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Control of S-phase periodic transcription in the fission yeast mitotic cycle

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In fission yeast, passage through START and into S-phase requires cyclin-dependent kinase (CDK) activity and the periodic transcription of genes essential for S-phase ('S-phase transcription'). Here we investigate the control of this transcription in the mitotic cell cycle. We demonstrate that the periodicity of S-phase transcription is likely to be controlled independently of CDK activity. This contrasts with the equivalent system in budding yeast. Furthermore, the CDK function required for S-phase acts after the onset of S-phase transcription and after the accumulation of cdc18p, a critical target of this transcriptional machinery. We investigate the role of individual components of the S-phase transcriptional machinery, cdc10p, res1p, res2p and rep2p, and define a new role for res2p, previously demonstrated to be important in the meiotic cycle, in switching off S-phase transcription during G2 of the mitotic cycle. We show that the presence of the *in vitro* bandshift activity DSC1, conventionally thought to represent the active complex, requires res2p and correlates with inactive transcription. We suggest that S-phase transcription is controlled by both activation and repression, and that res2p represses transcription in G2 of the cell cycle as a part of the DSC1 complex.
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Introduction

The periodic expression of genes required for S-phase is a common feature of cell cycle regulation in eukaryotes. It has been studied most thoroughly in the budding yeast *Saccharomyces cerevisiae* (Johnston, 1992; Koch and Nasmyth, 1994) and in mammalian cells (Martin *et al.*, 1995; Slansky and Farnham, 1996). In budding yeast, periodic transcription of S-phase genes is mediated by two transcriptional complexes, SWI4–SWI6 and SWI6–MBP1, which act through conserved promoter elements known as SCB and MCB sites respectively (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989; Lowndes *et al.*, 1991; Taba *et al.*, 1991; Dirick *et al.*, 1992; Koch *et al.*, 1993; for a recent review, see Breeden, 1996). A DNA-binding activity containing SWI6p and MBP1p, termed DSC1, that recognizes MCB elements, is thought to be involved in transcriptional activation (Verma *et al.*, 1991; Dirick *et al.*, 1992; Lowndes *et al.*, 1992a; Koch *et al.*, 1993). Periodic expression in budding yeast is controlled primarily by oscillations in cyclin-dependent kinase (CDK) activity through the cell cycle, although it is not fully understood how CDKs interact with the transcriptional complexes to regulate transcription. Transcription of genes containing SCB or MCB elements is activated in late G_1 by the CDK, CDC28–CLN3 (Tyers *et al.*, 1993; Dirick *et al.*, 1995). Transcription from SCB elements is then switched off in G_2 cells by the CDK activity of CDC28–CLB complexes (Koch *et al.*, 1996). An analogous control in mammalian cells involves the E2F–DP1 transcriptional complex which, although unrelated to SWI4/SWI6–MBP1, has a similar role. This is also regulated by CDKs, being activated by CDK4/6–cyclin D (reviewed in Martin *et al.*, 1995; Slansky and Farnham, 1996) and CDK2–cyclin E, and inactivated by CDK2–cyclin A activity in S-phase and G_2 (Xu *et al.*, 1994; Krek *et al.*, 1994).

In fission yeast, the S-phase transcriptional machinery is composed of cdc10p, res1p, res2p and rep2p proteins (Aves *et al.*, 1985; Lowndes *et al.*, 1992b; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995) and operates through MCB promoter elements. Target genes for the machinery include *cdc18*, *cdc22*, *cdt1* and possibly *cig2* (Gordon and Fantes; 1986; Kelly *et al.*, 1993; Hofmann and Beach; 1994; Obara-Ishihara and Okayama, 1994). DSC1, an endogenous bandshift activity found in fission yeast cell extracts (Lowndes *et al.*, 1992b), binds MCB sites and contains cdc10p and res1p (Lowndes *et al.*, 1992b; Caligiuri and Beach, 1993). Res2p also interacts with cdc10p to form an alternative complex which can bind specifically to MCB elements *in vitro* (Zhu *et al.*, 1994). Genetic analysis suggests that cdc10p/res1p is the major transcriptional regulator during the mitotic cell cycle (Tanaka *et al.*, 1992), and cdc10p/res2p during the meiotic cell cycle (Miyamoto *et al.*, 1994). The mechanism controlling periodic transcription is not understood, but it has been suggested that DSC1 plays a role in transcriptional activation, and that the formation of DSC1 and the onset of *cdc10*-dependent transcription are triggered by G_1 cdc2p activity (Reymond *et al.*, 1993). In addition, a role has been proposed for cdc10p in repression of transcription because a truncation of cdc10p causes elevated levels of transcription throughout the cell cycle (McInerny *et al.*, 1995). The level of cdc10p throughout the cell cycle is constant, indicating that oscillations in cdc10p do not control the periodicity of transcription (Simanis and Nurse, 1989).

