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Dual functions of *CDC6*: a yeast protein required for DNA replication also inhibits nuclear division

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The *Saccharomyces cerevisiae* gene *CDC6*, whose protein product is required for DNA replication, is transcribed only in late G_1 and S phases. We have discovered a critical reason why *CDC6* expression is regulated in this fashion. Constitutive *CDC6* transcription greatly delayed the initiation of M phase without effecting the G_1–S transition or growth rate. This occurred in both fission and budding yeasts. The *CDC6*-induced M phase delay was dependent on the *wee1/mik1* mitotic inhibitor kinases and was greatly accentuated in strains defective for the *cdc25/MIH1* mitotic inducer phosphatases, indicating that *CDC6* indirectly inhibits activation of the $p34^{cdc2/CDC28}$ M phase kinase. Thus *CDC6* appears to have an important and perhaps unique dual role in S phase, it is first required for the initiation of DNA replication and then actively participates in the suppression of nuclear division.

Key words: *CDC6*/cell cycle/mitotic control/yeast

Introduction

In recent years major advances have been made in understanding the cell cycle controls regulating the initiation of mitosis. Many of these studies have been carried out using the fission yeast *Schizosaccharomyces pombe*, although most features of the mitotic control appear to be highly conserved (Nurse, 1990). These studies have established that activation of a particular serine/threonine protein kinase is rate-limiting for the induction of mitosis. This kinase consists of a 34 kDa catalytic subunit and a 45–60 kDa regulatory subunit that in fission yeast are encoded by $cdc2^+$ and $cdc13^+$, respectively (Booher *et al.*, 1989; Moreno *et al.*, 1989). The $p56^{cdc13}$ protein is a member of the B-type cyclin family of proteins which are periodically degraded as cells exit M phase (Booher and Beach, 1988; Hagan *et al.*, 1988; Booher *et al.*, 1989; Moreno *et al.*, 1989). The $p34^{cdc2}$–cyclin complex is assembled in an inactive state prior to M phase. Activation of the $p34^{cdc2}$–cyclin kinase directly leads to the onset of mitosis. Characterization of the processes responsible for the inhibition of the $p34^{cdc2}$–cyclin kinase activity prior to the onset of M phase and its subsequent activation, hold the key to understanding the controls regulating the initiation of mitosis.

Biochemical analysis of fission yeast has determined that the $p34^{cdc2}$–cyclin kinase is maintained in an inhibited state during interphase by the phosphorylation of the Tyr15 residue of the $p34^{cdc2}$ subunit (Gould and Nurse, 1989). Studies of fission yeast mitotic control mutants, in particular cell division cycle (*cdc*) mutants that are unable to initiate M phase and wee mutants that undergo mitosis prematurely at an abnormally small size, have identified a small group of proteins playing key roles in regulating tyrosyl phosphorylation of $p34^{cdc2}$ (Nurse, 1975; Fantes, 1979; Russell and Nurse, 1986; Russell and Nurse, 1987a,b; Lundgren *et al.*, 1991). Two protein kinases, encoded by *wee1* and *mik1*, appear to be involved in promoting $p34^{cdc2}$ tyrosyl phosphorylation, perhaps by a direct mechanism (Russell and Nurse, 1987b; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Parker *et al.*, 1991). The *wee1* kinase plays the dominant role in this pathway, since a *mik1* deletion mutation imparts no phenotype in a $wee1^+$ background, whereas *wee1* deletion mutants exhibit a strong wee phenotype in a $mik1^+$ background. Counteracting the *wee1* and *mik1* kinases is $p80^{cdc25}$, which is the phosphatase that dephosphorylates Tyr15 of $p34^{cdc2}$ (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Millar *et al.*, 1991; Strausfeld *et al.*, 1991). A wee phenotype, reflecting an advancement of the onset of mitosis, can result either from down-regulating the rate of tyrosyl phosphorylation, as appears to be the case in *wee1* defective strains, or by increasing tyrosyl dephosphorylation activity, as occurs in strains that overproduce $p80^{cdc25}$.

We have been interested in identifying components of the mitotic control pathways in other species. In earlier studies we reported the discovery of the *Saccharomyces cerevisiae MIH1* gene, a budding yeast homolog of *cdc25* (Russell *et al.*, 1989). We also found that expression of *wee1* in *S.cerevisiae* delayed the onset of mitosis, providing additional support for the conclusion that the *cdc25/wee1* control network was conserved in *S.cerevisiae*. This led to an attempt to identify mitotic inhibitor genes in budding yeast. Here we report the cloning of a *S.cerevisiae* gene that is unrelated to *wee1* but whose product can function as a potent M phase inhibitor both in fission and budding yeasts. This gene was determined to be *CDC6*, whose product is required for S phase. Investigation of this puzzling observation revealed that *CDC6* acts as an inhibitor of the transition from G_2 to M phase when its pattern of cyclical transcription is perturbed. These findings show that periodic transcription of *CDC6* is required for normal cell cycle regulation and suggests that *CDC6* plays an active role in preventing nuclear division until S phase is complete.

Results

Isolation of a S.cerevisiae gene that delays the onset of mitosis when expressed in fission yeast

We used the fission yeast strain RE3 (*cdc2*-3w *wee1*-50) to select for *S.cerevisiae* genes capable of delaying the onset of mitosis. The *cdc2*-3w product is an activated form of

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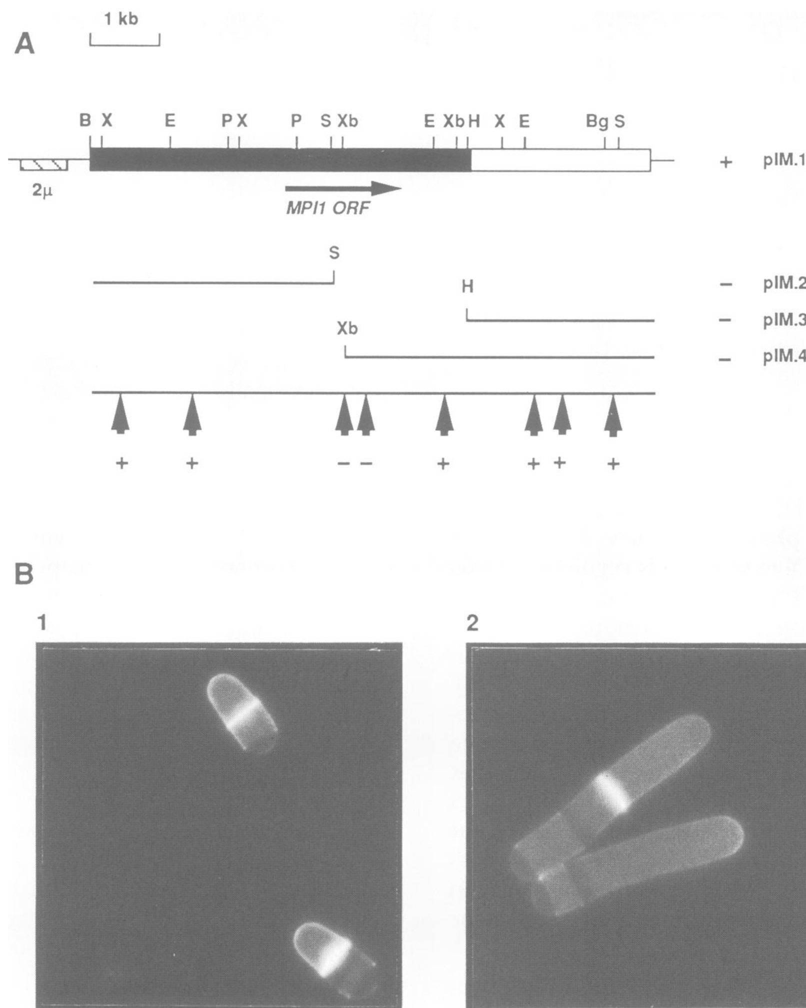

