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Cdc18 transcription and proteolysis couple S phase to passage through mitosis

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In fission yeast, cdc18p plays a critical role in bringing about the onset of S phase. We show that cdc18p expression is subject to a complex sequence of cell cycle controls which ensure that cdc18p levels rise dramatically as cells exit mitosis, before the appearance of CDK activity in G_1. We find that transcription of *cdc18*, together with the transcription of other cdc10p/res1p targets, is first initiated as cells enter mitosis and continues even in cells arrested in mitosis with highly condensed chromatin. However, cdc18p cannot accumulate during mitosis because it is targeted for proteolysis by mitotic cdc2p-protein kinase-mediated phosphorylation. On exit from mitosis, the cdc2p mitotic kinase activity falls, stabilizing cdc18p, which then rapidly accumulates. This combination of mitotic transcription and CDK-mediated proteolysis ensures that progression through mitosis simultaneously prepares cells for DNA replication. During S phase, *cdc18* transcription is then switched off, preventing the re-initiation of DNA synthesis until the completion of the next round of mitosis.

Keywords: *cdc18*/cell cycle/periodic transcription/proteolysis/S phase

Introduction

In eukaryotes, the decision to undergo DNA replication is subject to several controls. In late G_1, cells assess internal and external cues before committing themselves to a mitotic cycle and entry into S phase. These controls are best understood in the budding yeast *Saccharomyces cerevisiae*. In this organism, the Cln3p/Cdc28p protein kinase becomes activated early in G_1 (Tyers *et al*., 1993; Dirick *et al*., 1995) leading to an increase in the activity of two transcriptional complexes, Swi4p/Swi6p and Mbp1p/Swi6p (Andrews and Herskowitz, 1989; Nasmyth and Dirick, 1991; Ogas *et al*., 1991). Targets of this periodic transcription include the enzymatic functions necessary to bring about DNA replication, e.g. ribonucleotide reductase (Johnston and Lowndes, 1992; Breeden, 1996) and additional cyclin partners for Cdc28p which drive the initiation of S phase (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). In metazoan cells, Cdk4,6/cyclin D kinase activity accumulates in G_1, inactivating Rb (Sherr, 1995). This frees the E2F/DP1 transcription factor from repression, enabling the transcription of cyclins A and E. These cyclins, together with a CDK subunit, then initiate DNA replication.

Initiation requires conserved protein complexes acting at origins of replication (Stillman, 1996). The origin recognition complex (ORC) binds origins of replication throughout the *S.cerevisiae* cell cycle (Bell and Stillman, 1992; Diffley and Cocker, 1992). Then in G_1, Cdc6p and MCM proteins are recruited to the origin by the ORC complex (Cocker *et al*., 1996; Aparicio *et al*., 1997; Tanaka *et al*., 1997) giving rise to an extended 'pre-replicative' complex at the origin, as visualized by genomic footprinting (Diffley and Cocker, 1992; Diffley *et al*., 1994). Late in G_1, cyclin/CDK activity is required to catalyse the initiation of DNA synthesis, presumably by phosphorylating components at the origin (Stillman, 1996). After initiation, Cdc6p is lost from chromatin, while MCM proteins gradually dissociate during DNA synthesis (Chong *et al*., 1995; Kubota *et al*., 1995; Coleman *et al*., 1996; Aparicio *et al*., 1997).

The control of DNA replication in the fission yeast *Schizosaccharomyces pombe* shows both similarities and differences to that in *S.cerevisiae*. In *S.pombe*, the S-phase transcriptional machinery is composed of cdc10p, res1p, res2p and rep2p (Aves *et al*., 1985; Tanaka *et al*., 1992; Caligiuri and Beach, 1993; Miyamoto *et al*., 1994; Zhu *et al*., 1994; Nakashima *et al*., 1995; Baum *et al*., 1997). Cdc10p, res1p and res2p share homology with Swi4p, Swi6p and Mbp1p from budding yeast. The targets of this transcriptional machinery are expressed periodically in the cell cycle and include *cdc18*, *cdc22*, *cdt1* and *cig2* (Gordon and Fantes, 1986; Kelly *et al*., 1993; Hofmann and Beach, 1994; Obara-Ishihara and Okayama 1994). *Cdc18* is homologous to *CDC6* in *S.cerevisiae* and is thought to be a critical target of *cdc10* in fission yeast, as temperature-sensitive mutations in *cdc10* can be rescued by the ectopic expression of *cdc18* even at a high restrictive temperature (Kelly *et al*., 1993). Since the onset of S phase in fission yeast requires both periodic transcription and B-type cyclin/CDK activity, it is often assumed that the control of S-phase transcription in both yeasts is controlled in an analogous fashion. However, recent evidence suggests that this may not be the case because, in *S.pombe*, cdc10p/res1p mediated transcription is active in G_1, independently of the G_1 cdc2p protein kinase (Baum *et al*., 1997).

Because fission yeast cells are unable to replicate their DNA in the absence of *cdc18* (Kelly *et al*., 1993), while high-level over-expression of cdc18p drives cells into a cycle of continuous DNA synthesis without mitosis (Nishitani and Nurse, 1995; Muzi-Falconi *et al*., 1996), the periodic accumulation of cdc18p through the cell cycle is likely to be important for orderly cell cycle progression. This cyclic accumulation of cdc18p is controlled in part by periodic *cdc18* transcription (Kelly *et al.*, 1993). In addition, CDK activity promotes the proteolysis of cdc18p. This has been suggested to be important in S- and G_2-phase cells to prevent the re-initiation of DNA replication (Elsasser *et al.*, 1996; Jallepalli *et al.*, 1997; Lopez-Girona *et al.*, 1998). In *S.pombe* and human cells, dramatic reductions in G_2 CDK activity can also perturb the cell cycle, causing cells to re-enter S phase without first undergoing nuclear division (Broek *et al*, 1991; Hayles *et al.*, 1994; Moreno and Nurse, 1994; Itzhaki *et al.*, 1997). Moreover, in *S.cerevisiae*, G_2 CLB/CDK activity inhibits the formation of the pre-replicative complex (Piatti *et al.*, 1996; Tanaka *et al.*, 1997), and in *Xenopus* extracts CDK activity blocks the ability of MCMs to bind to DNA (Adachi and Laemmli, 1994; Hua *et al.*, 1997). Therefore, it is likely that ordered passage through the eukaryotic cell cycle is controlled by the interplay of components of the pre-replicative complex, in particular Cdc6p/cdc18p, with the cyclic activity of CDK–cyclin complexes.