Here we further investigate the mechanism controlling periodic S-phase transcription during the mitotic cell cycle in the fission yeast *Schizosaccharomyces pombe*. We show that CDK activity does not appear to play a role in the

regulation of S-phase transcription, that cdc10p and res1p are required for transcriptional activation and res2p for the G_2 repression of this transcription, whilst rep2p is important for maintaining the level of transcription. Finally, we demonstrate that the presence of DSC1 correlates with repression of transcription during the G_2-phase of the cell cycle.

Results

cdc2p does not appear to activate cdc18 transcription prior to S-phase

We first investigated whether cdc2p is required in G_1 for the activation of *cdc10*-dependent S-phase transcription. *cdc10*-dependent transcription was monitored by assessing the transcript levels of a target gene, *cdc18*, in cells proceeding towards S-phase in the presence and absence of *cdc2* function. This was done using the temperature-sensitive alleles of *cdc2* most severely compromised for progression through G_1, *cdc2-M26* and *cdc2-33* (Broek *et al.*, 1991; MacNeill *et al.*, 1991). Similar results were obtained with both alleles and data is shown for *cdc2-M26*.

In the first experiment (Figure 1), temperature-sensitive *cdc2-M26* cells were synchronized in G_1 by nitrogen starvation at 25°C. On re-feeding with nitrogen, S-phase began within 3 h at 25°C but did not take place at the restrictive temperature of 36.5°C. The *cdc18* transcript level was low in nitrogen-starved cells, but began to increase 1.5 h after the addition of nitrogen at 25°C, that is ~l h before the onset of S-phase (Figure 1A). This suggests that *cdc18* transcription is activated in small G_1 cells some time before they reach the critical size required for the onset of S-phase (Nurse, 1975; Nurse and Thuriaux, 1977). A similar increase in *cdc18* transcript level was observed at 36.5°C (Figure 1A), although cells were unable to enter S-phase at this temperature because they lacked *cdc2* function (see Figure 1C). Similar results were obtained using the other targets of *cdc10*, *cdc22* and *cdt1* (data not shown).

In order to assess whether residual *cdc2* kinase activity was likely to be present in these cells, the H1 histone kinase levels associated with cdc2p were determined in cdc2p immunoprecipitates from extracts of wild-type and *cdc2-M26* cells released from nitrogen starvation at 36.5°C. As demonstrated above, *cdc18* transcript accumulated in the *cdc2-M26* strain at 36.5°C, but cells failed to enter S-phase (Figure 1C and D). The H1 kinase assays were carried out at 36.5°C (Figure 1B) and quantified by phosphorimager analysis. In the *cdc2-M26* strain, H1 kinase activity at the time when *cdc18* transcript began to accumulate was only 0.13% of that seen in the wild-type strain. These data led us to conclude that cells released from nitrogen starvation in G_1 can activate *cdc10*-dependent transcription without significant cdc2p activity, although they require *cdc2* function for entry into S-phase.

In a second experiment, we studied the *cdc2* requirement for the activation of *cdc10*-dependent transcription in *cdc2-33* cells re-entering S-phase from G_2 (Figure 2). *cdc10* function is required for re-replicative DNA synthesis (Moreno and Nurse, 1994). Cells were arrested in G_2 at the restrictive temperature for *cdc2-33*, in the absence of nitrogen, and then subjected to a brief heat treatment at 49°C (Broek *et al.*, 1991). This procedure inactivates the

