation group, *mcs1*. The *mcs1-77* mutant also suppresses lethal inactivation of the Wee1 and Mik1 tyrosine kinases and thus delays mitosis independently of Cdc2 tyrosine phosphorylation. We have cloned *mcs1* by isolating suppressors of the cell cycle arrest phenotype of *mcs1-77 cdc25-22* cells and found that it encodes Res2, a component of the START gene–specific transcription factor complex MBF (also known as DSC-1). The *mcs1-77* mutant bears a single point mutation in the DNA-binding domain of Res2 that causes glycine 68 to be replaced by a serine residue. Importantly, two substrates of the anaphase-promoting complex (APC), the major B-type cyclin, Cdc13, and the anaphase inhibitor, Cut2, are unstable in G2-phase *mcs1-77* cells. Consistent with this, we observe abnormal sister chromatid separation in *mcs1-77 cdc25-22* cells at the restrictive temperature. Mutation of either Cdc10 or Res1 also deregulates MBF-dependent transcription and causes a G2 delay. We find that this cell cycle delay is abolished in the absence of the APC regulator Ste9/Srw1 and that the periodic expression of Ste9/Srw1 is controlled by the MBF complex. These data suggest that in fission yeast the MBF complex plays a key role in the inactivation of cyclin B and Cut2 destruction by controlling the periodic production of APC regulators.

INTRODUCTION

In eukaryotic cells, both the onset of DNA replication (S phase) and the initiation of mitosis (M phase) are triggered by members of the Cdk family in association with a regulatory cyclin subunit. Considerable efforts have been made in the past 10 years to understand exactly how Cdk/cyclin complexes are regulated. In the fission yeast, both major transitions are catalyzed by a single gene product, Cdc2 (Nurse and Bissett, 1981). Although Cdc2 associates with four distinct cyclins encoded by the *cdc13*, *cig1*, *cig2*, and *puc1* genes, only one of these, Cdc13, is indispensable for cell cycle progression and is sufficient to trigger both S phase and the initiation of mitosis in the absence of the other cyclins (reviewed by Fisher and Nurse, 1995; Stern and Nurse, 1996). Productive complex formation with the Cdc2 kinase requires phosphorylation of the catalytic subunit on a conserved threonine residue (Gould *et al.*, 1991). The activity of the Cdc2/Cdc13 complex periodically oscillates through the cell cycle, peaking in M phase, and is maintained in an inactive state in G1 by both direct binding of a Cdk inhibitor, Rum1, and proteolytic degradation of the cyclin subunit (Moreno *et al.*, 1989; Correa-Bordes and Nurse, 1995; Kominani *et al.*, 1998). Ubiquitination and subsequent degradation of cyclin B is initiated by a specialized multisubunit ubiquitin ligase complex called the anaphase-promoting complex (APC) (reviewed by Page and Hieter, 1999; Zachariae and Nasmyth, 1999). The APC responsible for cyclin B degradation is found in association with an adaptor protein called Ste9/Srw1 (a homologue of CDH1/Fizzy-related) (Yamaguchi *et al.*, 1997; Fang *et al.*, 1998; Kitamura *et al.*, 1998; Kominani *et al.*, 1998). The APC is also responsible for the degradation of Cut2, an inhibitor of the metaphase-to-anaphase transition, but in this case APC associates with a distinct adaptor protein called Slp1 (a homologue of CDC20/Fizzy) (Funabiki *et al.*, 1996; Matsumoto, 1997). The activity of the APC itself is cell cycle regulated in that ubiquitination and proteolysis of cyclin B occur only in mitosis and G1 (Amon *et al.*, 1994; Brandeis and Hunt, 1996; Iringer and Nasmyth, 1997). On passage through START, a key regulatory event in late G1, APC-mediated degradation is inhibited and the Cdc2/Cdc13 cyclin complex accumulates, increasing to a peak in late G2. The mechanisms that trigger cessation of APC-mediated degradation, however, are unknown.

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Although Cdc2 is required for the initiation of S phase, the activity of the complex is restrained in S phase and G2 by two functionally overlapping kinases, Wee1 and Mik1, which maintain an inhibitory phosphorylation of Cdc2 on a conserved tyrosine residue (Russell and Nurse, 1987; Lundgren *et al.*, 1991). At the initiation of mitosis, the Cdc2/Cdc13 complex is fully activated by tyrosine dephosphorylation of this residue by the Cdc25 phosphatase (Millar *et al.*, 1991). In addition to being required to induce S phase and mitosis, the presence or activity of the Cdc2/Cdc13 complex is believed to play an additional role in preventing the reinitiation of S phase during G2, because either deletion of *cdc13* or overexpression of the *rum1* Cdk inhibitor induces cells to undergo rereplication in the absence of an intervening mitosis (Hayles *et al.*, 1994; Correa-Bordes and Nurse, 1995). Conversely, hyperactivation of Cdc2/Cdc13 kinase induces premature entry into mitosis (or mitotic catastrophe), which results in cell death (Russell and Nurse, 1987). Together, these results have led researchers to propose a quantitative model to explain how the Cdc2/Cdc13 complex can trigger both S phase and mitosis in the correct sequential cell cycle order (Stern and Nurse, 1996).

Although this model may be relevant to the rapid early divisions of metazoan embryonic cells, it fails to explain how cell cycle–regulated transcription is coordinated with periodic alteration in Cdk/cyclin activity. In particular, passage through START requires the function of an essential transcription factor complex called MBF, also known as DSC-1, which controls the expression of a number of genes critical for S-phase initiation (Lowndes *et al.*, 1991, 1992). In fission yeast, the MBF complex, which contains the Cdc10 (Lowndes *et al.*, 1992), Res1 (Tanaka *et al.*,1992; Caligiuri and Beach, 1993), Res2 (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Ayté *et al.*, 1997; Zhu *et al.*, 1997), and Rep2 (Nakashima *et al.*, 1995) proteins, binds to *MluI* cell cycle box elements in the promoters of a small number of periodically expressed genes, which so far include *cdc18*, *cdc22*, *cdt1*, *cdt2*, and *cig2* (Caligiuri and Beach, 1993; Kelly *et al.*, 1993; Hofmann and Beach, 1994; Obara-Ishihara and Okayama, 1994; Zhu *et al.*, 1994). Of these, the *cdc18* gene appears to be a critical target because temperature-sensitive mutations of *cdc10* can be rescued by ectopic expression of *cdc18* (Kelly *et al.*, 1993). Not only is Cdc18 critical for S-phase initiation, but high-level expression can induce continuous DNA synthesis in the absence of an intervening mitosis (Nishitani and Nurse, 1995; Greenwood *et al.*, 1998). Thus, accurate periodic expression of *cdc18* is thought to be important for ensuring that cells initiate S phase only once per cell cycle (Nishitani and Nurse, 1995; Baum *et al.*, 1998). Although MBF was initially thought to be the transcriptionally active complex, recent analysis has indicated that it can be isolated by electrophoretic mobility shift assay only in G2, when periodic transcription of target genes is repressed (Reymond *et al.*, 1993; McInerny *et al.* 1995; Baum *et al.* 1997). The MBF complex disappears at the onset of mitosis and reappears during S phase of the next cell cycle, suggesting that it is directly or indirectly under the control of Cdk/cyclin activity (Reymond *et al.*, 1993; Baum *et al.* 1997). Paradoxically, however, the induction, maintenance, and repression of target genes appear not to require the Cdc2 kinase (Baum *et al.*, 1997). Thus, how cell cycle regulation of the MBF complex is coordinated with periodic activity of Cdk/cyclin complexes and the APC remains unclear.

To determine the mechanisms governing Cdc2/Cdc13 activity, we have focused on a number of mutants that display potent genetic interactions with *cdc2* and thus may encode novel regulators of the Cdc2/Cdc13 complex. In particular, *cdc2-3w wee1-50* cells, which express a dominantly active Cdc2 kinase and a temperature-sensitive Wee1 kinase, undergo premature and lethal mitotic initiation (Russell and Nurse, 1987). This phenotype is suppressed by mutations in one of six mitotic catastrophe suppressors (*mcs1–mcs6)* (Molz *et al.*, 1989). We and others have recently shown that the *mcs2* and *mcs6* genes code for a cyclin H–like molecule and its Cdk, respectively, which interact to form fission yeast Cdc2-activating kinase (Buck *et al.*, 1995; Damagnez *et al.*, 1995). In this paper, we demonstrate that *mcs1-77* bears a point mutation in the Res2 transcription factor, a component of the MBF complex. Mutations in Res2 and other components of the MBF complex cause a G2 delay that is exacerbated in cells bearing a partially defective Cdc25 phosphatase. We show that the Cdc13 cyclin B and the anaphase regulator Cut2 are unstable in *mcs1-77* cells. These results suggest that inactivation of the START gene–specific transcription factor complex is required for the timely inactivation of APC-mediated destruction of mitotic targets. We show that, at least in the case of Cdc13 cyclin B, this instability is due to ectopic expression of the APC regulator Ste9/Srw1, a previously unrecognized transcriptional target of the MBF complex.