Previously, it was thought that events leading to the onset of DNA replication in eukaryotes were initiated in G_1 in response to the re-accumulation of CDK activity. In this model, G_1 CDK–cyclin complexes activate S-phase transcription, leading to the accumulation of cdc18p/Cdc6p in late G_1 and the initiation of S phase. The same G_1 CDK activity was also shown to destabilize cdc18p/Cdc6p. This leaves it unclear how cdc18p is able to accumulate in the presence of G_1 CDK activity to perform its essential S-phase function. In this paper we re-assess the timing of periodic S-phase transcription in fission yeast. Unexpectedly, we show that *cdc18* is first transcribed early in mitosis in cells with condensed chromatin and in the presence of high levels of the mitotic kinase. Despite the accumulation of *cdc18* transcripts, cdc18p does not appear until cells exit mitosis, because it is destabilized by cdc2p phosphorylation of its N-terminus. Therefore, the combination of *cdc18* transcription during mitosis and of CDK-mediated cdc18p proteolysis gives rise to the timely production of cdc18p at the exit from mitosis. In view of this, the CDK-mediated proteolysis of cdc18p may function primarily to prevent the initiation of S-phase events within mitosis. Thus, controls over cdc18p expression contribute to orderly cell cycle progression by linking passage through mitosis to the setting-up of the subsequent S phase.

Results

cdc10-dependent transcription activated early in mitosis

The transcription of several components required for S phase in fission yeast such as *cdc18*, *cdc22* and *cdt1*, is dependent upon the transcription factor cdc10p. To determine the cell cycle timing of *cdc10*-dependent transcription, we followed transcription through a synchronous cell cycle. *cdc25-22* cells were incubated at the restrictive temperature of 36°C for 4 h to arrest them at the G_2/M boundary, and were then shifted to the permissive temperature of 25°C, releasing cells into mitosis and a subsequent cell cycle (Figure 1). The accumulation of *cdc18* mRNA was compared with two markers of progression through mitosis (Figure 1B), the presence of the cdc13p associated protein kinase and the state of the mitotic spindle. *cdc18* message levels rose within 20 min of the shift to the permissive temperature, just as the levels of cdc13p-associated H1 kinase and the percentage of cells with a short spindle were increasing (Figure 1B). *cdc18* expression persisted into G_1, decreasing as cells passed from G_1 into S and G_2 phase. Two other *cdc10* targets, *cdc22* and *cdt1*, showed a similar profile of expression (data not shown). These data suggest that a number of *cdc10* target genes are transcribed from early mitosis until late G_1/S-phase. Similar results were obtained using a synchronous culture of *cdc25-22* cells previously starved of nitrogen (data not shown). This starvation prevents cell growth and thereby rules out an effect of cell size on the timing of S-phase transcription.

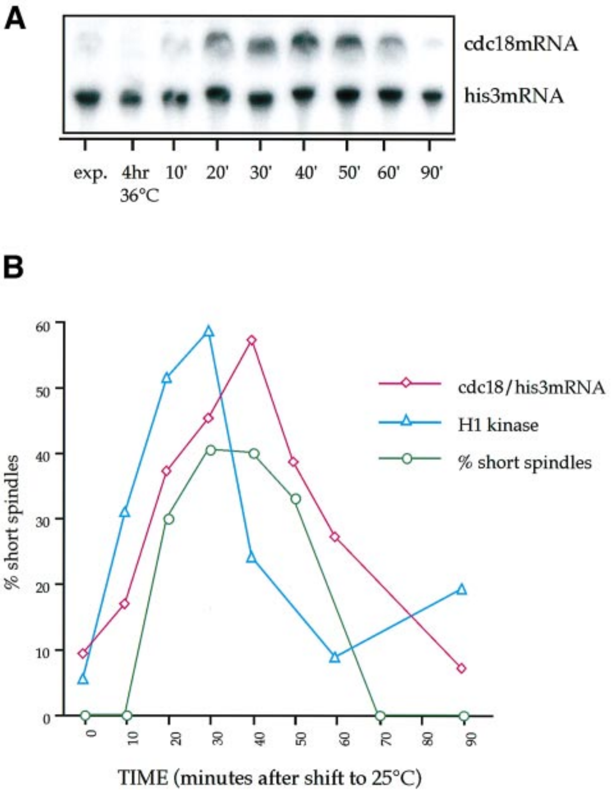

Fig. 1. *cdc18* is expressed in mitosis in cells released from a *cdc25* block. (**A**) *cdc18* mRNA levels were monitored through a synchronous mitosis. *cdc25-22* cells were shifted to 36°C for 4 h to arrest them at the G_2/M boundary and then synchronously released into mitosis and the subsequent round of the cell cycle at 25°C. (**B**) The Northern in (A) was quantified by PhosphorImager analysis and the ratio *cdc18/his3* plotted. Mitotic progression was monitored by measuring cdc13p-associated H1 histone kinase activity and by visualizing the percentage of cells with short spindles by immunofluorescence.

To verify that *cdc10*-dependent transcription is switched on early in mitosis, we next assayed transcript levels in cells arrested in mitosis using mutations in *nuc2* or *nda3* and by the expression of *cdc13*Δ*90*. *nuc2-663* cells are defective in the APC complex (or ‘cyclosome’) which controls the proteolysis of B-type cyclins and other specific targets (Kumada *et al.*, 1995; Ruderman and Hershko, 1995; King *et al.*, 1996). Since the APC is essential for the initiation of anaphase and the exit from mitosis, *nuc2*^{ts} cells accumulate at the metaphase–anaphase transition at the restrictive temperature, with high levels of mitotic