Fig. 1. *MPI1* rescues mitotic catastrophe in fission yeast. **(A)** Restriction enzyme map and subclone analysis of pIM.1 containing *MPI1*. Plus and minus symbols indicate the ability to rescue RE1 or RE3. Position and rescue activity of transposon inserts is shown at bottom. The fragment of DNA that has been sequenced is shown in dark, where the approximate localization of *MPI1* open reading frame is indicated by an arrow. Abbreviations on the map are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sph*I; Xb, *Xba*I; X, *Xho*I. **(B)** Photomicrographs of Calcofluor-stained *S.pombe* RE1 cells (*adh:cdc25*$^+$*:(ura4*$^+$*) wee1*-50 *leu1*-32 *ura4*-d18) transformed with pARTrMPI1 (1) or pARTMPI1 (2). In both cases samples were taken from exponentially growing cultures at 25°C.

p34^{cdc2} which causes a wee phenotype, in many respects *cdc2*-3w phenotypically mimics overexpression of *cdc25*$^+$ (Russell and Nurse, 1986). The wee phenotypes caused by *cdc2*-3w and the temperature sensitive *wee1*-50 mutation are additive, such that *cdc2*-3w *wee1*-50 double mutants undergo lethal premature onset of M phase when incubated at the restrictive temperature (35.5°C) (Russell and Nurse, 1987b). RE3 was transformed with a *S.cerevisiae* genomic DNA library constructed in a vector that can be shuttled between *S.cerevisiae* and *S.pombe*. Following selection for transformants at the permissive temperature (25°C, rescue of mitotic catastrophe was screened by incubating replica plates at 35.5°C. Plasmid DNA was recovered from three of the 11 transformants that continued cell division at 35.5°C. All three contained the same plasmid, designated pIM.1 (Figure 1A). Southern hybridization of total genomic DNA from the other eight transformants, using a probe derived from the pIM.1 insert, showed that they all had common DNA sequences and therefore all identified the same gene (data not shown). Plasmid pIM.1 efficiently rescued RE3 upon retransformation. In addition, pIM.1 also rescued strain RE1 [*adh:cdc25*$^+$*(ura4*$^+$*) wee1*-50], which exhibits a mitotic catastrophe phenotype as result of overexpression of the *cdc25*$^+$ mitotic inducer in combination with *wee1*-50 (Russell and Nurse, 1986). In both strains the pIM.1 transformants displayed a wee phenotype when grown at 35.5°C, while at 25°C they exhibited an elongated cell length phenotype, dividing ~30% longer than the wild type (~18 μm versus 14 μm).

These data suggested that a *S.cerevisiae* gene present in pIM.1 delayed the onset of mitosis when expressed in *S.pombe*. This gene was given the name *MPI1*, an acronym for *M* phase *i*nhibitor. Subclone complementation and transposon insertional mutagenesis analysis indicated that *MPI1* was located between the *Eco*RI sites located at positions 1.3 and 5.0 on the map (Figure 1A) and crossed the *Sph*I and *Xba*I sites located at positions 3.4 and 3.5. The DNA sequence of a 5.5 kb region was determined and a 1.5 kb open reading frame (ORF) was found to cross to the right through the *Sph*1(3.4) and *Xba*I(3.5) sites (Figure 1A). Translation initiating at the first methionine codon in the ORF would produce a protein of 513 amino acids with a M_r of 58 000. Previous to these studies, no candidate *S.cerevisiae* mitotic inhibitor gene had been reported, thus we expected

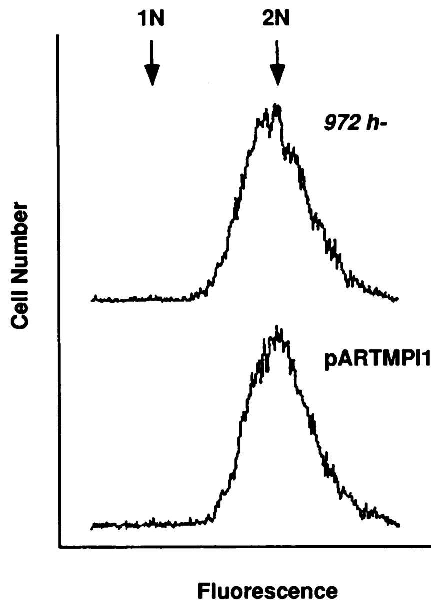

Fig. 2. *MPI1* expression causes a G_2 delay in fission yeast. DNA content analysis of log phase cells stained with propidium iodide and analyzed by flow cytometry. Top graph is 972h⁻ wild type *S.pombe* cells, bottom graph is a *leu1*-32 *ura4*-294 strain transformed with pARTMPI1. Similar results were obtained with a *leu1*-32 *ura4*-294 control transformed with pART1.

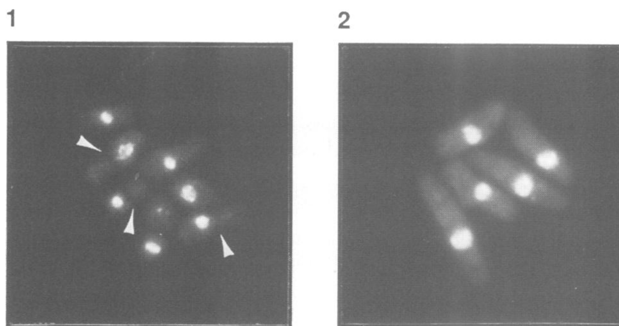

Fig. 3. Restoration of checkpoint control by *CDC6* expression. *adh:cdc25$^+$(ura4$^+$) leu1*-32 *ura4*-d18 cells transformed with pARTrMPI1 (**1**) or pARTMPI1 (**2**) were incubated with 10 mM hydroxyurea for 4 h fixed and then the DNA was stained with DAPI. Arrows indicate examples of septated cells.

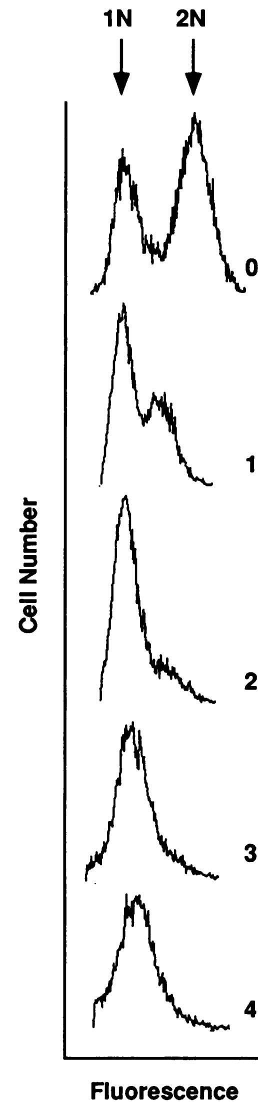

Fig. 4. *S.cerevisiae cdc6*-1 mutants arrest with 1N DNA content. An exponentially growing culture of a *cdc6*-1 mutant strain incubated at 23°C was shifted to the restrictive temperature (37°C). Samples were taken at 1 h intervals. DNA content analysis was performed by fluorescence activated cell sorting (FACS) in cells stained with propidium iodide. Morphological analysis showed that after 2 h at the restrictive temperature cells were arrested with the budding phenotype previously described for the *cdc6*-1 mutant (data not shown). This temperature sensitive phenotype was abolished in cells transformed with pIM.1.