MATERIALS AND METHODS

Media and General Techniques

Media and genetic methods for studying fission yeast have been reviewed (Moreno *et al.*, 1991). General DNA methods used standard techniques (Sambrook *et al.*, 1989). Cell length measurements were made with the use of log-phase cells with a Nikon (Garden City, NY) filar eyepiece drum micrometer at 1200× magnification. Transformations were regularly performed by means of the lithium acetate method (Moreno *et al.*, 1991) or by electroporation (Prentice, 1991) with the use of a Bio-Rad (Richmond, CA) Gene Pulser.

Analysis of DNA Content by Flow Cytometry

Samples containing ~10^6 cells were fixed with 70% ethanol, treated successively with RNase and pepsin, and stained with 50 mg/ml propidium iodide essentially as described previously (Corliss and White, 1981). DNA content was then analyzed with a Becton Dickinson (Franklin Lakes, NJ) FACScan and CELL Quest software (Becton Dickenson, Oxford, United Kingdom).

Isolation and Transposon Mutagenesis of the res2 Genomic Clone

A genomic library, pURB1 (Barbet *et al.*, 1992), was introduced into a *mcs1-77 cdc25-22 ura4-D18 h*$^-$ strain by lithium acetate transformation and plated on medium lacking uracil. A total of 60,000 transformants were screened. Transformants were replica plated twice to 30°C, and 89 complementing colonies were identified. DNA from these colonies was isolated by transformation into *Escherichia coli*. Plasmids encoding *cdc25* and *nim1* were identified by a combination of restriction mapping, PCR, and Southern blot analysis. The 42 remaining plasmids were found to be related by restriction mapping. The gene responsible for suppression was identified by transposon mutagenesis of the smallest genomic clone, pURB1-

mcs1, with the use of TnHIS3, as described previously (Sedgwick and Morgan, 1994). Complementing activity was tested by transforming transposed DNA into a *mcs1-77 cdc25-22 ura4-D18* strain that was assayed for growth at 30°C. Automated sequencing was performed with the Applied Biosystems (Foster City, CA) cycle sequencing kit with the use of previously described primers to the 5′ and 3′ ends of the transposon.

Sequencing of the mcs1-77 Mutation

The *res2* ORF was amplified from *mcs1-77* cells by PCR with the use of an Expand high-fidelity polymerase (Boehringer Mannheim, Indianapolis, IN) and cloned into pCRII (Invitrogen, Carlsbad, CA). Two independent PCR products were sequenced in both directions with the Applied Biosystems cycle sequencing kit with the use of T3 and T7 primers.

RNA Isolation and Hybridization

To isolate RNA, *Schizosaccharomyces pombe* cells were cultured in YEPD to exponential phase. Approximately 10 μg of total RNA was isolated and resolved by agarose gel electrophoresis before transfer to nitrocellulose for hybridization. Probing with [^{32}P]dCTP-labeled DNA was as described previously (Zhu *et al.*, 1997). A fragment of the *ste9/srw1* ORF was amplified by PCR with the 5′ oligonucleotide CTTAGTAGCCCTTTTATCAAAT and the 3′ oligonucleotide GATTCGCGACATCGCAAAA for use as probes. Similarly, a probe for *cdc18* was generated by PCR with the use of the 5′ oligonucleotide ATGGATGAATTTGATGGTTT and the 3′ oligonucleotide TTACCGTATTTTCATTGTACG. Probes for *cdc2* and *his3* were as described previously (Zhu *et al.*, 1997)

Electrophoretic Mobility Shift Assay

Cells were grown to midlog phase, harvested, washed with 1 ml of H_2O, resuspended in 40 μl of lysis buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 150 mM KCl, 0.1% Triton X-100, 25% glycerol, 1 M urea, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM EGTA, 1 mM tetrasodium pyrophosphate, 100 μM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate), and lysed as described above. Lysate (30 μg) was incubated in 20 μl of binding buffer containing 25 mM HEPES, pH 7.6, 34 mM KCl, 5 mM $MgCl_2$, and 2 μg of poly dIdC for 10 min at room temperature, and then for 20 min with 1 ng of [^{32}P]dNTP-labeled probe. Reactions were run on a 4% acrylamide gel in 0.5 M TBE (100 mM tris-borate, 2 mM EDTA), dried, and exposed for autoradiography.

Western Blot Analysis

Western blot analysis of cell extracts was performed as described previously (Buck *et al.*, 1995). Affinity-purified polyclonal antisera to Res2 were used at a 1:500 dilution and visualized with the use of anti-rabbit conjugated HRP. Autoradiographs were scanned with the use of a Molecular Dynamics Personal Densitometer (Amersham, Arlington Heights, IL). Anti-cyclin B polyclonal antibody (Alfa *et al.*, 1990) was used at a 1:1000 dilution and visualized with the use of anti-rabbit conjugated HRP. Anti-hemagglutinin 12CA5 antibody (BAbCO, Richmond, CA) was used at a 1:1000 dilution and visualized with the use of anti-mouse conjugated HRP.

RESULTS

Mcs1-77 Mutant Bypasses the Requirement for Cdc2 Tyrosine Phosphorylation

The fission yeast strain *cdc2-3w wee1-50* is viable at low temperature but undergoes a lethal premature entry into mitosis at the restrictive temperature for *wee1-50* (Russell and Nurse, 1987) (Figure 1). Seventeen mitotic catastrophe suppressors that were able to grow at the restrictive temperature of 37°C were identified (Molz *et al.*, 1989). Eleven of these were found to reside in a single complementation group, *mcs1*. We have characterized a mutant from this group, *mcs1-77* (Table 1). In addition to the Wee1 kinase, Cdc2 is phosphorylated on tyrosine 15 by the action of the Mik1 tyrosine kinase. Cells lacking both *wee1* and *mik1* also undergo a premature mitotic catastrophe (Lundgren *et al.*, 1991). To determine whether *mcs1-77* cells could proliferate in the complete absence of the Wee1 and Mik1 kinases, a *wee1-50* Δ*mik1 mcs1-77* strain was constructed and incubated at both the permissive and restrictive temperatures for *wee1-50*. Whereas a *wee1-50* Δ*mik1* strain underwent mitotic catastrophe at 34°C, a *wee1-50* Δ*mik1 mcs1-77* strain was still able to form colonies, although growth was poor (Figure 1). These results indicate that *mcs1-77* exerts a cell cycle delay in G2 that is independent of the tyrosine phosphorylation state of Cdc2.

Mcs1 Is Required for the G1-to-S Transition and Meiotic Progression

The *mcs1-77* allele was characterized further. After prolonged incubation at low temperature (19°C), *mcs1-77* cells elongate and undergo cell cycle arrest (Molz *et al.*, 1989). FACS analysis revealed that after 7 h of incubation at 19°C, a large proportion of *mcs1-77* cells delay in G1 before eventually arresting in G2 (Figure 2A). This suggests that Mcs1, like Cdc2, may be required for both the transition through START and the initiation of mitosis. However, cells did not arrest uniformly with a single nucleus but rather with abnormal chromatin structures, suggesting a chromosome segregation defect (Figure 2B). This phenotype is strikingly similar to that observed in cells that bypass the *cdc10* START gene (Marks *et al.*, 1992). We also found that *mcs1-77* cells displayed a profound meiotic defect that cosegregated with the cold-sensitive *cdc−* phenotype. In self-crosses, wild-type cells give rise to >94% four-spored asci, whereas *mcs1-77* cells produced asci with aberrant numbers of spores (Table 2). These result indicate that *mcs1* not only controls the mitotic cell cycle but also is required for meiotic progression.

mcs1-77 Contains a Point Mutation in the DNA-binding Domain of Res2

Consistent with a role for *mcs1* in controlling the G2/M transition, the *mcs1-77* mutant forms a conditional lethal genetic interaction with *cdc25-22* that encodes a temperature-sensitive version of the Cdc25 phosphatase (Molz *et al.*, 1989). Although *mcs1-77 cdc25-22* cells can proliferate at 28°C (Figure 3A), they undergo cell cycle arrest at the intermediate temperature of 31°C, whereas *mcs1-77* and *cdc25-22* single mutants are still able to form colonies. At this temperature, the *mcs1-77 cdc25-22* double mutants arrest as highly elongated cells, indicating G2 arrest (Figure 3). We made use of the observation that *mcs1-77 cdc25-22* double mutant cells arrest at 31°C to clone the *mcs1* gene. A genomic library was introduced into *mcs1-77 cdc25-22 ura4-D18* cells, and from a total of 60,000 transformants growing at 28°C, 89 plasmid-dependent colonies were isolated at 31°C. Forty-seven of these suppressors represented two known genes, *cdc25* (36 clones) and *nim1/cdr1* (11 clones). The restriction maps of the remaining 42 suppressors were found to be