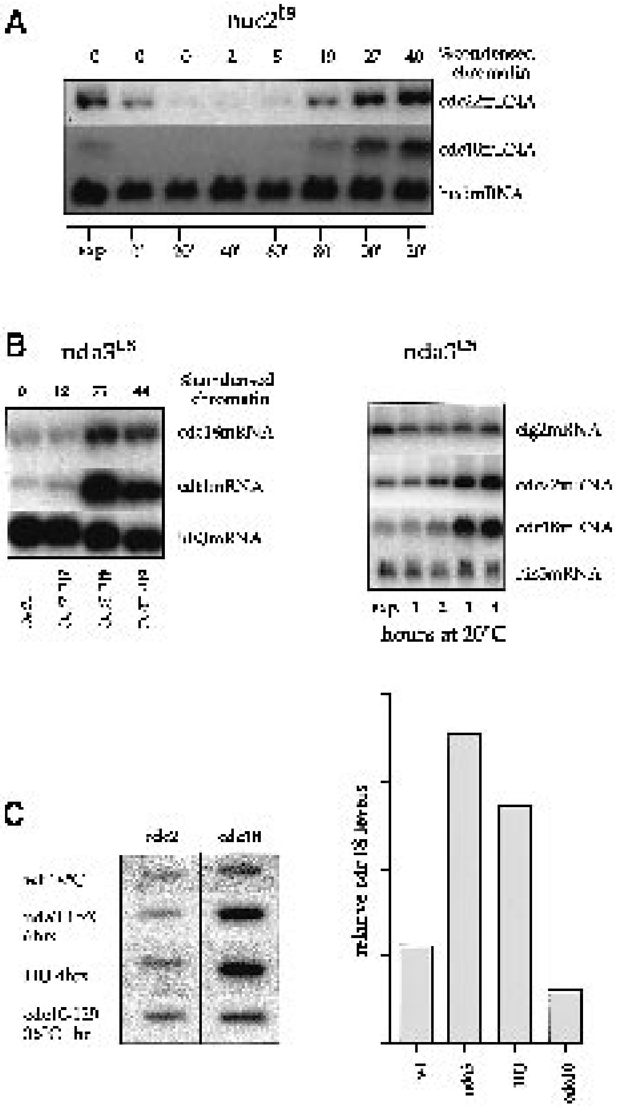

Fig. 2. *cdc10* targets are actively transcribed during mitotic arrest. (**A**) A synchronous culture of *nuc2-663* cells prepared by centrifugal elutriation at 25°C was shifted to 36°C. *cdc18*, *cdc22* and *his3* mRNAs were monitored as cells entered mitosis, and DAPI was used to quantify condensed chromatin. (**B**) An asynchronous population of *nda3-km311* cells grown at 32°C was shifted to a restrictive temperature, 20°C, to arrest cells at metaphase. A Northern was prepared and probed for *cdc18*, *cdt1* and *his3*. Cells with condensed chromatin were visualized using DAPI. The experiment was then repeated and a Northern probed for *cdc18*, *cdc22*, *cig2* and *his3* mRNAs. (**C**) A nuclear run-on experiment was used to assess ongoing transcription in a mitotic block. Nascent transcription was analysed in cells arrested at the onset of S phase by the addition of 11 mM HU, in *nda3-km311* and wild-type cells after 6 h at 18°C, and in *cdc10-129* cells at 36°C. Radiolabelled RNA was isolated and hybridized to ssDNA. *cdc18*, *cdc2* and *ura4* probes in both sense and anti-sense orientations, together with vector alone, were used to determine the background signal. Raw data is presented together with quantification by PhosphorImager analysis.

cdc2p/cdc13p kinase activity and highly condensed chromatin (Kumada, *et al.*, 1995). To arrest synchronously *nuc2*^{ts} cells in mitosis, early G_2 cells were isolated by centrifugal elutriation at 25°C, then shifted to 36°C and sampled as they entered the mitotic block. *cdc18* and *cdc22* transcripts appeared as cells accumulated condensed chromatin, visualized using DAPI (Figure 2A). Histone transcript levels did not increase (data not shown), indicating that cells did not leak through the block into S phase.

The accumulation of *cdc10*-dependent transcripts during a metaphase block was confirmed using a cold-sensitive mutation in β-tubulin, *nda3-km311*, which arrests cells at the metaphase–anaphase transition as the result of a spindle checkpoint, with high levels of cdc2p/cdc13p kinase activity and condensed chromatin (Umesono *et al.*, 1983; Hiraoka *et al.*, 1984). The arrest is transient at 20°C and more prolonged at 18°C. *cdc18*, *cdt1* and *cdc22* mRNAs accumulated 3–4 h after *nda3* cells were shifted to a restrictive temperature (20°C), in parallel with the proportion of cells containing condensed chromatin (Figure 2B). The levels of *cdc18*, *cdt1* and *cdc22* mRNAs fell as cells leaked through the mitotic block. *cig2* transcript levels were not elevated during the arrest, suggesting that *cig2* may be subject to different cell cycle controls (Figure 2B).

To test whether *cdc18* is actively transcribed during mitosis, a nuclear run-on experiment was carried out (Humphrey *et al.*, 1994). Cells were permeabilized and transcriptional elongation assayed by the incorporation of radioactive UTP into nascent mRNA strands. Ongoing *cdc18* transcription was compared in exponentially growing cells and in cells arrested in mitosis and normalized to levels of *cdc2* transcription (see Materials and methods for details). In cells arrested at the *nda3*^{cs} block or in S phase (in HU), the rate of ongoing transcription was elevated compared with that of control wild-type cells. As expected, *cdc18* transcription was reduced in *cdc10-129* cells at 36°C (Figure 2C). These results indicate, unexpectedly, that *cdc18* is actively transcribed during mitosis, leading to the accumulation of *cdc18* mRNA.

To show that the mitotic expression of *cdc18* results from *cdc10*-dependent transcription, a strain was constructed carrying the *nda3*^{cs} mutation together with *cdc10-129* (Figure 3A). Cells were first arrested in mitosis by incubation at 18°C for 6 h and thiabendazole was added to delay mitotic exit. Cells were then shifted to 36°C to inactivate *cdc10*^{ts} in the continued presence of thiabendazole. The temperature shift had no effect on the levels of *cdc18* mRNA in control cells, but in cells carrying a *cdc10*^{ts} allele, *cdc18* mRNA levels fell to background within 5 min of the shift to 36°C. This demonstrates that the high levels of *cdc18* mRNA observed in mitotic cells result from active, *cdc10*-dependent transcription. This result was confirmed by arresting cells in mitosis using over-expression of the non-degradable B-type cyclin, *cdc13Δ90* (Figure 3B) (Murray *et al.*, 1989). In this situation cells enter mitosis, but cannot exit into G_1 or decondense their chromosomes, since the cdc2p/cdc13p mitotic kinase cannot be inactivated. Upon induction of *cdc13Δ90* expression in the wild type, cells underwent mitotic arrest and *cdc18* mRNA rose to levels similar to those observed in an HU-induced arrest (Figure 3B, lanes 1–4). To confirm that this was the result of ongoing *cdc10*-dependent transcription, *cdc13Δ90* was expressed in *cdc10*^{ts} (Figure 3B, lanes 5 and 6) and *res1Δ* backgrounds (Figure 3B, lanes 7–9). In the *cdc10-129* strain, upon induction of *cdc13Δ90* at 25°C, *cdc18* mRNA levels became elevated as cells entered the mitotic arrest, but then decreased to low levels after the shift to 36°C. In the *res1Δ* strain, in which S-phase transcription is constitutive through the cell cycle (Baum *et al.*, 1997),