MPI1 to define a new genetic locus, perhaps encoding the budding yeast homolog of *S.pombe wee1*. Surprisingly, *MPI1* protein was found to have no sequence similarity to $p107^{wee1}$. Moreover, *MPI1* protein did not contain protein kinase consensus sequences, nor did it have significant homology to proteins available in databases at that time.

The absence of any sequence similarity between *MPI1* and *wee1* proteins prompted us to do several experiments to prove more convincingly that *MPI1* inhibited the initiation of M phase. We first sought to confirm that the cell cycle delay phenotype imparted by pIM.1 was due to expression of *MPI1* protein. The *MPI1* open reading frame was cloned into the fission yeast expression vector pART1, placing *MPI1* expression under the control of the strong *adh* promoter. This plasmid was named pARTMPI1. As a control we constructed pARTrMPI1, having the *MPI1* open reading frame in the reverse orientation. Plasmid pARTMPI1 rescued the mitotic catastrophe phenotype of RE1 and RE3 strains. When grown at 25°C, both RE1 and RE3 transformants exhibited an elongated cell length phenotype, dividing ~60% longer than the wild type (Figure 1B). Plasmid pARTMPI1 increased cell division size of wild type cells to 22.7 ± 0.4 μm, ~1.5 times longer than the normal size at division. Plasmid pARTrMPI1 exhibited no ability to rescue RE1 and RE3, nor did it have any effect on cell size at division. These experiments proved that *MPI1* was responsible for rescuing mitotic catastrophe in RE1 and RE3 strains.

In the second experiment we sought to determine whether *MPI1* acted to influence the mitotic control regulating the rate of transit through G_2 phase. In theory the mitotic catastrophe phenotype exhibited by RE1 and RE3 strains potentially could be rescued by delaying the execution of an event early in the cell cycle, for example the transit from G_1 to S phase. To ascertain whether this might be case for the *MPI1*-induced cell cycle delay, we performed

fluorescence-activated flow cytometry analysis of a *leu1*-32 *ura4*-294 strain transformed with pARTMPI1 and then fixed and stained with the DNA-binding reagent propidium iodide. Wild type cells growing under optimal conditions exhibit a single peak at the 2N DNA content because daughter cells physically separate as S phase is completed. The pARTMPI1 transformants exhibited a single DNA peak at the 2N DNA content, identical to wild type cells and to the *leu1*-32 *ura4*-294 strain transformed with pART1 (Figure 2). This indicated that the *MPI1*-induced cell cycle delay occurred after S phase. Fluorescence microscopy showed that the frequency of cells having either condensed chromosomes or two nuclei was equivalent in strains transformed with pARTMPI1 and control plasmids, indicating that progression through the mitosis and cell septation was not delayed as a consequence of *MPI1* expression. These observations strongly indicated that the *MPI1*-induced cell cycle delay occurred in G_2 phase, consistent with the idea that the protein product of *MPI1* functions as a mitotic inhibitor.

We next asked whether *MPI1* reversed the lethality observed when either *cdc25*-overproducer or *cdc2*-3w strains are grown in the presence of hydroxyurea. These strains die when DNA synthesis is inhibited by hydroxyurea because the initiation of mitosis is no longer dependent on the completion of DNA replication, unlike wild type cells (Enoch and Nurse, 1990). Both strains harboring the control plasmid (pARTrMPI1) showed the hydroxyurea suicide phenotype previously described (Enoch and Nurse, 1990). Microscopic observation showed that transformation of pARTMPI1 into *cdc25*-overproducer or *cdc2*-3w strains very effectively rescued the hydroxyurea-induced mitotic suicide phenotype (Figure 3). These cells arrested in hydroxyurea as unseptated cells with a single nucleus. Furthermore, we did not observe a significant loss of viability in pARTMPI1 transformed *cdc2*-3w or *cdc25*-overproducer strains. In both strains >90% of the pARTMPI1 transformant cells formed colonies after removal of hydroxyurea following 4 h of treatment, whereas only 30% of the pARTrMPI1 transformant cells did so. These results showed that expression of *MPI1* generates a mitotic inhibition signal that in this assay is epistatic to strong overexpression of *cdc25* and by the mutation *cdc2*-3w.

MPI1 inhibits the onset of mitosis by a cdc25-independent mechanism

In fission yeast the timing of mitosis is determined by controls regulating tyrosyl phosphorylation of $p34^{cdc2}$ (Gould and Nurse, 1989). Therefore *MPI1* was likely to function either by inhibiting tyrosyl dephosphorylation catalyzed by the *cdc25* phosphatase, or by activating the presumptive *wee1/mik1* tyrosyl phosphorylation pathway. To evaluate the former possibility we transformed pARTMPI1 into strains partially or completely defective in *cdc25* activity. In the first experiment we noted a striking additive genetic interaction between *MPI1* expression and partial loss of *cdc25* activity. Strains having *cdc25*-22, which when grown at the permissive temperature of 25°C are normally ~1.25 times longer than wild type (~18 μm versus ~14 μm), were approximately three times longer than wild type when transformed with pARTMPI1 and grown at 25°C (~43 μm). Statistically accurate cell size measurements of *cdc25*-22 cells transformed with pARTMPI1 could not be determined because a large proportion of the cells were inviable, having undergone a cell cycle arrest. At 30°C pARTMPI1 caused an even more dramatic phenotype, resulting in a uniform cell cycle arrest. The *cdc25*-22 cells transformed with the control plasmid pARTrMPI1 grew well at 30°C, having a doubling time of ~3 h and dividing at ~20 μm.

These results, showing strong additive genetic interactions between *MPI1* expression and partial loss of *cdc25* activity, suggested that *MPI1* delayed the onset of M phase by counteracting the *cdc25* M phase induction process. To evaluate whether *MPI1* M phase inhibition activity acts independently of *cdc25*, we examined the effect of *MPI1* expression in a *cdc25* deletion strain. Strains having a deleted version of *cdc25* and the *cdc2*-3w allele divide at 18 μm (Russell and Nurse, 1987b). We attempted to transform *cdc25*::*ura4 cdc2*-3w cells with pARTMPI1. Three separate transformations failed to yield any viable transformants. Concurrent transformations of the same strain with pART1 yielded >500 transformants in each experiment. Likewise, concurrent transformation of *cdc2*-3w cells with pARTMPI1 yielded many thousands of transformants. These results indicate that *MPI1* can inhibit the onset of mitosis in strains lacking $p80^{cdc25}$.

MPI1 does not rescue mitotic catastrophe in wee1⁻ mik1⁻ double mutants

Having shown that *MPI1* delays mitosis by a mechanism that can function in the absence of *cdc25*, we next considered the possibility that *MPI1* counteracts *cdc25* by stimulating the *wee1/mik1* mitotic inhibition process that leads to tyrosyl phosphorylation of $p34^{cdc2}$ (Russell and Nurse, 1987b; Featherstone and Russell, 1991; Lundgren *et al.*, 1991). If *MPI1* acts primarily by stimulating *wee1* and *mik1* activity, then *MPI1* expression should not rescue the lethal mitotic catastrophe in a *wee1*$^-$ *mik1*$^-$ double mutant. Indeed, a *wee1-50 mik1::LEU2* strain transformed with a plasmid carrying the *adh:MPI1* construct underwent mitotic catastrophe with the same kinetics as seen in control strains, including one transformed with a plasmid having *MPI1* in the reverse orientation relative to the *adh* promoter (see Materials and methods). *MPI1* expression had no effect on cell division size in a *wee1* deletion strain. These results indicate that the M phase delay signal generated by *MPI1* is dependent on the *wee1/mik1* inhibitory pathway, either directly or indirectly.