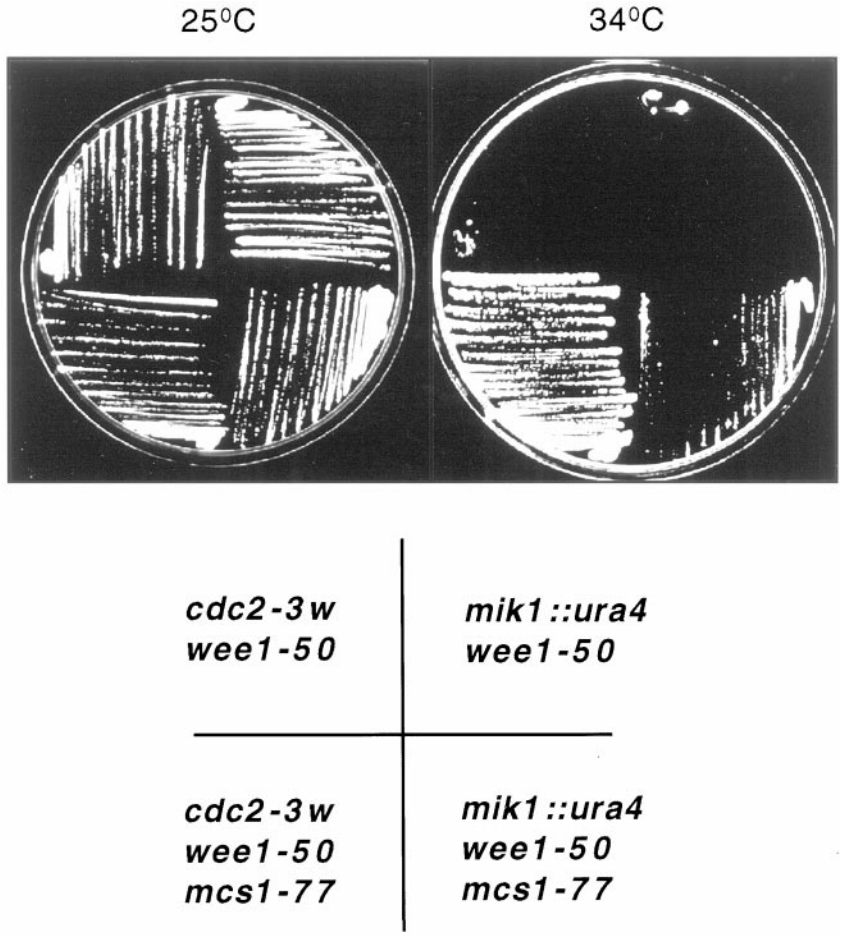

Figure 1. *mcs1-77* cells bypass the requirement for the Wee1 and Mik1 tyrosine kinases. *cdc2-3w wee1-50*, *cdc2-3w wee1-50 mcs1-77*, *mik1::ura4 wee1-50*, and *mik1::ura4 wee1-50 mcs1-77* cells were grown on YEPD at 25°C and then streaked on the same medium at either 25°C (left) or 34°C (right), and growth of the colonies was examined after 4 d at these temperatures.

related. To localize the region responsible for suppression of the *mcs1-77 cdc25-22* cell cycle arrest, one of these clones (pURB1-mcs1) was subjected to transposon mutagenesis (Sedgwick and Morgan, 1994). Surprisingly, noncomplementing insertions were found to reside in a known gene, *res2*, that encodes a component of the START gene–specific transcription factor complex MBF (Figure 4A). Notably, two transpositional insertions in *res2* that remove the C-terminal 67 and 151 amino acids were still able to rescue *mcs1-77 cdc25-22* cells at the restrictive temperature. Because the C-terminal domain of Res2 is required for interaction with Cdc10 and to confer periodic transcriptional regulation to the MBF complex, this suppression is due to heteroallelic complementation, suggesting that the MBF complex contains more than one molecule of Res2 (Zhu *et al.*, 1997).

Cells deleted for *res2* undergo a cold-sensitive cell cycle arrest in G1 and are defective in premeiotic DNA synthesis, which results in aberrant spore formation (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Ayté *et al.*, 1997; Zhu *et al.*, 1997). This persuaded us to determine the relationship between the *mcs1-77* mutation and the *res2* genomic clone. To achieve this, *mcs1-77* cells were crossed to *res2::ura4* mutants, and the progeny were scored for cold-sensitive cell cycle arrest. Because this cross also gave rise to inviable asci, it was performed with cells expressing *res2* from an episomal plasmid. The progeny from >2000 spores plated were all found to undergo a cold-sensitive cell cycle arrest, indicating that *mcs1-77* and *res2* are allelic. To localize the mutation in *res2*, genomic DNA from *mcs1-77* cells was amplified by PCR, and the product was sequenced. *mcs1-77* cells were found to contain a single point mutation in the DNA-binding domain of *res2*, resulting in the mutation of glycine 68 to a serine residue (Figure 4B) (Xu *et al.*, 1994). These results indicate that Res2, which has been implicated as a repressor of Cdc10-dependent gene transcription in G2, plays an unexpected additional role in controlling the timing of mitotic initiation.

Mcs1-77 Cells Are Partially Defective in Mitotic Gene Transcription

Our previous analysis suggested that *mcs1-77* cells are phenotypically indistinguishable from Δ*res2* cells. To discover how a component of the START gene–specific transcription factor complex can be implicated in the G2/M transition, we characterized the *mcs1-77* mutation in more detail. Consistent with the nature of the *mcs1-77* mutation, we observed that antibodies to Res2 were able to detect a full-length protein in Western blot analysis of cell extracts from *mcs1-77* but not Δ*res2* cells (Figure 5A). However, using a fragment of the *cdc22* promoter in electrophoretic mobility shift assays

Table 1. Strains used in this study

Strain	Genotype	Reference/source
PR 109	*h*$^-$	P. Russell
SP 820	*mcs1-77 ura4*$^+$ *h*$^+$	Molz *et al.* (1989)
JM 1344	*mcs1-77 his7-366 h*$^-$	This study
JM 1346	*mcs1-77 ade6-M216 h*$^+$	This study
NT 9	*cdc10-129 h*$^-$	N. Jones
Y 337	*cdc10-c4 h*$^-$	L. Johnston
PN 1359	*res1*::*ura4 ade6-M210 h*$^-$	P. Nurse
NT 38	*res2*::*ura4 ade6-M210 h*$^-$	N. Jones
PN 1404	*rep2*::*ura4 h*$^-$	P. Nurse
JM 1272	*rad3*::*ura4 ade6-704 h*$^-$	A. Carr
JM 2059	*ste9*::*ura4 h*	T. Toda
PR 12	*cdc2-3w wee1-50 h*$^-$	P. Russell
SP 763	*cdc2-3w wee1-50 mcs1-77 h*$^-$	Molz *et al.* (1989)
PR 337	*mik1*::*ura4 wee1-50 h*$^-$	P. Russell
JM 2038	*mik1*::*ura4 wee1-50 mcs1-77*	This study
JM 1351	*cdc25-22 ade6-M210 his7-366 h*$^+$	Shieh *et al.* (1997)
JM 1518	*mcs1-77 cdc25-22*	This study
JM 1604	*mcs1-77 cdc25-22 rad3*::*ura4*	This study
JM 2060	*mcs1-77 cdc25-22 ste9*::*ura4*	This study
HF 178	*cut2-364*::*cut2*$^+$*-HA-LEU2 ura4*$^+$ *h*$^-$	M. Yanagida
JM 1603	*mcs1-77 cdc25-22 cut2-364*::*cut2*$^+$*-HA-LEU2*	This study

All strains were *leu1-32 ura4-D18* unless indicated otherwise.

with extracts of either *mcs1-77* or Δ*res2* cells, we were unable to detect the MBF complex (Figure 5B). To examine expression of the MBF target gene *cdc18*, mRNA was extracted from cells that were synchronized in S phase by incubating in the presence of hydroxyurea and then released. Under these conditions, expression of *cdc18* in Δ*res2* cells was found to be invariant through the cell cycle compared with wild type, whereas in Δ*rep2* cells expression was substantially decreased and periodic, as observed previously (Figure 5C) (Baum *et al.*, 1997). Under the same conditions, expression of *cdc18* in *mcs1-77* cells was seen to fluctuate, although the maximal levels and rates of accumulation were decreased significantly with respect to wild type (Figure 5C). Thus, *mcs1-77* is not a null allele of the *res2* gene.

Cells Lacking the MBF Complex Are Delayed in G2

The MBF transcription factor complex, which contains the Res2, Cdc10, Res1, and Rep2 proteins, can be isolated from fission yeast cell lysates only when S phase transcription is repressed in G2 (Baum *et al.* 1997; Zhu *et al.*, 1997). Mutations in genes that prevent the appearance of this complex, however, have different effects on cell cycle–regulated transcription of target genes. For example, deletion of *res2* or removal of the C-terminal 61 residues in the Cdc10 protein (*cdc10-c4*) causes high constitutive expression of target genes, whereas deletion of *res1* causes low constitutive expression of target genes (McInerny *et al.*, 1995; Baum *et al.*, 1997). Regardless, Δ*res2*, *cdc10-c4*, and Δ*res1* cells undergo a nonconditional cell cycle arrest in combination with *cdc25-22*, a partially defective form of the Cdc25 phosphatase (Table 3). Conversely, the MBF complex can be isolated from Δ*rep2* cells in which transcription is also periodic but maximal accumulation is greatly diminished (Figure 5C) (Baum *et al.*, 1997). Deletion of *rep2* has no effect on the timing of mitotic initiation alone or in combination with *cdc25-22* (Table 3). These results suggest a strong correlation between deregulation of MBF-dependent transcription and a delay in the timing of mitotic initiation. The reason why inactivating mutations in *res1* were not found in the screen for mitotic catastrophe suppressors in the *cdc2-3w wee1-50* strain is probably that this screen was performed at 37°C, at which temperature *res1*$^-$ cells are inviable (Molz *et al.*, 1989).