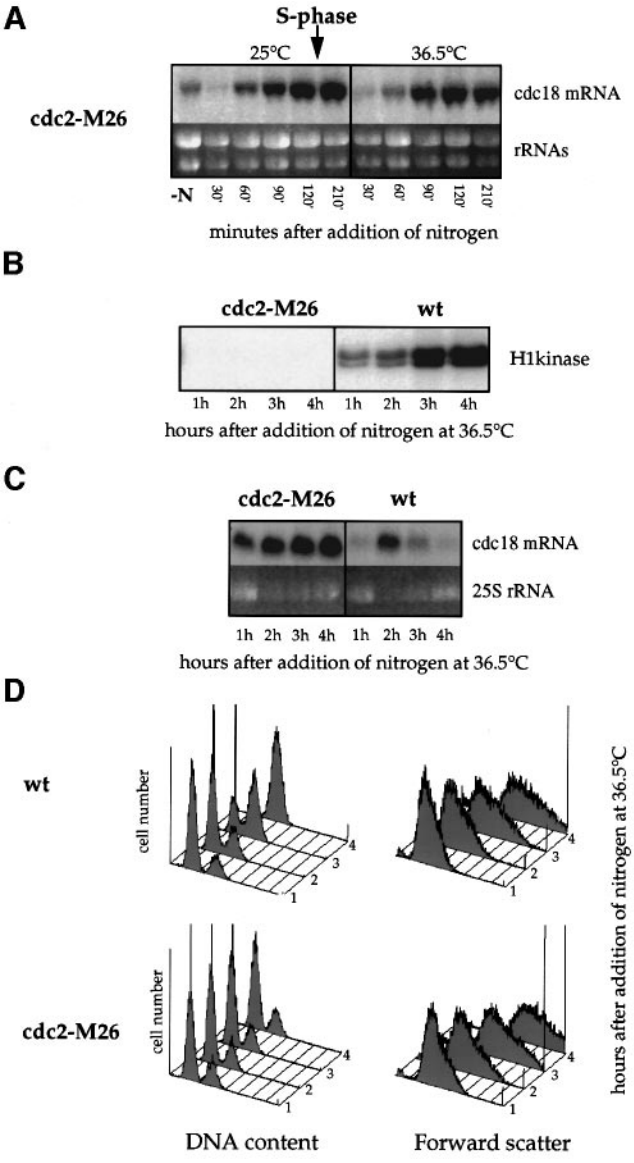

Fig. 1. *cdc2* function is not required for the activation of *cdc10*-dependent transcription following release from nitrogen starvation. *cdc2-M26* cells were arrested in G_1 by nitrogen starvation and re-fed at 25 or 36.5°C. (**A**) A Northern blot was probed for *cdc18*; rRNA was visualized in the gel using EtBr to control for sample loading. At 25°C, cells entered S-phase after 3 h (arrow), whereas at the restrictive temperature cells remained in G1. (**B**) Wild-type and *cdc2-M26* cells in G_1 were re-fed with nitrogen at 36.5°C. H1 kinase assays were carried out at 36.5°C using cdc2p immunoprecipitates from wild-type and *cdc2-M26* cells. (**C**) A Northern blot is shown of samples taken from the experiment in (B), the blot was probed for *cdc18*, and EtBr used to visualize rRNA. (**D**) Samples from (B) were taken for FACS analysis. Forward scatter (which represents cell mass) and DNA content are shown on separate histograms.

G_2 form of cdc2p and thereby allows cells to undergo an additional round of DNA synthesis. At the permissive temperature, 28.5°C, cells underwent an additional round of S-phase, 4–5 h after the re-addition of nitrogen (Figure 2B). However, when cells were incubated at 36.5°C, with functionally inactive *cdc2*, they failed to re-replicate their DNA. After the heat treatment, the level of *cdc18* mRNA was very low, but it increased to a peak level sufficient to bring about S-phase, 4 h after the addition of nitrogen at the permissive temperature. A similar increase in the