MPI1 is identical to CDC6, a gene required for DNA replication

Shortly after these experiments were completed we looked again for matches between *MPI1* sequence and those new sequences that had recently been deposited in sequence databases. Much to our surprise, we found that the *MPI1* open reading frame was identical to that of the *S.cerevisiae CDC6* gene (Zhou *et al.*, 1989). Genetic and TAFE−gel electrophoresis mapping confirmed that the *MPI1* M phase inhibitor gene was indeed *CDC6*.

CDC6 had none of the properties expected of a mitotic inhibitor gene. A genetic description of *CDC6* was first provided by Hartwell and his colleagues (Hartwell, 1976). Their data showed that *CDC6* was required for proper DNA replication, although the position in the cell cycle where $cdc6^{ts}$ mutants arrest was left uncertain. Because this issue was of some importance for the interpretation of our results, we used fluorescence activated flow cytometry of propidium

iodide stained cells to analyze the DNA content of *cdc6*-1 cells that were incubated at the restrictive temperature. This analysis showed that within 2 h after the temperature shift-up >90% of the cells had a 1N DNA content, consistent with arrest in G_1 or early S phase (Figure 4). Upon longer incubation at the restrictive temperature there was a slow shift of the 1N peak to the right, towards 2N. This is seen in most *cdc* mutants and is believed to reflect either leakiness of the mutation or increased background fluorescence occurring as a consequence of increasing cell size.

Deregulated CDC6 expression delays the onset of mitosis in S.cerevisiae

We were thus faced with the seemingly paradoxical observation that *CDC6*, a *S.cerevisiae* gene apparently involved with DNA replication, delays the initiation of mitosis when present on a multicopy plasmid in *S.pombe*. It appeared that this effect must be due either to improper function of *CDC6* in *S.pombe*, to abnormally high *CDC6* expression or to deregulated *CDC6* expression. If the latter hypothesis was correct, we would predict that deregulated expression of *CDC6* in *S.cerevisiae* would also cause a delay of M phase. As the first step to test this hypothesis, we determined whether the expression of *CDC6* is cell cycle regulated in *S.cerevisiae*. We suspected that *CDC6* transcription would normally be restricted to G_1 and S phases because the upstream non-coding sequences of *CDC6* contained the sequence ACGCGTNA, the so-called *Mlu*I motif, required for G_1 and S phase specific pattern of transcription for other genes involved in DNA replication (McIntosh *et al.*, 1988; Gordon and Campbell, 1991; Wittenberg and Reed, 1991). To confirm this, *CDC6* mRNA levels were monitored in a culture synchronized in G_1 phase by treatment with α-factor mating pheromone, released