Mitotic Delay Is Not Due to Activation of the DNA Damage or Replication Checkpoint

All of the known targets of the MBF complex, including *cdc22*, *cdc18*, *cdt1*, and *cig2*, are important for the initiation of S phase. Aberrant initiation of DNA replication forks causes activation of a DNA replication checkpoint that delays the onset of mitosis. This signal is dependent on the activity of the Rad3 kinase, which is also required to inhibit mitosis when DNA is damaged. To determine whether deregulation of the MBF complex delays mitosis by activating either the DNA damage or DNA replication checkpoint, we examined the effect of deleting *rad3* in *mcs1-77 cdc25-22* cells. The resulting *rad3::ura4 mcs1-77 cdc25-22* triple mutant underwent cell division at the same length as *mcs1-77 cdc25-22* cells at all temperatures, indicating that the mitotic delay observed in *mcs1-77* cells is not due to activation of the DNA damage or replication checkpoint (Table 4). Similar results were obtained when other components of these pathways were deleted, including *chk1* (our unpublished results).

The mcs1-77 Mutant Causes Instability of Cyclin B and Cut2 in G2

Activation of the Cdc2/Cdc13 complex is the rate-limiting factor responsible for M-phase initiation in fission yeast.

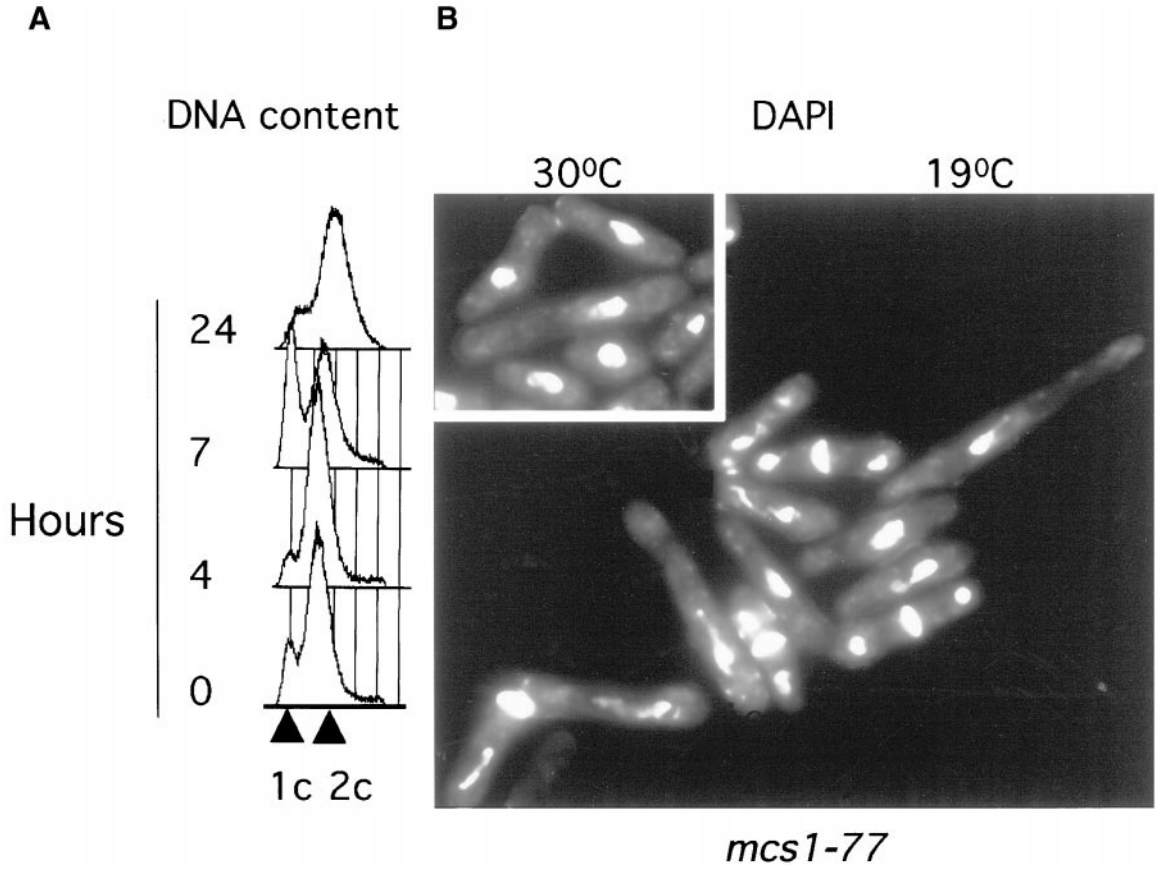

Figure 2. *mcs1-77* cells arrest at low temperature in G1 with abnormal chromatin structures. (A) *mcs1-77* cells transiently arrest in G1. Log-phase cultures of *mcs1–77* cells growing in Edinburgh minimal medium (EMM) medium at 30°C were analyzed for DNA content after shift to 19°C for the times indicated. (B) *mcs1-77* cells cell cycle arrest at low temperature with abnormal chromatin structures. Log-phase cultures of *mcs1-77* cells growing in EMM medium at 30°C were transferred to the same medium at either 30°C (inset) or 19°C for 24 h. After this time, cells were stained with DAPI to visualize nuclei.

Because transcription of *cdc13* or *cdc2* was unaffected in *mcs1-77* cells (our unpublished results), we focused on the Cdc2/Cdc13 protein complex itself. The level of the Cdc13 protein oscillates through the cell cycle, reaching a peak in late G2 (Moreno *et al.*, 1989). This is due in part to the fact that Cdc13 is unstable in M and G1 phase and stabilizes after cells pass through START. To determine whether *mcs1-77* affects either the steady-state level and/or the stability of Cdc13, *cdc25-22* and *mcs1-77 cdc25-22* cells were synchronized in late G2 and cell extracts were probed for the presence of Cdc13. As the results in Figure 6 show, the steady-state level of Cdc13 was decreased significantly in *mcs1-77* cells. To determine whether this is due to a decreased protein synthesis rate or to an increased rate of degradation, cells were incubated in the presence of the protein synthesis inhibitor cycloheximide for various times and the level of Cdc13 was monitored. No change in the level of Cdc13 was observed in *cdc25-22* cells at the restrictive temperature, even after protein synthesis was inhibited for prolonged periods (Figure 6). This confirms that instability of cyclin B is normally confined to the M and G1 phases of the cell cycle. In contrast, we observed a more rapid decrease in the level of Cdc13 in *mcs1-77 cdc25-22* cells under the same conditions (Figure 6). This indicates that cyclin B destruction is not suppressed in G2-phase *mcs1-77* cells.

Because Cdc13 degradation is triggered by the action of the APC, we asked whether other known substrates of the APC were also unstable in *mcs1-77* cells. In particular, the anaphase inhibitor Cut2, like cyclin B, is normally degraded only in the M and G1 phases of the cell cycle. To monitor Cut2 levels, a chromosomal C-terminally hemagglutinin-tagged *cut2* gene was introduced into *cdc25-22* and *mcs1-77 cdc25-22* cells. We found that the steady-state level of Cut2

Table 2. Effect of *mcs1-77* mutation on ascospore formation

	Spores per ascus (%)				
Strain	0	1	2	3	4
Wild type	0	1.5	2.5	1.5	94.5
mcs1-77	38	17	31	4	13