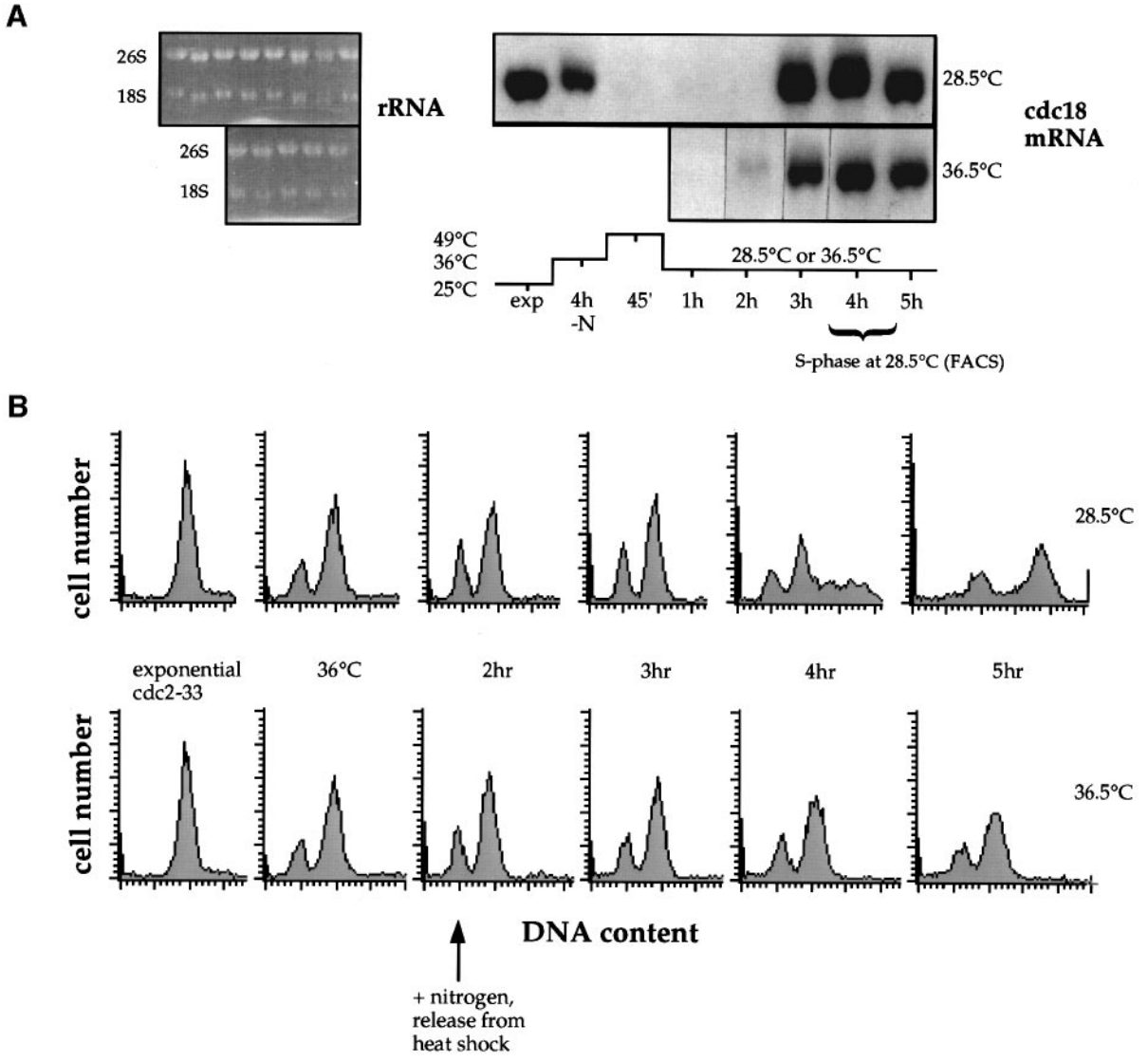

Fig. 2. *cdc2* function is not required for the re-activation of *cdc10*-dependent transcription following re-entry into S-phase from G_2. *cdc2-33* cells were arrested at G_2–M, subjected to a heat shock to induce them to re-enter S-phase and re-fed at the permissive or restrictive temperature for *cdc2-33* (28.5°C or 36.5°C). (**A**) The Northern blot was probed for *cdc18* mRNA; rRNA is shown as a loading control; exp denotes exponentially growing cells. (**B**) Fixed samples were analysed by FACS analysis to determine the timing of S-phase as cells with a 2C DNA content re-replicate their DNA, resulting in a 4C peak.

level of *cdc18* transcript was seen at 28.5 and 36.5°C, i.e. in both the presence and absence of *cdc2* function (Figure 2A). Similar results were obtained using the strain *cdc2-M26* (data not shown).