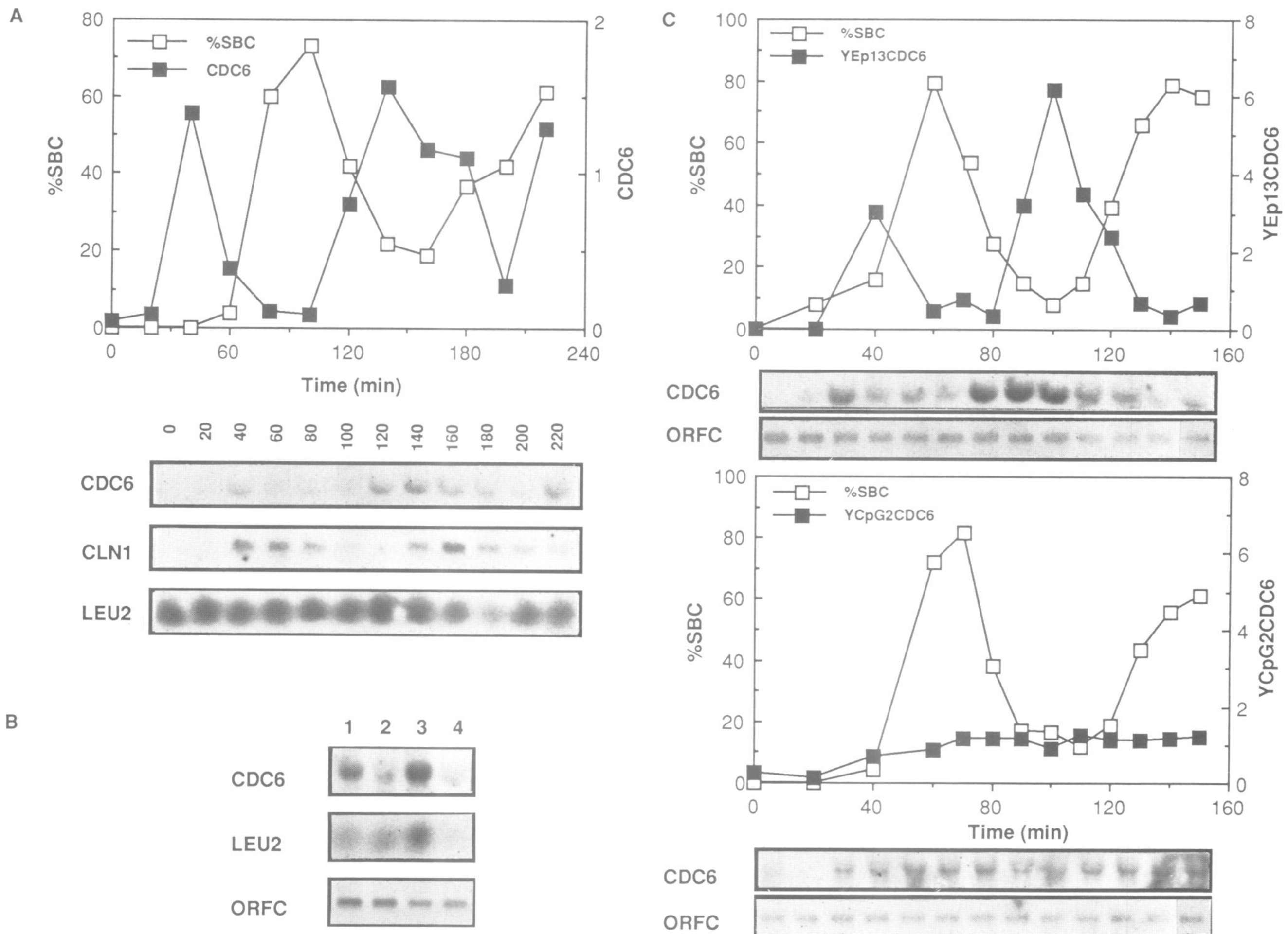

Fig. 5. Periodic expression of *CDC6* mRNA. **(A)** A *S.cerevisiae* culture (GCY11, 15Dau *bar1::LEU2*) was arrested in G_1 by treatment with α-factor, then released synchronously into the cell cycle by washing out the α-factor. Samples were taken at intervals of 20 min. Ten micrograms of total RNA (as determined by OD_{260}) from each sample were analyzed by Northern blot using *CDC6*, *LEU2* and *CLN1* probes. The top panel indicates the relative amount of small budded cells (SBC, open squares) and appearance of *CDC6* mRNA normalized to *LEU2* transcript (filled squares). It is interesting to note that in the second cycle the reappearance of *CDC6* mRNA slightly precedes that of *CLN1* mRNA. The significance of this is not understood. **(B)** Northern analysis of the relative abundance of *CDC6* mRNA in *S.cerevisiae* GCY11 strain harboring YCpG2CDC6 with galactose (lane 1) or glucose (lane 2), pIM.1 (lane 3) and wild type (lane 4). Controls of *LEU2* and *ORFC*, a constitutive mRNA from a gene located upstream of *CDC6* (unpublished data), are also shown. It should be noted that pIM.1 does not contain the N-terminal region of *ORFC*, hence *ORFC* mRNA is not overexpressed in pIM.1 transformants. **(C)** Total RNA was purified from synchronous cultures obtained by α-factor treatment in a *bar1⁻* strain harboring pIM.1 (YEp13 derivative) or YCpG2CDC6 to determine cell cycle periodicity of the *CDC6* transcript. Northern analysis was performed and the relative amount was quantified by laser densitometry and normalized to *ORFC*, because this gene proved to be constant in the different transformants used where *LEU2* proved not to be (see Figure 5B) due to the different number of copies of the *LEU2* gene carried by YEp13 and YCpG2 transformants. Panels indicate budding index in synchronous cultures of cells harboring pIM.1 (top) and YCpG2CDC6 (bottom) respectively, where open squares indicate small budded cells and filled squares indicate relative appearance of *CDC6* mRNA.

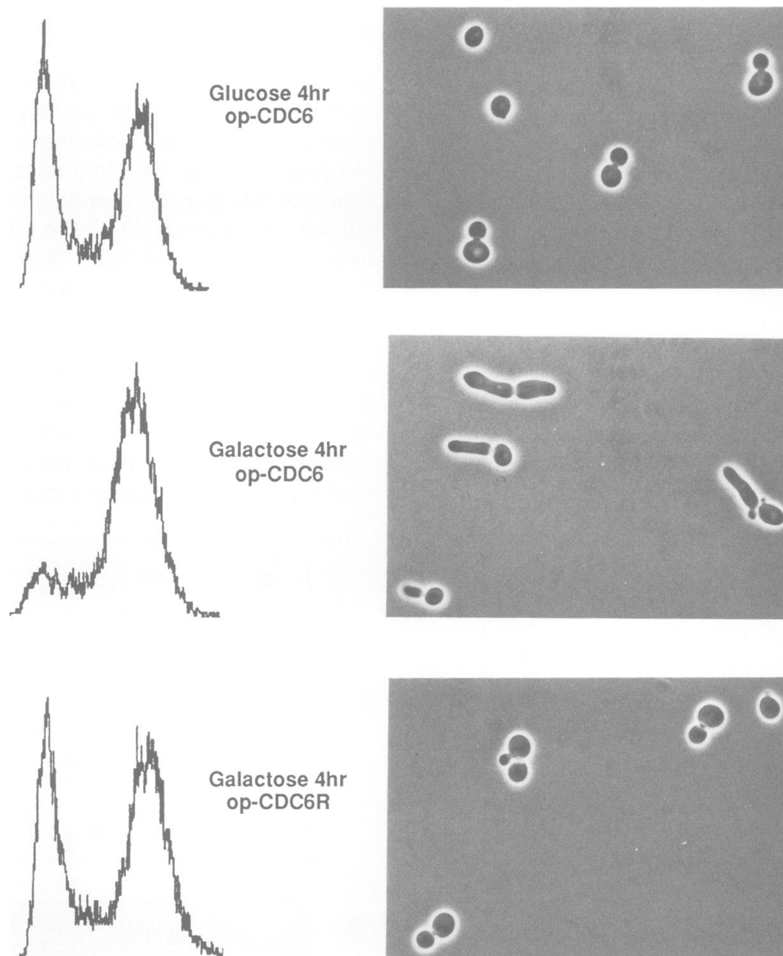

Fig. 6. Constitutive *CDC6* expression in *S.cerevisiae* delays the onset of mitosis. Flow cytometry analysis of DNA content of log phase cells of *S.cerevisiae* 15Dau strain harboring YCpG2CDC6 (top panels) and YCpG2rCDC6 (bottom). Photomicrographs of cells described above are also shown. Cells were cultured at 30°C using glucose or galactose as carbon source (as indicated).

from the arrest by inoculation in fresh medium and then sampled at intervals of 20 min. Northern analysis showed that the *CDC6* mRNA oscillated periodically, peaking around the time of bud emergence, which in most strains corresponds to late G_1 or early S (Figure 5A). *CDC6* mRNA cell cycle periodicity data have also recently been reported by Zhou and Jong (1990).

Assuming that *CDC6* expression was regulated primarily at the level of transcription, a prediction of our model was that constitutive *CDC6* expression would delay the onset of M phase in *S.cerevisiae*, whereas an equal level of periodic *CDC6* overexpression would have a smaller impact on the timing of mitosis. We transformed *S.cerevisiae* strain GCY11 with two plasmids, YCpG2CDC6, a single copy centromere plasmid having *CDC6* under the control of the *GAL1* promoter and pIM.1, a high copy vector having *CDC6* expressed by its own promoter. pIM.1 caused the *CDC6* mRNA signal to increase 12-fold above wild type in asynchronous cultures (Figure 5B). Generation time, cell size and DNA content profiles of the pIM.1 transformed cells were not different from control cells transformed with YEp13, the vector from which pIM.1 was derived. Northern analysis of a pIM.1 transformant synchronous culture confirmed that the normal cell cycle periodicity of *CDC6* mRNA levels was maintained (Figure 5C). In contrast, constitutive *CDC6* expression (Figure 5C) driven by the *GAL1* promoter had a significant impact on cell size and cell cycle profile. Nearly 100% of the cells divided with very large buds (Figure 6). The fraction of cells having a 2N DNA content increased to 85% in cells constitutively expressing *CDC6*, as compared to 52% in a control culture transformed with a similar plasmid having *CDC6* inserted in the reverse orientation (Figure 6). Of the large budded cells, the large majority (>80%) had a single nucleus located in the mother cell, indicating that the cell cycle delay occurred prior to the onset of M phase (data not shown). These phenotypes were not simply a consequence of *CDC6* overexpression, because in asynchronous cultures the level of *CDC6* mRNA in the YCpG2CDC6 transformants was less than half of that seen in pIM.1 transformed cells (Figure 5B, lanes 1 and 3). These data show that moderate, unrestricted transcription of *CDC6* causes a strong cell cycle delay in budding yeast, either in G_2 or possibly in late S phase, whereas a higher level of regulated expression has no detectable phenotype.

To investigate whether the *CDC6*-induced cell cycle delay occurred after the completion of S phase, we measured the cell number increase in YCpG2CDC6 transformants after the addition of the DNA synthesis inhibitor hydroxyurea. As shown in Figure 7, YCpG2CDC6 transformants grown in galactose underwent a 92% increase in cell number after the addition of hydroxyurea, whereas the same cells grown

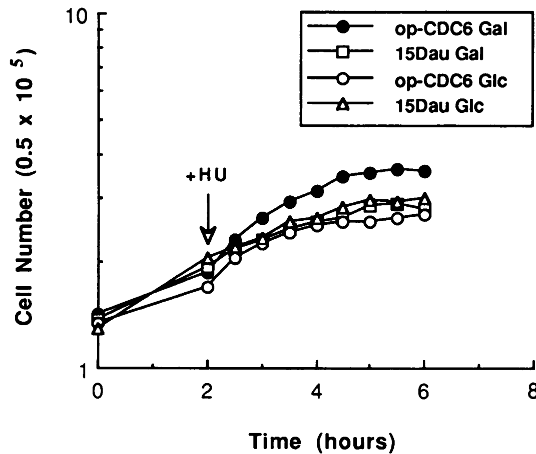

Fig. 7. *CDC6*-induced cell cycle delay occurs after the completion of S phase. Strain 15Dau untransformed (control) or transformed with YCpG2CDC6 (as indicated), was grown at 30°C in YPRAF medium to mid-log phase. At t = 0 time galactose (Gal) or glucose (Glc) were added and the cells were incubated for an additional 2 h before adding hydroxyurea (HU).