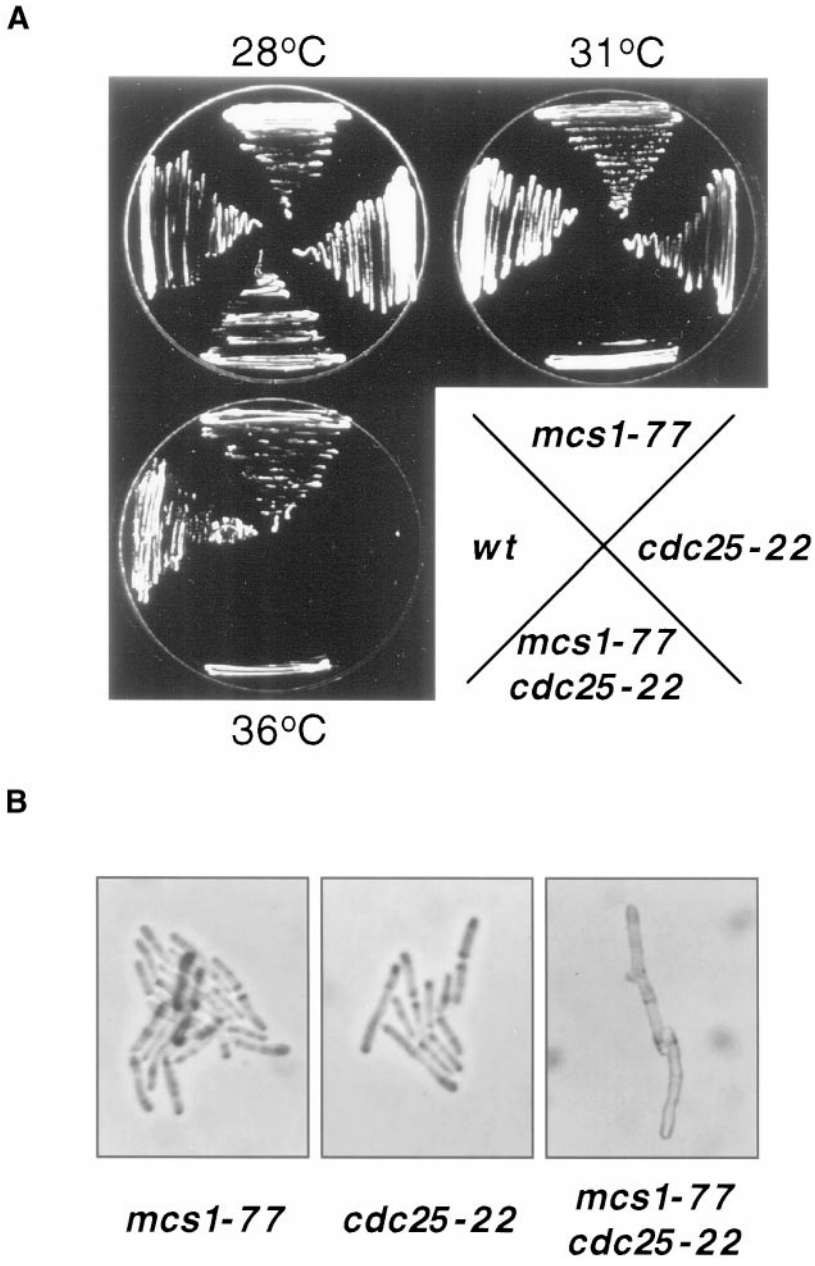

Figure 3. *mcs1* delays the initiation of mitosis. (A) *mcs1-77* is synthetically lethal with *cdc25-22*. Wild-type, *mcs1-77*, *cdc25-22*, and *mcs1-77 cdc25-22* cells were grown on YEPD at 28°C and then streaked onto YEPD plates at 28, 31, or 36°C, and the growth of the strains was monitored after 3 d at these temperatures. (B) *mcs1-77 cdc25-22* arrested cells are highly elongated. *mcs1-77* cells were crossed to *cdc25-22* cells, and the resulting asci were subjected to tetrad dissection on YEPD plates at 31°C. Colonies from a tetratype that were genotypically *mcs1-77* (left), *cdc25-22* (middle), or *mcs1-77 cdc25-22* (right) were visualized after 2 d of growth at 31°C.

was higher in *cdc25-22* cells at the restrictive temperature than in *mcs1-77 cdc25-22* cells (Figure 6A). This can also be accounted for by a dramatically increased rate of protein degradation observed when protein synthesis was blocked in the presence of cycloheximide (Figure 6). These results suggest that APC-mediated degradation is deregulated when the MBF complex is compromised. Destruction of Cut2 during mitosis is required for the separation of sister chromatids into two daughter cells (Funabiki *et al.*, 1996). To determine the effect of Cut2 degradation in vivo, nuclei from either *cdc25-22* or *mcs1-77 cdc25-22* cells were stained with DAPI after shift to the restrictive temperature for 4 h and the number of binucleate cells or cells with abnormal chromatin structures were determined. At the permissive temperature of 25°C, 19% of *cdc25-22* cells were binucleate compared with 38% of *mcs1-77 cdc25-22* cells. At the restrictive temperature, *cdc25-22* cells arrested with a single elongated nucleus, with only 1.5% displaying binucleate or segregated DNA (Figure 6C). In contrast, 47% of *mcs1-77 cdc25-22* cells arrested, with a large number of either binucleate cells or cells showing aberrantly segregated chromosomes, under the same conditions (Figure 6C). These results are consistent with the inability of *mcs1-77* cells to maintain sister chromatid cohesion in the G2 phase of the cell cycle. Together, these data suggest that APC-mediated degradation is ectopically activated in cells lacking the MBF complex.

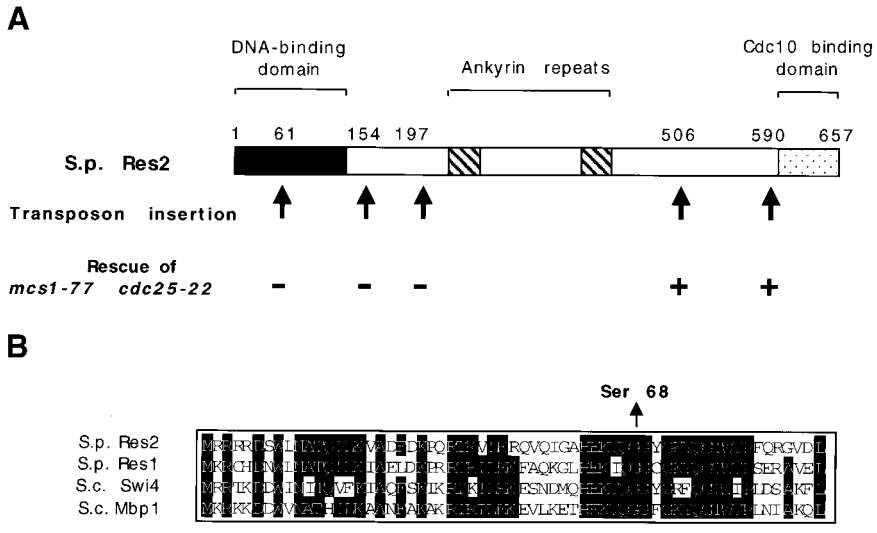

Figure 4. *mcs1-77* encodes a point mutant in the DNA-binding domain of Res2. (A) Localization of inactivating transposon insertions in a genomic clone containing Res2. Simultaneous localization and sequencing of complementing regions was performed by transposon mutagenesis of the pURB1-Res2 genomic clone. The structure of the Res2 protein is shown with the N-terminal DNA-binding domain (black bar), central ankyrin repeats (gray bar), and the C-terminal Cdc10 interaction domain (hatched bar). Arrows indicate the location of transposon insertions, and the ability of the transposed plasmid to rescue *mcs1-77 cdc25-22* cells at 31°C is shown as either rescue (+) or no rescue (−) after 3 d of growth on Edinburgh minimal medium (EMM) plates lacking uracil. (B) Sequence of the DNA-binding domain of Res2 from wild-type and *mcs1-77* cells. Alignment of amino acids 28–89 of *Schizosaccharomyces pombe* (S.p.) Res2 protein with homologous regions of S.p. Res1 and *Saccharomyces cerevisiae* (S.c.) Mbp1 and S.c. Swi4 transcription factors. The arrow indicates the change of glycine 68 to a serine residue in *mcs1-77* cells.

Deletion of Ste9/Srw1 Bypasses the G2 Delay in mcs1-77 Cells

Degradation of both cyclin B and Cut2 by the APC requires association with WD40-domain containing adaptor proteins, which in fission yeast are encoded by the *slp1* (fizzy) and *ste9/srw1* (fizzy-related) genes. We noted that *ste9/srw1* was initially identified as a multicopy suppressor of the mitotic catastrophe phenotype of Δ*mik1 wee1-50* cells at high temperature (Yamaguchi *et al.*, 1997; H. Okayama, personal communication). This prompted us to determine whether the G2 delay in *mcs1-77* cells is due to deregulated activity of APC^{Ste9}, resulting in ectopic degradation of cyclin B. To do this, we examined the effect of deleting *ste9/srw1* in *mcs1-77 cdc25-22* cells. At the permissive temperature, the resulting *ste9::ura4 mcs1-77 cdc25-22* triple mutant underwent cell division at 20.5 ± 0.2 μm compared with 28 ± 0.3 μm for *mcs1-77 cdc25-22* cells and failed to arrest in G2 when incubated at 31°C (Figure 7A). These results indicate that perturbation of the MBF complex causes a G2 delay that is due in part to deregulation of APC^{Ste9}, resulting in ectopic degradation of cyclin B. To analyze this directly, *mcs1-77 cdc25-22* or *ste9::ura4 mcs1-77 cdc25-22* cells were arrested in G2 at high temperature and the stability of Cdc13 was assessed after addition of cycloheximide. Whereas the steady-state level of Cdc13 decreased in *mcs1-77* cells, it was unchanged in *mcs1-77 ste9::ura4* cells during the same period (Figure 7B). It should be noted that 35% of *ste9::ura4 mcs1-77 cdc25-22* cells were either binucleate or displayed some aberrant chromosome structures (our unpublished results), indicating that Ste9/Srw1 is not the sole target by which the MBF complex controls APC activity.