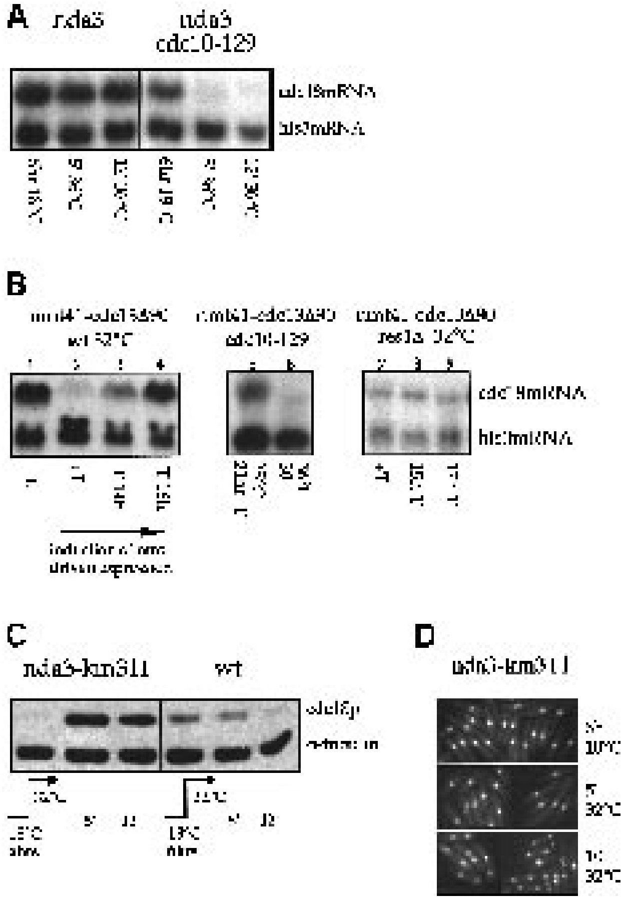

Fig. 3. The mitotic expression of *cdc18* is dependent on *cdc10* function and cdc18p cannot accumulate until exit from mitosis. (**A**) *nda3* and *nda3 cdc10-129* cells were arrested in mitosis for 6 h at 18°C, and 150 µg/ml of thiabendazole was added prior to a shift to 36°C. *cdc18* and *his3* transcript levels were monitored by Northern analysis. (**B**) Wild-type, *cdc10-129* and *res1*Δ strains were used containing an integrated copy of *cdc13Δ90* behind the *nmt41* promoter. Wild-type cells were assayed 14 and 16 h after thiamine removal (2–4 h after the start of derepression). Cells arrested in HU for 4 h were used for comparison. *res1*Δ cells were sampled after 15 and 17 h after thiamine removal, while *cdc10-129* cells were grown for 21 h at 25°C and then shifted to 36°C for 30 min to inactivate *cdc10^ts^*. Northern blots were used to assess the levels of *cdc18* and *his3* mRNA. (**C**) Exponentially growing *nda3-km311* and wild-type cells grown at 32°C were shifted to 18°C for 6 h and then to 36°C. Protein samples were analysed by Western blotting using anti-cdc18p and anti-α-tubulin antibodies. (**D**) A similar experiment presented to show the synchrony of anaphase obtained using this procedure; nuclei were observed by DAPI staining of fixed cells.

no increase in *cdc18* mRNA was seen during mitotic arrest. We conclude that *cdc10*-dependent transcription is active from early mitosis through until G_1/S phase.

Since *cdc10*-dependent transcription is active in a metaphase arrest, we explored whether *cdc10* is able to complete its essential S-phase function during the mitosis of the previous cell cycle. In order to do this, *nda3-km311*, *cdc10-129* and *nda3-km311* cells were arrested in metaphase for 6 h at 18°C and then shifted to 36°C to inactivate *cdc10^{ts}*. Subsequently, whereas *cdc10^{+}* cells underwent DNA replication at 36°C, *cdc10^{ts}* cells were unable to do so (FACS data not shown). Therefore, *cdc10* cannot complete its S-phase function during a mitotic arrest.

We next investigated whether the high levels of *cdc18* transcription during mitosis result in the accumulation of cdc18p. A mitotic arrest was induced by shifting *nda3-km311* mutant cells to 18°C for 6 h. Cells were then shifted to 32°C to release them into the subsequent cycle (Figure 3C). Cdc18p was undetectable during the mitotic block, in spite of the elevated levels of *cdc18* mRNA (Figure 3A). However, cdc18p accumulated to high levels within 5 min of release from the mitotic arrest, at the same time at which the majority of cells initiated anaphase. There was no concominant increase in *cdc18* mRNA levels (data not shown). *nda3* cells were stained 5 and 10 min after release from the mitotic block with DAPI to visualize anaphase (Figure 3D). In the wild-type control, the only observed change was a decrease in cdc18p levels after the temperature shift, possibly due to the transient mitotic arrest brought about by a shift to higher temperatures (Nurse, 1975) (Figure 3C, right-hand panel). The inability of cdc18p to accumulate in a metaphase arrest may explain why the *cdc10* function is not completed in cells arrested in an *nda3* block.

Mitotic instability of cdc18p is regulated by cdc2p phosphorylation

To test whether upstream regulatory regions of the *cdc18* gene are important in bringing about this increase in cdc18p levels on exit from mitosis, either through effects on transcription or translation, the *cdc18* ORF was fused with the weak regulatable *nmt81* promoter. This construct was introduced into an *nda3^{cs}* mutant deleted for the endogenous *cdc18* gene, and a block-and-release experiment was performed. Like the endogenous full-length *cdc18* gene, cdc18p expressed from the *nmt* promoter was barely detectable during mitotic arrest, but accumulated rapidly after release (Figure 4B). Therefore, the rapid accumulation of cdc18p upon mitotic exit is probably the consequence of regulated proteolysis. In agreement with this, it has recently been shown that cdc18p is subject to proteolytic control (Jallepalli *et al*., 1997) mediated by ubiquitination, targeting the protein to the proteosome (Kominami and Toda, 1997).

Cdc18p contains six *cdc2* phosphorylation consensus sites, five located in the N-terminal region (Figure 4A). Phosphorylation of these sites by the S-phase cdc2p kinase destabilizes cdc18p in exponential cultures and during S phase (Jallepalli *et al*., 1997). To investigate whether cdc2p-dependent phosphorylation is important for regulating cdc18p levels during mitosis, we constructed several mutant forms of cdc18p (Figure 4A), either lacking the entire N-terminus, containing five *cdc2* consensus phosphorylation sites (S/T–P–X–K/R), or carrying mutations in these *cdc2* sites.

First, we investigated whether phosphorylation of the N-terminal portion of cdc18p plays a role in determining cdc18p levels during mitosis. In initial experiments, two N-terminal truncations were constructed, both of which lacked five of the six cdc2 consensus phosphorylation sites (Figure 4A). These truncated versions of cdc18p were constitutively expressed from the weak *nmt81* promoter, at close to endogenous levels, in an *nda3^{cs}* mutant strain. Cells were then subjected to an *nda3* block and release protocol (as in Figure 3C). Although the endogenous cdc18p was unable to accumulate in mitosis, removal of the N-terminal region containing the cdc2 consensus sites