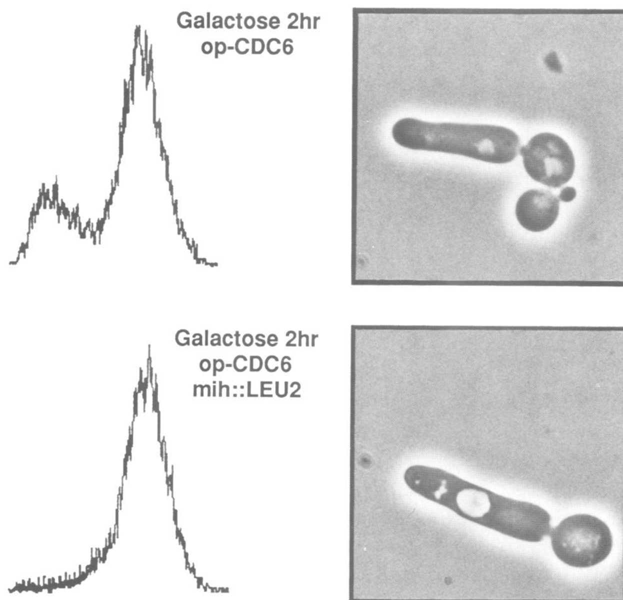

Fig. 8. *MIH1* function is required to overcome the G_2 arrest induced by *CDC6* constitutive expression. Flow cytometry analysis of DNA content in *S.cerevisiae* 15Dau *CDC6::GAL1:CDC6(URA3)* cells overproducing *CDC6* in a *MIH1* background (top) or a *mih1::LEU2* background (bottom) after 2 h of induction in galactose.

in glucose and thus not overexpressing *CDC6* underwent a 54% increase in cell number after the addition of hydroxyurea. These data indicate that the *CDC6*-induced cell cycle delay occurred after the completion of S phase.

Constitutive CDC6 expression causes G_2 arrest in cells lacking the MIH1 tyrosyl phosphatase

We next addressed the question of whether the *CDC6*-induced M phase delay occurs by similar regulatory pathways in *S.cerevisiae* and *S.pombe*. Our studies of fission yeast had shown that the *CDC6*-induced M phase delay is more pronounced in strains partially defective for *cdc25* function. In *S.cerevisiae* a $p80^{cdc25}$ homolog is encoded by *MIH1* (Russell *et al.*, 1989). Deletion of *MIH1* has little effect in most laboratory strains, but *mih1* deletion does make *S.cerevisiae* strains highly sensitive to *wee1* expression. In fact, *wee1* expression causes a G_2 arrest in *mih1* deletion strains (Russell *et al.*, 1989). If *CDC6* inhibited the onset of mitosis by a similar mechanism in the two yeasts, then we would expect a similar genetic interaction involving *CDC6* expression and defects in *MIH1* should occur in *S.cerevisiae mih1* deletion strains. To confirm this we disrupted *MIH1* in a strain having a *GAL1::CDC6* construct integrated at the *CDC6* locus. Induction of *CDC6* expression as a result of growth in galactose media caused the cessation of cell division within 2 h. These arrested cells soon acquired extremely long buds (Figure 8), closely resembling the phenotype obtained by expression of *wee1* in *mih1* deletion background (Russell *et al.*, 1989). Flow cytometry analysis confirmed that these cells arrested with 2N DNA content (Figure 8). This strongly argues that the mechanism by which *CDC6* delays mitosis is conserved in budding and fission yeasts. Specifically, these results show that in *S.cerevisiae CDC6* inhibits mitosis by a mechanism that is counteracted by *MIH1*, which by analogy to the findings in fission yeast (Millar *et al.*, 1991) is presumed to be the phosphatase that dephosphorylates key inhibitory phosphorylation sites of $p34^{CDC28}$.

The CDC6-induced G_2 delay is RAD9-independent

In the budding yeast *S.cerevisiae*, it has been shown that the checkpoint control involved in inhibiting mitosis in response to X-ray induced DNA damage is dependent on the *RAD9* product (Weinert and Hartwell, 1988, 1990; Hartwell and Weinert, 1989) The *RAD9* pathway is also responsible for the inhibition of mitosis when DNA replication is prevented or delayed by a number of *cdc* mutants. Mutants blocked in S phase, e.g. mutants alleles of the *CDC9*-encoded DNA ligase, will undergo a lethal mitosis in a *rad9* mutant background (Hartwell and Weinert, 1989). We determined whether the *CDC6*-induced G_2 delay involved the activation of the *RAD9*-dependent checkpoint control. We disrupted *RAD9* in two different strains having *GAL1::CDC6* integrated at different loci. When shifted to media containing galactose as the sole carbon source, *rad9::URA3* or *rad9::HIS3* disruptants showed the characteristic *CDC6*-associated G_2 delay phenotype . We did not observe any difference with the control having *GAL1::CDC6* in a *RAD9* wild type background. Thus, we concluded that the G_2 delay caused by *CDC6* expression is independent of the *RAD9* pathway.

Discussion

The aim of this work was to identify *S.cerevisiae* genes whose protein products inhibit the initiation of mitosis. We employed a functional screen identical to that used to clone the fission yeast mitotic inhibitor genes *wee1* and *mik1*, namely rescue of a *S.pombe* conditional mitotic catastrophe strain with a genomic DNA library (Russell and Nurse, 1987a; Lundgren *et al.*, 1991). Notwithstanding possible complications arising from differences in mRNA transcription mechanisms of the two yeasts, our expectation was that we would most likely clone homologs of fission yeast *wee1*. This screen failed to identify a *wee1* homolog. Instead, the single *S.cerevisiae* gene that was identified as an M phase inhibitor was *CDC6*, whose protein product is required for DNA replication.

It was initially difficult to rationalize why a protein required for DNA replication should also function as an inhibitor of mitosis. We considered three alternative explanations for this conundrum. First, we proposed that the *CDC6* protein functioned aberrantly in the foreign environment of fission yeast, somehow interfering with progression through G_2 phase. A prediction of this model was that overexpression of *CDC6* in *S.cerevisiae* would have no effect on the timing of mitosis. We eliminated this model by showing that *CDC6* overexpression in *S.cerevisiae* driven by the *GAL1* promoter caused a dramatic G_2 delay.

Having shown that the *CDC6* protein has an inherent ability to inhibit the onset of mitosis and suspecting that *CDC6* expression was normally limited to G_1 and S phases, we next considered whether the mitotic delay effect seen in *S.cerevisiae* was simply due to overexpression of *CDC6*, or did it require aberrant regulation of *CDC6* expression during the cell cycle. We found that transformation of *S.cerevisiae* with a high copy plasmid harboring *CDC6* resulted in abundant *CDC6* overexpression that maintained normal cell cycle periodicity. *CDC6* transcripts appeared soon after bud emergence and then disappeared before mitosis. This pattern of *CDC6* overexpression had no detectable effect on mitotic timing. In contrast to this, we found that a lower level of constitutive *CDC6* expression caused a dramatic G_2 delay. We estimate that the level of *CDC6* mRNA in cells that constitutively expressed *CDC6* was below the peak of *CDC6* mRNA levels seen in wild type cells. This strongly indicates that the periodic transcription of *CDC6* is critical for proper regulation of cell cycle progression, in particular for regulating the timing of mitosis.