Cell Cycle–regulated Expression of Ste9/Srw1 Is Controlled by the MBF Complex

We postulated that the MBF complex could control APC^{Ste9} activity by directly regulating the transcription of the *ste9/srw1* gene. We noted that the promoter region of *ste9/srw1* contains two elements that correspond to the consensus core sequence (A/TCGCGA/T) recognized by the MBF complex. To determine whether *ste9/srw1* is cell cycle regulated, a *cdc25-22* strain was synchronized by blocking in G2 at the restrictive temperature of 35.5°C and then releasing to 25°C. Northern blot analysis of total RNA isolated from this culture at various times after release indicated that the mRNA for *ste9/srw1* was strongly cell cycle regulated and peaked just before S phase, which occurs as the division septa are laid down (Figure 8A). Notably, the peak of *ste9/srw1* expression was exactly coincident with that of *cdc18*, a known MBF target (Figure 8A). To determine whether the expression of *ste9/srw1* is regulated by the MBF complex, wild-type cells or cells lacking either Res1 or Res2 were synchronized by blocking in S phase with hydroxyurea and then releasing to G2. Northern blot analysis showed that in wild-type cells the expression of both *ste9/srw1* and *cdc18* was high in S phase but declined as cells entered G2 (Figure 8B). Importantly, no periodicity of either *cdc18* or *ste9/srw1* expression was observed in either Δ*res1* or Δ*res2* cells under the same conditions, strongly indicating that the MBF complex regulates expression of *ste9/srw1* (Figure 8B). Under the same conditions, periodic transcription of *ste9/srw1* was also deregulated in *mcs1-77* cells, but to a lesser extent than Δ*res2* cells (our unpublished results).

DISCUSSION

We have characterized mutants that bypass the lethal hyperactivation of Cdc2 in fission yeast to identify novel mechanisms by which Cdc2/cyclin B activity is controlled. In a previous genetic screen, 11 of 17 mutants that suppress this phenotype were found to reside in a single complementation group, *mcs1* (Molz *et al.*, 1989). We have characterized the *mcs1-77* mutant and found it to contain a point mutation in the DNA-binding domain of Res2, a component of the S

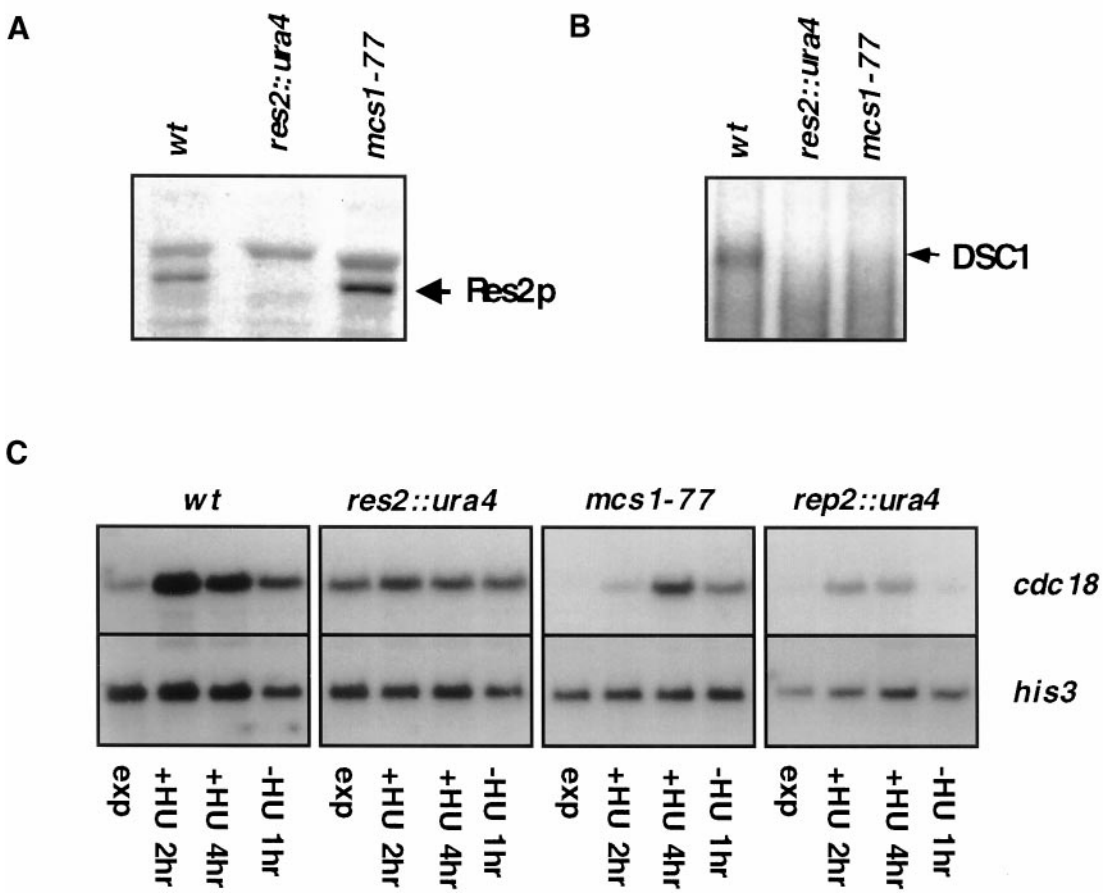

Figure 5. *mcs1-77* cells are partially defective in periodic transcription of *cdc18*. (A) Expression of Res2 in *mcs1-77* cells. Ten micrograms of cell extract prepared from log-phase cultures of wild-type (wt), *res2::ura4*, or *mcs1-77* cells were probed for the presence of Res2 polypeptide with the use of anti-Res2 antiserum after SDS-PAGE and Western blotting. (B) The DSC1 (MBF) complex cannot be isolated from *mcs1-77* cells. Electrophoretic mobility shift assays were performed with the labeled *cdc22* probe with the use of extracts derived from log-phase cultures of wild-type (wt), *res2::ura4*, or *mcs1-77* cells. (C) Expression of *cdc18* mRNA in *mcs1-77* cells. Log-phase cultures growing in YEPD at 30°C of wild-type (wt), *res2::ura4*, *rep2::ura4*, or *mcs1-77* cells were incubated in the same medium containing 2 mM hydroxyurea for the times indicated and then incubated in YEPD for an additional 1 h after extensive washing. Total RNA was extracted, and equal quantities were separated by electrophoresis and probed with the use of DNA specific to the *cdc18* gene. Represented blots were probed with the same probe and for the same length of time. Reprobing was performed with *his3*-specific probes to verify approximately equal loading of RNA.

phase–specific transcription factor complex MBF. The MBF complex, which contains the Cdc10, Res1, Res2, and Rep2 proteins, is responsible for the periodic transcription of a number of genes essential for the onset of S phase. Mutations in other MBF components that prevent the periodic appearance of the complex in G2 also display a synthetic genetic interaction with *cdc25-22*, suggesting that the MBF complex plays a role in determining the timing of mitotic initiation.

We have shown previously that *mcs2* and *mcs6*, mutants of which bypass the mitotic catastrophe phenotype of *cdc2-3w wee1-50*, code for components of a Cdk-activating kinase that controls Cdc2 phosphorylation on threonine 167 (Buck *et al.*,

Table 3. Mutation of MBF components causes a G2 delay

Strain	Wild type	*Cdc25-22*
Wild type	+++	+++
mcs1-77	+++	*cdc*−
res2::*ura4*	+++	*cdc*−
res1::*ura4*	+++	*cdc*−
cdc10-c4	+++	*cdc*−
cdc10-129	+++	*cdc*−
rep2::*ura4*	+++	+++

Table 4. Mitotic delay is not dependent on Rad3 kinase

Strain	28°C	31°C
mcs1-77 cdc25-22	+++	cdc−
mcs1-77 cdc25-22 rad3::*ura4*	+++	cdc−