We conclude that *cdc10*-dependent transcription is activated in G_1 cells, and re-activated in cells re-entering S-phase from G_2, in the absence of significant *cdc2* function. We cannot rule out the possibility that although cells in these experiments were unable to enter S-phase at the restrictive temperature, sufficient residual cdc2p activity remained to activate *cdc10*-dependent transcription. However, this seems unlikely given the low levels of *in vitro* H1 kinase activity detectable in the $cdc2^{ts}$ strains at the restrictive temperature.

cig1, cig2 and cdc13 are required after cdc10 function to bring about the onset of S-phase

Three B-type cyclins act together with cdc2p in G_1 of the mitotic cycle to bring about S-phase (Fisher and Nurse, 1996). To determine whether these B-type cyclins are required downstream of *cdc10* function in G_1, we used a strain containing the temperature-sensitive *cdc10-V50* mutation in which two of the cdc2p cyclin partners, cig1p and cig2p, were deleted, with the remaining partner, cdc13p, placed under control of the thiamine-repressible promoter (Fisher and Nurse, 1996). This allows us to manipulate *cdc10*-dependent transcription and CDK activity independently. As a control strain, *cdc13::nmt cdc10-V50* $cig1^+cig2^+$ was used. Cells were shifted to the restrictive temperature for 3 h to arrest the majority of cells in G_1, and then *cdc13* was switched off by the addition of thiamine. After a further 1 h at 36°C, cells were shifted back to 25°C, re-activating *cdc10* function in the absence of the B-type cyclins needed to drive entry into S-phase (Figure 3C). Under these conditions, as a result of the instability of cdc13p in early G_1 cells (Hayles *et al.*, 1994), cdc13p levels were reduced to 2% of the level seen in the exponential population (data not shown).