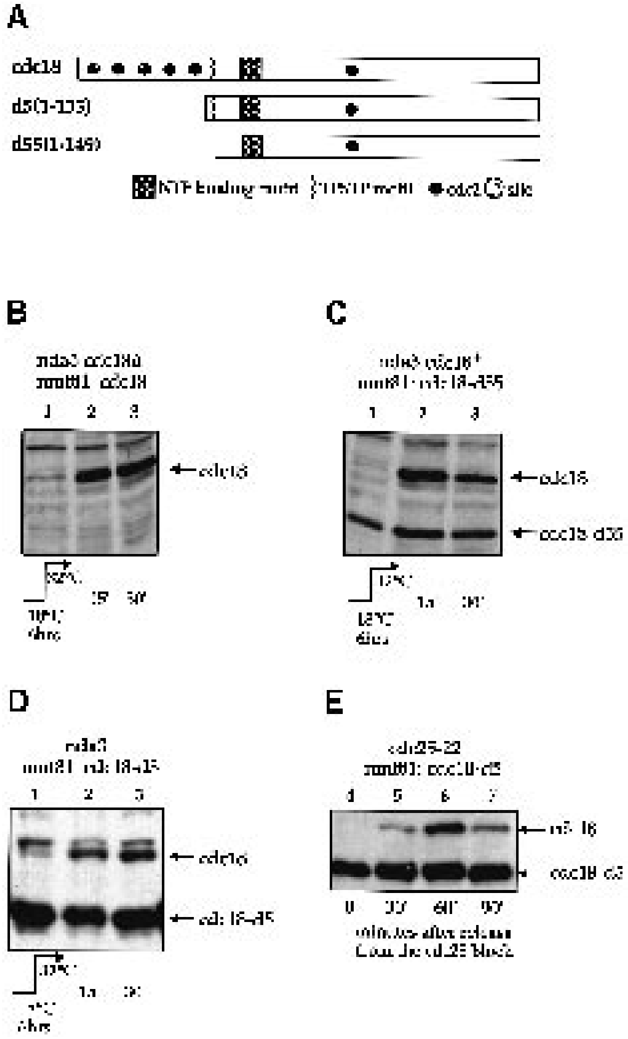

Fig. 4. The cdc18p N-terminus is required for the protein's periodic instability. (**A**) A schematic is shown of cdc18p and the truncated versions of the protein used in the following experiments. All five N-terminal cdc2 consensus phosphorylation sites are contained within the first 135 N-terminal amino acids. (**B**) Cells constitutively expressing their only copy of cdc18p from the *nmt81* promoter on a plasmid were subjected to an *nda3* block-and-release protocol, (lanes 1–3). Cells were released at 32°C after 6 h at 18°C, and Westerns probed with anti-cdc18p antibody. (**C**–**D**) *nda3-km311* cells ectopically expressing truncated versions of cdc18p lacking the N-terminal (C) 149 amino acids (d55) and (D) 135 amino acids (d5) from the *nmt81* promoter were subjected to the same *nda3* block and release protocol. (**E**) An alternative synchronization procedure was used in which *cdc25-22* cells expressing *cdc18-d5* were followed through a synchronous mitosis, G_1 and S phase. In all cases, the levels of cdc18p were monitored using a polyclonal antibody raised against cdc18p.

in *cdc18-d55* was sufficient to stabilize the protein in the *nda3* block (Figure 4C, lane 1). Furthermore, the level of *cdc18-d55* protein did not increase significantly upon release into G_1 (Figure 4C lanes 2–3). The less extensive truncation of *cdc18*, *cdc18-d5*, behaved similarly (Figure 4D). The observation that cdc18p lacking its N-terminal portion is stable during mitosis was confirmed using a *cdc25-22* mutant to synchronize cells (Figure 4E). *cdc18-d5* protein was expressed from the *nmt* promoter during a block-and-release experiment. While wild-type cdc18p was absent in G_2 and accumulated transiently in G_1 of the subsequent cycle (peaking 60 min after release from the block), the truncated version remained at constant levels throughout the cell cycle. This shows that the N-terminus is required for the periodic instability of cdc18p, targeting cdc18p for degradation in mitosis. In additional experiments, the full-length protein or N-terminal portions of cdc18p were shown by co-immunoprecipitation to bind to cdc2p *in vivo*, while the C-terminal truncations used in this experiment were unable to bind cdc2p (data not shown), suggesting that the N-terminus of the protein mediates the association of cdc18p with cdc2p. It is also possible that the N-terminus is required for the interaction of cdc18p with the proteolytic machinery itself (Drury *et al*., 1997).

Next, two cdc18p mutants were constructed to test whether the N-terminal cdc2 sites mediate the mitotic instability of cdc18p, one in which the five N-terminal cdc2 sites were mutated to a non-phosphorylatable alanine (*cdc2-5**) and a second version carrying mutations at all six cdc2 sites (*cdc2-6**) (Figure 4A). We confirmed previously published data which show that mutation of these cdc2 sites stabilizes cdc18p (Jallepalli *et al*., 1997) and that cdc2p activity is required for phosphorylation of these consensus sites *in vivo* (Jallepalli *et al*., 1997; Lopez-Girona *et al*., 1998). Myc-tagged cdc18p constructs were ectopically expressed in wild-type cells from the *nmt* promoter. When the *nmt* promoter was switched off, wild-type tagged cdc18p disappeared within 30 min (Figures 5A) but the *cdc2-5** and *cdc2-6** mutant proteins were still detectable 120 min after the addition of thiamine. Therefore, mutation of the cdc2 consensus phosphorylation sites within cdc18p stabilizes the protein. To confirm that cdc18p phosphorylation is dependent upon the cdc2p protein kinase *in vivo*, these same constructs were then expressed in a $cdc2^{ts}$ strain. At 25°C, when the cdc2p kinase was active, the mobility of wild-type cdc18p was shifted towards higher molecular weights. In contrast, the *cdc2-5** and *cdc2-6** mutant proteins, at both the permissive and restrictive temperatures, ran with the same mobility as the wild-type cdc18p in cells with inactive *cdc2* function (Figure 5B). This implies that cdc2p phosphorylates the wild-type protein at some or all of these consensus phosphorylation sites *in vivo*.

It is possible that different mechanisms mediate cdc18p turnover at different times during the cell cycle. Therefore, to observe the effect of these mutations on the mitotic instability of cdc18p and on its accumulation through the cell cycle, *nda3-km311* cells expressing myc-tagged proteins from the *nmt* promoter were subjected to a mitotic block and release (Figure 5C). While the endogenous and tagged wild-type cdc18p behaved similarly, mutation of the cdc2 sites stabilized cdc18p during mitosis. Also, the levels of mutant cdc18p did not increase further upon the release into G_1 of the subsequent cycle. This result was confirmed in a *cdc25* block and release experiment (Figure 5D). Again, while the endogenous or myc-tagged wild-type cdc18p did not accumulate until 40 min after release from G_2, (coinciding approximately with the decrease in mitotic kinase activity in Figure 1B), the *cdc2-6** mutant form of cdc18p remained stable during mitosis and throughout the subsequent cycle. Therefore, the *cdc2* consensus phosphorylation sites in the cdc18p N-terminus are required for the mitotic instability of cdc18p.