CDC6 is a member of a class of genes in *S.cerevisiae* that are periodically transcribed during late G_1 and S phases. All of these genes encode proteins involved in DNA replication, either DNA synthesis enzymes or proteins required for the biosynthesis of DNA precursors. Members include *POL1* and *POL3* encoding DNA polymerases (Johnston *et al.*, 1987; Bauer and Burgers, 1990), *PRI1* and *PRI2* encoding DNA primases (Foiani *et al.*, 1989; Johnston *et al.*, 1990) and *CDC8* encoding thymidine kinase (White *et al.*, 1987) to name only a few. All of these genes have one or more copies of the *MluI* motif (ACGCGTNA) located in their promoter regions. There is strong evidence indicating that the *MluI* motif plays an important role in determining the periodic pattern of expression during the cell cycle (McIntosh *et al.*, 1988; Pizzagalli *et al.*, 1988; Gordon and Campbell, 1991). The discovery of this class of genes has led to speculation regarding the purpose of the periodic pattern of expression. Three hypotheses have been proposed (Wittenberg and Reed, 1991). The first suggests that periodic expression may play a role in regulating entry into and progression through S phase. We found that constitutive expression of *CDC6* had no observable effect on the rate of progression through G_1 and S phases, although a subtle change would not have been detected. A second explanation has been based on an argument of energetic efficiency, suggesting that it would be more advantageous to periodically produce and degrade certain proteins required for S phase than to maintain them throughout the cell cycle. In this scenario one might expect that constitutive expression of *CDC6* would cause growth rate to slow, although again the effect might be subtle. In any case, we saw no effect on growth rate as a consequence of constitutive *CDC6* expression.

A third hypothesis, also unproven until now, proposes that some genes whose products are involved with DNA replication may be cyclically expressed because their functions in some way interfere with the execution of cell cycle events occurring after DNA replication is complete. Although one could imagine a variety of scenarios by which this might occur, the most plausible are those involving a role of cell cycle controls that make the initiation of mitosis dependent upon the completion of DNA replication (Weinert and Hartwell, 1988; Hartwell and Weinert, 1989). Our data are consistent with this model. We observed that *CDC6* expression in *S.pombe* exhibited a remarkable ability to rescue the hydroxyurea suicide phenotype in certain *wee* mutants. A reasonable interpretation of this result may be that *CDC6* protein function is responsible for generating a checkpoint control signal that inhibits the initiation of mitosis. In this model *CDC6* has two important functions. In the first instance *CDC6* has an essential S phase function, and in the absence of *CDC6* activity, new rounds of DNA replication are not initiated. Secondly, during S phase *CDC6* generates a signal that inhibits the onset of mitosis.

If *CDC6* does have a second role as a mitotic inhibitor, one might ask why *cdc6* defective mutants do not undergo mitosis without completing S phase. Two explanations come to mind. First, execution of *CDC6* function in late G_1 or early S phases may be necessary to proceed to a point where DNA replication checkpoint controls become necessary to delay mitosis. Secondly, there is increasing evidence of redundant checkpoint controls in *S.cerevisiae*. In particular, *RAD9*-deficient mutants retain the capacity to prevent the initiation of mitosis when DNA synthesis is inhibited by hydroxyurea (Hartwell and Weinert, 1989).

We should also consider whether *CDC6* is likely to be unique among *MluI* motif genes in its ability to inhibit the onset of M phase. The fact that we identified *CDC6* plasmids in all 11 RE3 transformants that were analyzed suggests that this particular screen with this genomic DNA library was fairly exhaustive. It is possible that other DNA replication genes would cause a similar phenotype if they were constitutively expressed at a sufficient level. Other *MluI* motif genes, whose products could also function as M phase inhibitors, may not have been detected either because they were expressed periodically due to partial conservation of the *MluI* motif transcription controls, or perhaps their promoters were simply nonfunctional in fission yeast. Divergence of transcription initiation mechanisms between fission and budding yeasts, which has been documented (Russell, 1983), may also explain why this screen failed to identify a *S.cerevisiae* homolog of *wee1*. In view of these results it would clearly be worthwhile to screen for M phase inhibitors using a library in which cDNA copies of yeast genes are expressed from a regulated promoter.

Disregarding possible complications involving divergence of transcription processes, evidence exists suggesting that *CDC6* may play a particularly important role in a DNA replication checkpoint control. It has been shown that *cdc6-1* diploids exhibit an extremely high rate of chromosome loss when grown at a semi-permissive temperature (Hartwell and Smith, 1985), higher than any other *cdc* mutant that was tested. This may indicate that when grown at the semi-permissive temperature, *cdc6-1* mutants complete the *CDC6* DNA replication function while failing to fully activate a checkpoint control.

Our results strongly suggest that *CDC6* delays the onset of mitosis by a mechanism that involves inhibition of the activation of p34^{cdc2}–cyclin M phase kinase. Flow cytometry verified that the delay occurred after S phase, whereas microscopic analysis revealed that progression through M phase was not delayed. This suggests that the *CDC6*-induced cell cycle delay occurs in G_2 phase, before activation of the p34^{cdc2}–cyclin kinase. Consistent with this conclusion, the *CDC6*-induced cell cycle delay in *S.pombe* was found to be strongly additive with mutations causing partial loss of the activity of the *cdc25* phosphatase, which activates the p34^{cdc2}–cyclin M phase kinase. The same additive interaction was observed in *S.cerevisiae* mutants lacking *MIH1*, the budding yeast homolog of *cdc25* (Russell *et al.*, 1989). The simplest interpretation of these interactions is that defects in *cdc25/MIH1* and expression of *CDC6* both delay the execution of the same rate-limiting step in late G_2, namely activation of p34$^{cdc2/CDC28}$–cyclin M phase kinase.

Materials and methods

Strains and plasmids

All *S.pombe* strains were derived from wild type 972h$^-$. All the strains used in this study were *leu1*-32 and *ura4*-294 or *ura4*-d18 except as noted. The genotype of the strains used in the present work were: RE1, *adh::cdc25$^+$ (ura4$^+$) wee1*-50; RE3, *cdc2*-3w *wee1*-50; *cdc25*-22; *cdc2*-3w; *adh::cdc25$^+$ (ura4$^+$)*; *cdc25::ura4$^+$ cdc2*-3w; and *wee1*-50 *mik1::LEU2*.

The genotype of the *S.cerevisiae* strains utilized in this study were: J16D, *MAT*a *trp1 leu2 ura3 his$^-$*; 15Dau, a *MAT*a derivative of BF264-15D (Cole *et al.*, 1990); *MAT*a *cdc6*-1 *trp1 leu2 ura3 his$^-$*; and GCY11, 15Dau *bar1::LEU2*.

To clone *MPI1*, a *S.pombe* RE3 strain (Russell and Nurse, 1987b) was transformed with a *S.cerevisiae* library made into YEp13 (Nasmyth and Reed, 1980) that carries the *LEU2* gene which is able to rescue the *leu1*-32 point mutation of fission yeast. The transformants were incubated at 25°C and then shifted to the restrictive temperature. The complete DNA sequence of a 5.5 kb fragment containing *MPI1* (*CDC6*) was determined (as indicated in Figure 1), including 3 kb of 5′ flanking sequence and 1 kb of 3′ flanking sequence. The open reading frame of *CDC6* was identical to that reported by Zhou *et al.* (1989), although there were numerous differences in the 5′ flanking region. The sequence of the 2.5 kb fragment containing *CDC6* open reading frame, 5′ and 3′ possible control elements has been submitted to the EMBL/GenBank/DDBJ (accession number X65299).