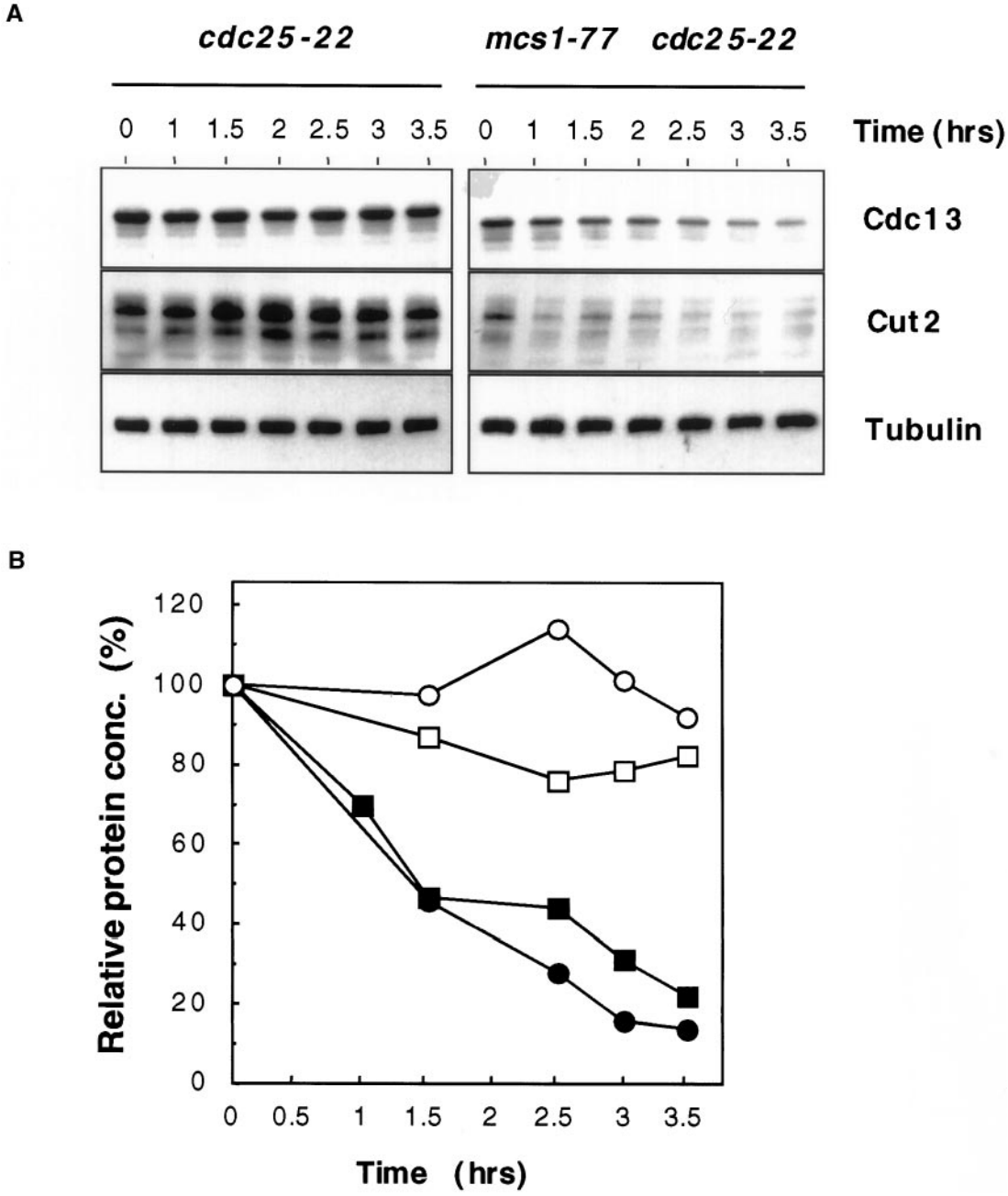

Figure 6. Cdc13 and Cut2 are unstable in *mcs1-77* cells in G2 phase. (A) Stability of cyclin B and Cut2 proteins in *mcs1-77 cdc25-22* cells. Log-phase cultures of either *cdc25-22 cut2-364::cut2*$^+$*-HA-LEU2* or *mcs1-77 cdc25-22 cut2-364::cut2*$^+$*-HA-LEU2* cells growing in YEPD at 28°C were shifted to 35.5°C for 3 h and then incubated in the same medium containing 100 μg/ml cycloheximide at 35.5°C for the times indicated. Cell extracts were prepared and probed for the presence of either Cdc13, with the use of affinity-purified anti-Cdc13 polyclonal antibodies, or Cut2, with the use of anti-hemagglutinin mAbs. (B) Quantification of cyclin B and Cut2 stability from A. The abundance of Cdc13 (squares) and Cut2 (circles) was expressed as a percentage of the initial level after the addition of cycloheximide to either *cdc25-22 cut2-364::cut2*$^+$*-HA-LEU2* (○, □) or *mcs1-77 cdc25-22 cut2-364::cut2*$^+$*-HA-LEU2* cells (●, ■). (C) Aberrant chromosome segregation in *mcs1-77 cdc25-22* cells. Log-phase cultures of either *cdc25-22* or *mcs1-77 cdc25-22* cells growing in YEPD at 25°C were shifted to 36°C for 3 h. Cells were then fixed and stained with DAPI to visualize chromatin.

1995; Damagnez *et al.*, 1995). Our observation that the *mcs1-77* mutation can bypass the loss of Wee1 and Mik1 tyrosine kinases indicates that Res2 also controls the activity of the Cdc2/Cdc13 complex, but by a mechanism that is independent of Cdc2 tyrosine phosphorylation. This is consistent with our finding that the G2 delay observed in

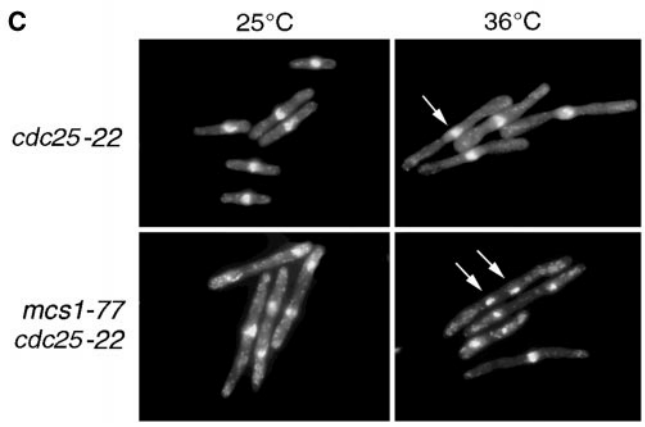

Figure 6 (cont).

mcs1-77 cells is not dependent on activation of either the DNA damage or DNA replication checkpoint pathway, because these act through Cdc2 tyrosine phosphorylation. Instead, we have found that the rate of degradation of the major cyclin B, Cdc13, is increased in *mcs1-77* cells blocked at the G2/M transition. Cdc13 is targeted for degradation by the APC, a specialized E3 ubiquitin ligase (Page and Hieter, 1999; Zachariae and Nasmyth, 1999). Because APC-mediated degradation ceases after cells pass START, we suggest that repression of the MBF complex may play a key role in the inactivation of APC at this time. We considered that this may be due to either cell cycle–regulated production or modification of an APC subunit(s) or adaptor proteins such as Ste9/Srw1 (Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998; Kominani *et al.*, 1998). In particular, we focused our attention on *ste9/srw1*, because it had been isolated previously as a multicopy suppressor of the mitotic catastrophe phenotype of Δ*mik1 wee1-50* cells at high temperature (Yamaguchi *et al.*, 1997; H. Okayama, personal communication). This persuaded us to examine whether the G2 delay observed in

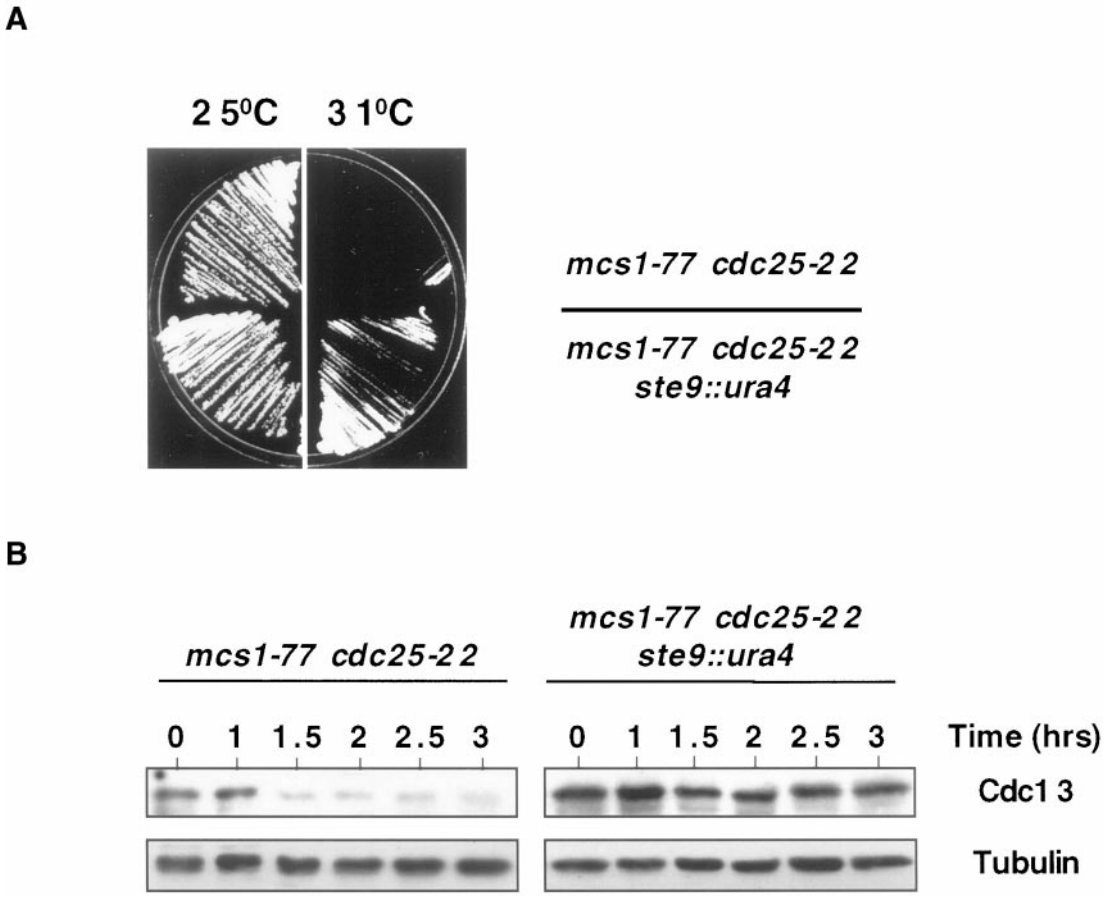

Figure 7. Instability of Cdc13 in *mcs1-77* cells is due to the activity of APC^{Ste9} (A) Deletion of Ste9/Srw1 rescues the cell cycle delay in *mcs1-77* cells. *mcs1-77 cdc25-22* or *ste9::ura4 mcs1-77 cdc25-22* cells were grown on YEPD at 25°C and then streaked onto YEPD plates at either 25 or 31°C, and the growth of the strains was monitored after 3 d at these temperatures. (B) Deletion of Ste9 stabilizes Cdc13 cyclin B in *mcs1-77* cells. Log-phase cultures of either *mcs1-77 cdc25-22* or *ste9::ura4 mcs1-77 cdc25-22* cells growing in YEPD at 28°C were shifted to 35.5°C for 3 h and then incubated in the same medium containing 100 μg/ml cycloheximide at 35.5°C for the times indicated. Cell extracts were prepared and probed for the presence of Cdc13 with the use of affinity-purified anti-Cdc13 polyclonal antibodies.