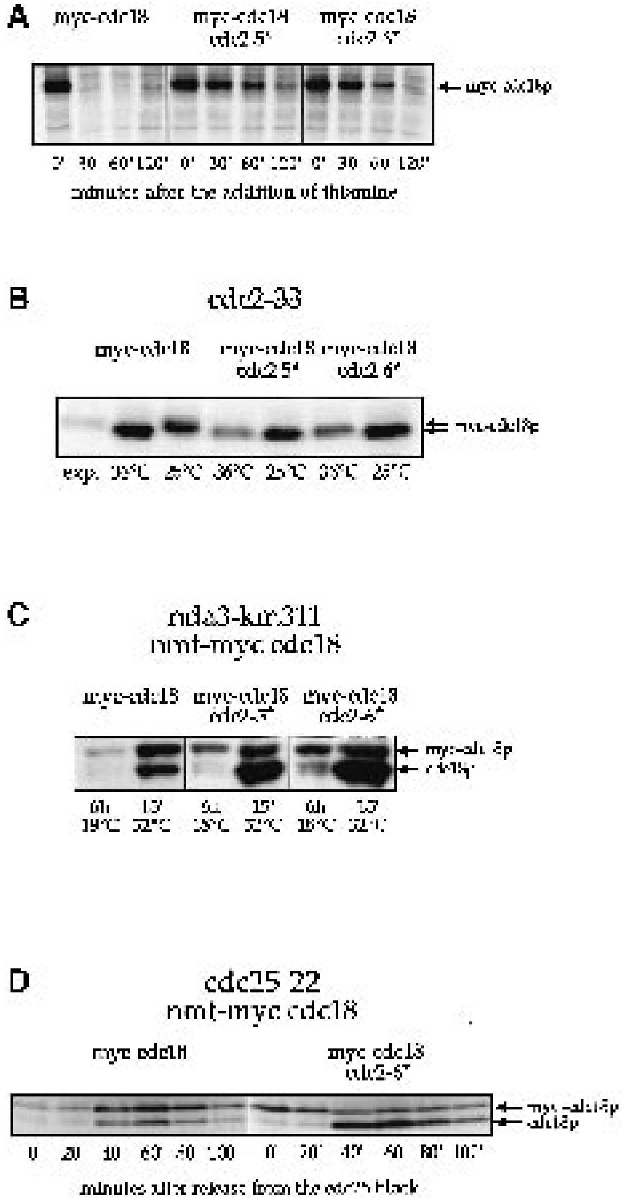

Fig. 5. Mutation of the cdc2 consensus phosphorylation sites contained within cdc18p prevents its periodic instability. (**A**) Myc-tagged cdc18p and tagged cdc2 site mutant versions of the protein were expressed from the *nmt81* promoter; *cdc18*, *cdc18-cdc2-5** and *cdc18-cdc2-6**. Thiamine was added, and the level of myc-cdc18p assessed using anti-myc antibody. (**B**) The ability of cdc2p to phosphorylate cdc18p at the consensus cdc2 sites was assessed *in vivo*. *cdc2-33* cells were transformed with myc-tagged wild-type and cdc2 consensus site mutant versions of cdc18p behind the *nmt81* promoter. Cells were shifted to the restrictive temperature for 30 min to remove cdc2p activity. The wild type was shifted back to 25°C for 30 min to re-activate the cdc2p kinase. The Western was probed with anti-myc antibodies to visualize the tagged protein. The same constructs were expressed in (**C**) *nda3-km311* (**D**) and *cdc25-22* cells. Cdc18p was monitored using a polyclonal anti-cdc18p antibody in an *nda3* block and after the release (C), and in a *cdc25* block-and-release experiment (D).

Discussion

The work presented in this paper investigates the regulatory mechanisms controlling the periodic expression of cdc18p, a key regulator of the decision to initiate DNA replication. We have found the following. (i) Transcription of *cdc18* is activated as cells enter mitosis, leading to an accumulation of *cdc18* mRNA during mitosis and G_1. (ii) Remarkably, transcription of *cdc18* continues even in cells blocked in metaphase with highly condensed chromosomes. (iii) This *cdc18* transcription is accompanied by the mitotic transcription of other *cdc10*-dependent genes, such as *cdc22* and *cdt1*, and requires the continued activity of the cdc10p 'start' transcription factor (homologous to the budding yeast Swi6p 'start' transcription factor). This means that so called 'start' transcription is initiated during mitosis, not in late G_1 as previously thought. (iv) Despite the presence of high levels of *cdc18* mRNA during mitosis, cdc18p is unable to accumulate until the exit from mitosis. (v) Cdc18p is stabilized by mutation of the consensus cdc2 phosphorylation sites located in its N-terminus, leading to the accumulation of cdc18p during mitosis and throughout the cell cycle. Furthermore, phosphorylation of these sites is dependent upon cdc2p activity *in vivo*. Therefore, the high level of the cdc2p/cdc13p protein kinase activity in mitotic cells prevents cdc18p from accumulating. However, since *cdc18* is already being actively transcribed and translated in metaphase, cdc18p can accumulate as soon as the cdc2p mitotic kinase activity falls. In this way, the combined regulation of *cdc18* transcription and cdc2p-mediated proteolysis leads to the transient accumulation of cdc18p immediately following the exit from mitosis, before the re-accumulation of CDK activity in late G_1.

The finding that *cdc10*-dependent transcription is activated in cells with condensed chromatin is surprising since it is widely accepted that active transcription cannot take place in mitotic chromatin. In metazoan nuclei, there is a clear general inhibition of transcription during mitosis (Johnson and Holland, 1965). In these cells transcription is switched off by the cdc2p mitotic kinase (Hartl *et al.*, 1993; Gottesfeld *et al.*, 1994; White *et al.*, 1995; Leresche *et al.*, 1996; Gebara *et al.*, 1997). Mitotic CDK activity has been shown to inhibit RNA polymerase function directly, both *in vivo* and *in vitro* (Hartl *et al.*, 1993; Gottesfeld *et al.*, 1994; White *et al.*, 1995; Leresche *et al.*, 1996; Segil *et al.*, 1996; Gebara *et al.*, 1997) and is able to clear certain transcription factors from the mitotic chromatin (Roberts *et al.*, 1991; Martinez-Balbas *et al.*, 1995; Segil *et al.*, 1996). In fission yeast, however, there is no evidence to suggest that transcription is highly repressed during mitosis, since the rate of RNA synthesis is maintained during passage through mitosis in synchronous cultures (Creanor and Mitchison, 1982; Elliot, 1983) and in cells arrested in mitosis (Novak and Mitchison, 1986, 1987). Nevertheless, it is surprising to find that the transcription of specific genes is activated as fission yeast cells enter mitosis and maintained in cells arrested in mitosis with highly condensed chromatin. Therefore, specific mechanisms may exist to enable cdc10p to activate transcription during mitosis. First, transcription factors need to target sites within condensed chromatin. This problem could be circumvented if cdc10p were bound to the DNA in G_2 cells in an inactive state (McInerny *et al.*, 1995; Baum *et al.*, 1997), thereby marking the promoters of *cdc10* targets for potential activation upon entry into mitosis. Alternatively, active promoters might be maintained in a more decondensed state throughout mitosis. In addition, cdc10p must also recruit active RNA pol II to promoters in mitotic chromatin and local decondensation