To construct plasmids pARTMPI1 and pARTrMPI1, the *MPI1* ORF was amplified by PCR using as template pIM.1 under the conditions described before (Sadhu *et al.*, 1990) except that the hybridization temperature was 55°C. The 26mer 5′ oligonucleotide had the sequence: 5′-ATGGATCCT-TC**ATG**TCAGCTATACCA-3′ where the underlined sequence contains *Bam*HI restriction enzyme site and the 3′ proximal 15 nucleotides correspond to the beginning of *MPI1* ORF (from the ATG in bold). The 23mer 3′ oligonucleotide had the sequence 5′-ATGGATCCCCCACCTCCCCCTA-A-3′. The underlined sequence contains a *Bam*HI site and the reverse complement of the 3′ proximal 15 nucleotides correspond to the 3′ end of the 5.5 kb sequenced fragment. The reaction products were digested with *Bam*HI and separated by agarose gel (1%) electrophoresis. The 1.8 kb electroeluted band was cloned into the *Bam*HI digested vector pART1 (Russell, 1989). Plasmids pIRT2Uadh:MPI1 and pIRT2Uadh:rMPI1 were made by inserting the 2.7 kb *Sph*I–*Eco*RI fragment carrying *adh::MPI1* of pART1MPI1 into *Sph*I and *Eco*RI cut pIRT2U (Russell, 1989) and selecting for recombinant plasmids with the *Eco*RI fragment containing the *ars1* element. To construct plasmids YCpG2CDC6 and YCpG2rCDC6, the same *Bam*HI-cut PCR fragment described above was inserted into *Bam*HI-digested vector YCpG2. Integrative vectors YIpG2CDC6, YIpG2rCDC6, YIpG3CDC6 and YIpG3rCDC6 were constructed by ligating the *Bam*HI *CDC6* PCR fragment into the single *Bam*HI site of YIpG2 or YIpG3 respectively. YIpG2 bears the *LEU2* gene (Lew *et al.*, 1991). YIpG3, is a pUC18 derivative which carries the 0.7 kb *Bam*HI–*Eco*RI *GAL1* promoter fragment and the 1.2 kb *Hin*dIII fragment containing the *URA3* gene (Russell *et al.*, 1989). Integrative transformations in *S.cerevisiae* were targeted into the *leu2* or the *CDC6* loci. Three different PCR reactions were carried out in order to ensure the reproducibility of the G_2 delay caused by deregulated expression of *CDC6*. From each one a number of clones (at least 4) were inserted in the appropriate plasmids and tested in *S.cerevisiae* and *S.pombe* for G_2 delay. All of them rendered the described phenotype.

rad9 deletions strains were constructed in 15Dau *leu2::GAL1:CDC6 (LEU2)* or 15Dau *CDC6::GAL1:CDC6(URA3)* haploid backgrounds transforming them with plasmids pTW030 *rad9::URA3* and pTW033 *rad9::HIS3* cut with *Not*I (Weinert and Hartwell, 1990). Approximately one-third of the generated prototrophs for uracil had the correct genomic structure, as determined by Southern hybridization (data not shown). Only one-sixth of the histidine prototrophs were *rad9* deletions, a fact that might be explained for the high frequency of reversion of the *his3* mutant allele.

Culture conditions and general techniques

All media used in growing *S.pombe* strains have been described by Moreno *et al.* (1991). *S.cerevisiae* cultures were grown in YEPD (1% Difco Yeast Extract, 2% Difco Bacto Peptone, 2% Glucose) except when selecting for plasmids, in these cases cultures were grown in EMM with supplemented amino acids. General molecular genetic techniques were performed as described by Sambrook *et al.* (1989) and Moreno *et al.* (1991).

DNA sequence analysis was performed by the dideoxy nucleotide method (Sanger *et al.*, 1977). DNA restriction fragments were cloned in pBluescript (Stratagene). Unidirectional deletions of the DNA fragments cloned in pBluescript were made with Exonuclease III and Mung Bean nuclease following a modification of Henikoff's protocol (Henikoff, 1984). Sequence similarities were searched using IFIND and FASTDB programs from Intelligenetics, Inc. (CA) searching through SWISS-PROT (University of Geneva, Switzerland) and PIR (Protein Identification Resource maintained by the National Biomedical Research Foundation, Washington D.C.) protein Data Banks.

The DNA content of individual cells was measured using a Beckman Dickinson FACS IV Analyzer was used. Cells were prepared for flow cytometry by the method of Hutter and Eipel (Hutter and Eipel, 1979) staining them with propidium iodide.

Galactose induction experiments

Yeast cells were grown in YPRAF (1% Difco Yeast Extract, 2% Difco Bacto Peptone, 2% Raffinose) at 30°C until the cultures reached mid-log phase. Cell concentration was determined and adjusted to 1×10^6 cells/ml with YPRAF and then galactose was added to a final concentration of 2.5%.

Hydroxyurea-induced cell cycle arrest

Log phase cultures were grown in EMM at 29°C. Hydroxyurea was added to a final concentration of 10 mM and the culture was further incubated at the same temperature during 4 h (Enoch and Nurse, 1990). Cells were fixed and stained with DAPI as previously described (Moreno *et al.*, 1989).

Cell division size measurements

The length of cells at division was determined by using cells growing in early log phase ($1-2 \times 10^6$ cells/ml) in EMM liquid media at 29°C with shaking. Cell size was then determined by microscopic observation of living cells using a phase contrast microscope and a 100× objective with a micrometer. At least 21 cells were measured in each sample.

Cell cycle synchronization by α-factor treatment

Mating pheromone α-factor synchronization experiments were made growing 2 l of a *bar1$^-$* strain to a density of $A_{600} = 0.5$ in YEPD media followed by a treatment with 40 ng/ml of α-factor for 200 min at 23°C. Cells were collected by centrifugation, washed once in fresh media and reinoculated into 1 l of YEPD. Cells were further incubated with shaking for 220 min at the same temperature. Samples were taken at intervals of 20 min for morphological examination to determine the budding index and for RNA preparation. When the synchronization experiment involved strains harboring plasmids (pIM.1 or YCpG2CDC6) cells were cultured in selective media supplemented with 2.5% galactose until they reached a density of $A_{600} =$ 0.2, then collected by centrifugation and inoculated into YEPGAL (1% Difco Yeast Extract, 2% Difco Bacto Peptone, 2.5% Galactose). α-Factor was added when cells reached a density of $A_{600} = 0.5$ and samples were taken following the protocol described above.

RNA purification and Northern analysis

RNA was purified from yeast using a technique previously described (Moreno *et al.*, 1991). Total RNA was separated on 1.2% agarose gels with formaldehyde and transferred to nitrocellulose filters and the hybridization performed as recommended (Sambrook *et al.*, 1989). Probes were radiolabelled with [α-^{32}P]dATP using the random primed DNA labelling kit (Pharmacia) following manufacturer's instructions. DNA probes were: *CDC6*, the 0.8 kb *Pst*I–*Xba*I fragment internal to the open reading frame (Figure 1A); *CLN1*, the 0.7 kb *Nco*I–*Eco*RI [Wittenberg *et al.*

(1990)]; *LEU2*, the 1.4 kb *Cla*I−*Sal*I fragment derived from YEp13 (Nasmyth and Tatchell, 1980); and *ORFC*, the 1.2 kb *Bam*HI−*Eco*RI fragment derived from pIM.1 (Figure 1A).

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