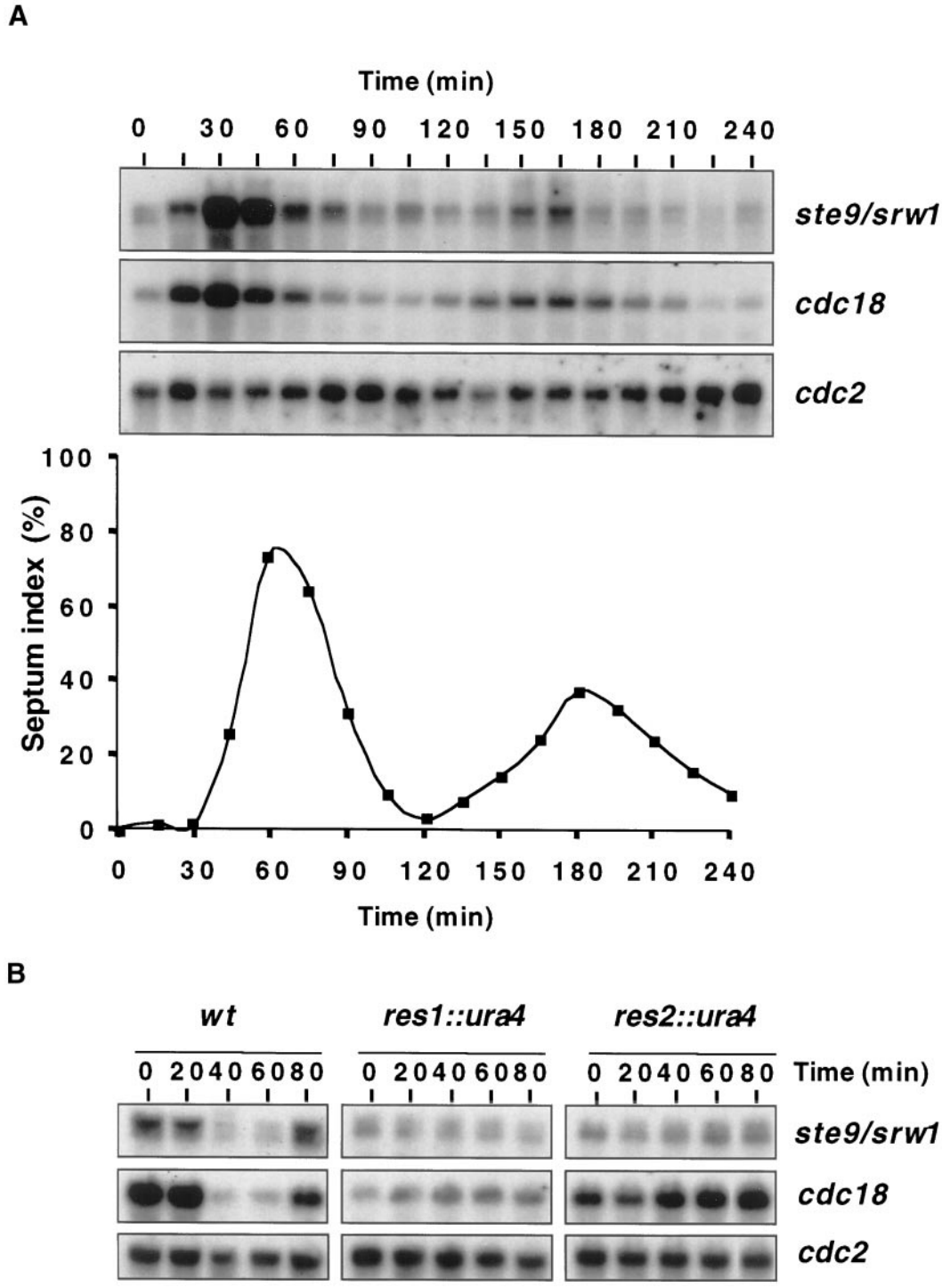

Figure 8. MBF complex controls periodic transcription of *ste9/srw1*. (A) Transcription of *ste9/srw1* is cell cycle regulated. A log-phase culture of *cdc25-22* cells growing in YES was arrested in G2 by incubation at 36.5°C for 4 h. Cells were shifted to 25°C and collected for Northern blot analysis and examined microscopically for the appearance of septa at the times indicated. Total RNA was extracted, and equal quantities were separated by electrophoresis and probed with the use of DNA specific to the *ste9/srw1* and *cdc18* genes. Reprobing with *cdc2*-specific probes verified approximately equal loading of RNA. (B) Res1 and Res2 control transcription of *ste9/srw1*. Log-phase cultures of wild-type, *res1::ura4*, or *res2::ura4* cells growing in YES were incubated in the presence of 11 mM hydroxyurea for 4 h and then washed and resuspended in fresh YES lacking hydroxyurea for the times indicated. Cells were harvested, and total RNA was extracted. Equal quantities of RNA were separated by electrophoresis and probed with the use of DNA specific to the *ste9/srw1* and *cdc18* genes. Reprobing with *cdc2*-specific probes verified approximately equal loading of RNA.

mcs1-77 cells was due to ectopic activity of APC^{Ste9}, and we found that this was indeed the case. Furthermore, we found that the expression of Ste9/Srw1 was profoundly cell cycle regulated and that this periodicity was regulated by the MBF complex. This leads to a simple explanation of how the *mcs1-77* mutation rescues the mitotic catastrophe of a *cdc2-3w wee1-50* strain: ectopic expression of Ste9/Srw1 leads to enhanced degradation of Cdc13 in G2, thereby reducing the effective concentration of active Cdc2/Cdc13 kinase.

Intriguingly, a recent report has suggested that the accumulation of cyclin B1 in mammalian cells is triggered by the E2F-mediated expression of cyclin A, which in association with Cdk2 phosphorylates and inactivates APC^{Cdh1} (Lukas *et al.*, 1999). Although we cannot formally rule out the possibility that MBF-mediated accumulation of the Cig2 cyclin in fission yeast may contribute to the accumulation of Cdc13 through phosphorylation and inactivation of APC^{Ste9}, deletion of Cig2 did not rescue the lethality observed *in cdc2-3w wee1-50* cells at high temperature. Therefore, we would argue that repression of START-dependent transcription of Ste9/Srw1 may be more important for the inactivation of APC^{Ste9} and thus the accumulation of cyclin B. It should be noted that levels of CDH1 also fluctuate in mammalian cells, being present primarily in G1, although it is not known whether this is due to regulation by E2F (Kramer *et al.*, 2000). Regardless, our results highlight a role for the S phase–specific gene transcription factor complex in the timely inactivation of cyclin B destruction as cells pass through S phase.

In addition to Cdc13, we found that another APC substrate, the anaphase inhibitor Cut2, was also unstable in *mcs1-77* cells. Consistent with this, we observed a high frequency of chromosomal abnormalities in *mcs1-77* cells in G2, as assessed by DAPI staining of nuclei. However, we found that deletion of *ste9/srw1* had little effect on the frequency of chromosomal abnormalities observed in *mcs1-77* cells and by inference Cut2 stability. Because ubiquitination of Cut2 is not regulated by APC^{Ste9} but rather by a related complex, APC^{Slp1}, our results suggest that the MBF complex has an additional role in controlling APC activity (Funabiki *et al.*, 1996; Matsumoto, 1997; Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998; Kominani *et al.*, 1998). One obvious possibility is that the MBF complex may control the periodic transcription of one or more components or activators of the APC^{Slp1} complex.

It is now well recognized that the APC plays a central role in controlling the destruction of key cell cycle regulators and, as a consequence, the order and timing of cell cycle progression in all eukaryotes. Deregulation of this activity is very likely to contribute to the chromosome instability and missegregation associated with the uncontrolled proliferation of many tumor cells. This highlights the need to understand how various forms of the APC are cell cycle regulated and how APC activity is restrained in response to cellular damage. Importantly, the composition of the fission yeast APC and the mechanism by which it is inactivated upon spindle damage closely resemble those observed in higher eukaryotes (reviewed by Peters, 1999). Thus, it is highly likely that cell cycle cues governing APC activity, such as those described in this paper, may also be operative in higher eukaryotes.

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