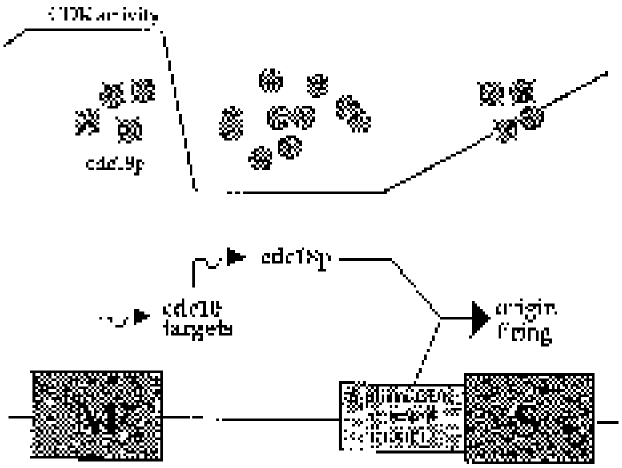

Fig. 6. A model for the control of *cdc10*-dependent transcription and the stability of cdc18p through the cell cycle. *cdc10*-dependent transcription becomes active in metaphase. Although *cdc18* is expressed in metaphase, cdc18p cannot accumulate until cells leave mitosis. Cdc18p can act as soon as cells enter G_1in preparation for the subsequent S phase. When cells in G_1 have reached a sufficient size to pass 'start', B-type cyclins re-accumulate. cdc2p/cyclin B complexes can then, in the presence of *cdc10* targets, initiate S phase. This simultaneously destabilizes cdc18p, preventing origin refiring. During S phase, *cdc10*-dependent transcription is switched off, contributing to the block over endo-reduplication.

or breathing of the chromatin may be required to enable the RNA polymerase to traverse the gene. Although the mechanisms controlling the periodicity of *cdc10*-dependent transcription remain unresolved, this study makes it clear that the activation of cdc10p/res1p is likely to be linked to mitotic events, not events, e.g. growth, during G_1. Chromatin condensation *per se* is probably not responsible for the activation signal because the ectopic expression of *NimA*, which induces condensation without mitosis (O'Connell *et al*., 1994), does not lead to an increase in *cdc18* transcript levels (data not shown). One possibility is that the mitotic activation of *cdc18* transcription is brought about by phosphorylation of a component(s) of the cdc10p complex by cdc2p at the onset of mitosis.

CDC6, the budding yeast homologue of *cdc18* was initially thought to be expressed in late G_1 (Zhou and Jong, 1990), but more recently has been shown to be expressed at telophase (at the exit from mitosis) (Zwerschke *et al*., 1994; Piatti *et al*., 1995) in an *SWI5*-dependent manner (Piatti *et al*., 1995), and to be under the transcriptional regulation of Mcm1p (McInerny *et al*., 1997). From these data it has been proposed that *CDC6* is expressed twice during the cell cycle, first at the exit from mitosis and then again at the end of G_1 (Piatti *et al*., 1995; Detweiler and Li, 1997). The second burst of *CDC6* transcription may be redundant because the M/G_1 burst suffices for S phase, even in cells which pause in G_1 (Piatti *et al*., 1995). This is reminiscent of the situation in *S.pombe*, in which *cdc18* mRNA appears to accumulate in G_1 upon re-entry into the cell cycle after nitrogen starvation (Baum *et al*., 1997), and in mitosis in cycling cells (this study). The different technical approaches required to follow transcription through the cell cycle (in cells exiting mitosis or during re-growth from G_1 arrest) may yield different profiles of expression, and in fact, two peaks of *CDC6*/*cdc18* expression have not been observed within a single cell cycle in synchronous cultures of either organism. Moreover, we have preliminary results suggesting that in budding yeast, *CDC6* is expressed to high levels in cells arrested at metaphase, i.e. in mitosis itelf, using temperature-sensitive mutations in *cdc20* and *cdc16* or by the addition of nocodazole (data not shown). Further investigation is required to firmly establish whether 'start' transcription factors assist in the mitotic expression of *CDC6* in *S.cerevisiae*. However, it seems that in both yeasts, S-phase genes are first transcribed in mitosis, suggesting a conserved function for this window of gene expression. Whether this is likely to be the case for transcription of S-phase genes (e.g. by E2F) in mammalian cells remains to be seen.

Despite the accumulation of *cdc18* transcripts, cdc18p is unstable and cannot accumulate during mitosis. However, an N-terminal truncation of cdc18p removing five of the six S/T–P–X–R/K consensus target sites for cdc2p-mediated phosphorylation within the protein, or mutation of these motifs, stabilizes cdc18p in mitosis. Therefore, the mitotic instability of cdc18p is conferred upon the protein by these N-terminal sites. The cdc2p protein kinase is likely to be directly responsible for this phosphorylation, since cdc18p has been shown to interact with cdc2p (Jallepalli and Kelly, 1996; Leatherwood *et al*., 1996; Brown *et al*., 1997; Jallepalli *et al*., 1997; Lopez-Girona *et al*., 1998), an interaction which is mediated by the N-terminus (H.Nishitani, unpublished data). Finally, cdc2p has also been shown to phosphorylate cdc18p on these sites *in vivo* (Jallepalli *et al*., 1997; Lopez-Girona *et al*., 1998; this study). Therefore cdc2p phosphorylates cdc18p at the five N-terminal cdc2 consensus sites destabilizing the protein during mitosis. The pop1p complex may direct phosphorylated cdc18p to the proteosome (Kominami and Toda, 1997). Like cdc18p, Cdc6p in *S.cerevisiae* is also subject to proteolysis via ubiquitin ligation, catalysed by the Cdc4p complex (Piatti *et al*., 1996; Drury *et al*, 1997), the counterpart to the pop1p complex in *S.pombe* (Kominani and Toda, 1997). Since Cdc4p binds to the N-terminus of Cdc6p (Drury *et al*., 1997), additional sequences in the N-terminal region of cdc18p may also be important for the interaction of cdc18p with the pop1p complex. If, as previously thought, cdc18p was first synthesized in late G_1, it is not clear how cdc18p, which is destabilized by cdc2p, could carry out its S-phase function. This dilemma is resolved by the finding that *cdc18* is first transcribed in mitosis. In this case, CDK-mediated proteolysis is likely to be important to inhibit the initiation of S-phase events during mitosis itself.

Cdc6p has been shown to act at the G_1/M boundary, at which time it is required to set up the 'pre-replicative complex' (Piatti *et al*., 1995; Cocker *et al*., 1996). If cdc18p acts in a similar way, then CDK control over cdc18p stability establishes a direct link between the end of mitosis and DNA replication 'licensing' (Blow and Laskey, 1988). Given the conserved function of the *cdc18* homologue, *CDC6*, in promoting DNA replication in metazoa, and the requirement for CDK activity to inhibit endo-reduplication in mammalian cells, it is possible that expression of cdc18p/Cdc6p at the exit from mitosis is also conserved in all eukaryotes. Later in the cycle, cdc18p is degraded as renewed cdc2p protein kinase activity drives the initiation of DNA replication (Jallepalli *et al*., 1997), while *cdc10* is inactivated during S phase (Baum *et al*., 1997). This prevents the re-initiation of S phase until cells pass through the subsequent round of mitosis, since as a result there is little or no cdc18p present in G_2 cells.

In conclusion, periodic transcription and CDK-regulated proteolysis act together in fission yeast to confine cdc18p function to the period from the end of mitosis until late G_1 (Figure 6). This contributes to the control ensuring that a single round of DNA replication follows each mitosis and leads to a simple scenario in which the pre-replicative state is defined by both the presence of cdc18p and the absence of cdc2p protein kinase activity, and the post-replicative state by high cdc2p kinase levels and low levels of cdc18p.

Materials and methods

Schizosaccharomyces pombe strains and methods

All strains were derived from *972h*⁻ and *975h*⁺. All experiments were carried out in EMM2 minimal media, and growth conditions are as described previously (Moreno *et al*., 1991). Unless otherwise stated, *nda3-km311* strains (Umesono *et al*., 1983) were grown to exponential phase at 32°C and *nda3-km311 cdc10-129* mutants at 28.5°C; *nuc2-663* (Kumada *et al*., 1995), *cdc10-129* (Nurse and Bissett, 1981), *cdc25-22* (Fantes *et al*., 1979) and *cdc2-33* (Nurse and Bissett, 1981) strains were grown at 25°C, *res1*Δ strains (Tanaka *et al*., 1992) at 30°C. The *cdc13*Δ*90* and *NimA* integrants were derived from strains described previously (O'Connell *et al*., 1994; Stern and Nurse, 1997). Different-strength thiamine-regulatable promoters, *nmt1*, *nmt41* and *nmt81*, were used (Maundrell, 1990, 1993). To generate the N-terminally truncated cdc18p mutants, *cdc18-d5* (amino acids 1–135) and *cdc18-d55* (1–149), the C-terminal fragments were amplified by PCR and subcloned into Rep81 and Rep41. The six threonine residues (at positions 10, 46, 60, 104, 134 and 374) were mutated to alanine using the Bio-Rad mutation kit and checked by sequencing. The myc-tagged versions were constructed using pRMH41 (a gift from Tony Carr).

Thiamine was used at 5 μg/ml, hydroxyurea (HU) at 11 mM and thiabendazole (TBZ), in DMSO, at 150 μg/ml. Cells were prepared for FACS and stained for propidium iodide as previously described (Sazer and Sherwood, 1990). Microtubules were detected in methanol-fixed cells by immunofluorescence using the TAT1 antibody (a gift from K.Gull) and DNA visualized using DAPI, as described previously (Moreno *et al*., 1991). Chromatin condensation was assessed visually.

Biochemical analysis

Western blotting was carried out as described previously (Moreno *et al*., 1991), using extracts from boiled cells. The following antibodies were used for Western blotting: cdc18p polyclonal antibody at 1:1500 (Nishitani and Nurse, 1995), α-tubulin monoclonal antibody at 1:10 000 (Sigma), myc monoclonal antibody at 1:1000 and a cig2p polyclonal antibody at 1:1000 (Stern and Nurse, 1997).

H1 kinase assays were carried out using calf thymus Histone H1 (Sigma) as a substrate, as previously described (Correa-Bordes and Nurse, 1995). Kinase was precipitated from 500 μg of protein using 5 μl of polyclonal cdc13p antisera, SP4, followed by the addition of protein A–Sepharose (Pharmacia Biotech.).

Transcript analysis

For RNA preparation and Northern blot analysis, cultures were washed in STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3 pH 8) and frozen on dry ice. RNA was prepared using glass bead lysis in 0.1 M EDTA, 0.1M NaCl, 0.05M Tris pH 8.0, in the presence of phenol:chloroform:isoamyl alcohol (Gibco-BRL) and 0.4% SDS. RNA was precipitated after two phenol extractions by the addition of NH_4OAc to 2.5 M and 2.5 vol. EtOH. Ten micrograms of sample RNA, as measured by an OD of 260/280, were denatured in 1× MOPS, 8% formaldehyde and 67% formamide and run on a formaldehyde, 1.2% agarose gel in 1× MOPS. The RNA was transferred by Northern blotting in 10× SSC onto a GeneScreenPlus membrane (DuPont). Probes for blotting were prepared by random oligo-priming with [α-^{32}P]dATP using a Prime-It Kit (Stratagene). The membrane was hybridized overnight in 1% SDS, 10% dextran sulfate and 1M NaCl, and washed in 1% SDS 2× SSC. The following template DNAs were used to generate probes: a *cdc18* fragment from REP3X-*cdc18* (Nishitani and Nurse, 1995), a *cig2* fragment from a genomic *cig2* clone in pAL-SK (S.Moreno), a *his3* fragment from a pKS *his3* plasmid and a histone *H2B* fragment from pSJM211 (Matsumoto and Yanegida, 1985). *cdt1* and *cdc22* probes were made from PCR fragments amplified from genomic DNA.

For the nuclear run-on, 2×10^8 cells were filtered and washed with 5 ml ice cold TMN buffer (10 mM Tris–HCl, 5 mM $MgCl_2$, 100 mM NaCl) and resuspended in 950 μl cold H_2O, and 50 μl of 10% sarcosyl was added. The detergent was removed after 20 min and cells were resuspended in 120 μl of 'run-on' buffer (50 mM Tris–HCl pH 7.9; 80 mM $MgCl_2$; 500 mM KCl; 1 mM DTT; 1 mM rATP, 0.5 mM rGTP and rCTP; 100 units of RNase inhibitor (Sigma) and 100 μCi rUTP-α-^{32}P). The run on was carried out at 30°C for 10 min. Cells were then washed once in TMN buffer and broken to isolate labelled RNA as for a Northern blot. Isolated RNA was precipitated in the presence of 200 μg tRNA and washed in 70% ethanol. RNA was then dissolved in 100 μl 1× TE, denatured at 95°C for 3 min, and then added to the prehybridized membrane in 3 ml of hybridization buffer (Humphrey *et al*., 1994) and incubated at 65°C for 2 days. Five micrograms of single-stranded DNA probe were absorbed onto the nylon membrane using a slot blot and cross-linked using the STRATAGENE Stratalinker. *cdc18* KS was constructed from *nmt1-cdc18* cDNA (Nishitani and Nurse, 1995), *ura4* KS from Rep4, (Maundrell, 1993) and *cdc2* KS from the *cdc2* ORF (Correa-Bordes *et al*., 1997). Single-stranded DNA was prepared from *cdc18* KS$^+$, *cdc18* SK$^+$, *ura4* KS$^+$, *ura4* KS$^-$, *cdc2* KS$^+$, *cdc2* KS$^-$, KS$^+$ and KS$^-$ plasmids, by infecting XL1-Blue with M13KO7 bacteriophage. Single-stranded phage DNA was purified using the Qiagen M13 kit. Bands were quantified by PhosphorImager analysis, the background subtracted (using the value of KS$^+$ or KS$^-$, as appropriate) and the *cdc18/cdc2* value determined. (The relative values of *cdc18/ura4* and *cdc18/cdc2* were similar.)

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