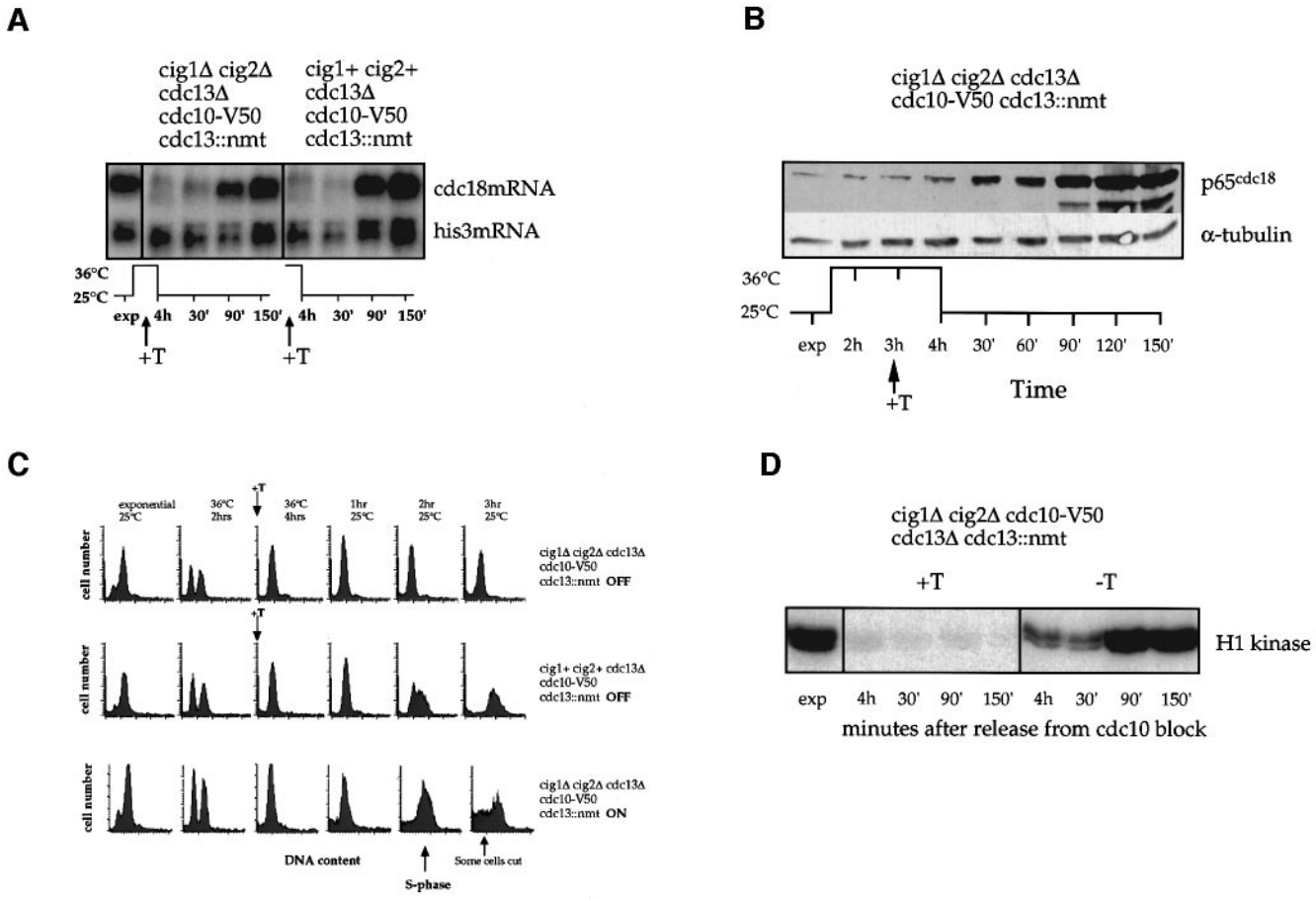

Fig. 3. *cig1*, *cig2* and *cdc13* functions are required after *cdc10* function in G_1 to bring about S-phase. In order to manipulate *cdc10* and CDK activity independently in G1, *cdc13::nmt cdc10-V50 cig1*Δ *cig2*Δ and *cdc13::nmt cdc10-V50 cig1*$^+$*cig2*$^+$ strains were used. Cells were arrested in G1 by incubation at 36°C, the non-permissive temperature for *cdc10-V50*, for 3 h. *cdc13* was then switched off in half the cells by the addition of thiamine. After an additional hour at 36°C, cells were shifted back to 25°C, reactivating *cdc10*. (**A**) A Northern blot for samples from both strains in the presence of thiamine was probed for *cdc18* mRNA; *his3* was probed as a control. (**B**) A Western blot of extracts from the strain lacking all three cyclins (*cdc13::nmt cdc10-V50 cig1*Δ *cig2*Δ in the presence of thiamine) was probed with antibodies for cdc18p, cdc13p (data not shown) and α-tubulin. (**C**) Fixed cells were taken for FACS analysis. Cells lacking all three cyclins were unable to enter S-phase after release from the *cdc10* block. (**D**) Extracts from *cdc13::nmt cdc10-V50 cig1*Δ *cig2*Δ cells in the presence and absence of thiamine were used in an H1 kinase assay.

The level of *cdc18* transcripts increased within 1.5 h of the release from the *cdc10* block, with similar kinetics and to a similar level in the presence or absence of G1 cyclins (Figure 3A). In this experiment, cdc18p levels were also monitored and found to be at a low in exponential cells (when most cells are in G2 of the cycle) and in G1 cells in the absence of the *cdc10* function. However, within 2 h of shift to 25°C (Figure 3B), cdc18p accumulated to a high level, exceeding that seen in *cig1*$^+$*cig2*$^+$ cells which enter S-phase (protein data not shown). These elevated levels of cdc18p were not sufficient to drive cells into S-phase in the absence of B-type cyclin partners for *cdc2* (Figure 3C).

Next we assessed the residual cdc2p kinase activity in the cyclin deletion strain upon release from the *cdc10* block, in the presence or absence of ectopic *cdc13* (Figure 3D). The level of cdc2p-associated H1 kinase activity in the absence of the three B-type cyclins was shown to be 1.5% of that in the control cells which express *cdc13* from the *nmt* promoter and enter S-phase (Figure 3C).

In conclusion, this experiment shows that the B-type cyclins, which are responsible for the vast majority of the measurable H1 kinase activity in G1 cells, act after the onset of *cdc10*-dependent transcription and after the accumulation of cdc18p, to bring about entry into S-phase. It is conceivable, nevertheless, that other cyclin partners for cdc2p are present in these cells which are unable to make a significant contribution to the overall H1 kinase activity, but can activate *cdc10*-dependent transcription.

cdc2 function is not required in S-phase or in G2 for the appropriate control of periodic cdc10-dependent transcription

The previous experiments suggest that *cdc2* function is not required for the onset of *cdc10*-dependent transcription. To investigate the control of *cdc10*-dependent transcription during S-phase and in G2 cells, we first asked whether *cdc10*-dependent transcription was active in cells arrested at the onset of S-phase by hydroxyurea (HU). Wild-type and *cdc10-129* cells were arrested with HU and then shifted to 36°C for 30 min to inactivate *cdc10* [HU prevented cells from entering S-phase, fluorescence-activated cell sorting (FACS) data not shown]. While both wild-type and *cdc10-129* cells that were arrested at the permissive temperature had elevated *cdc18* transcript levels (Figure 4A, lanes 2 and 5), cells at the restrictive

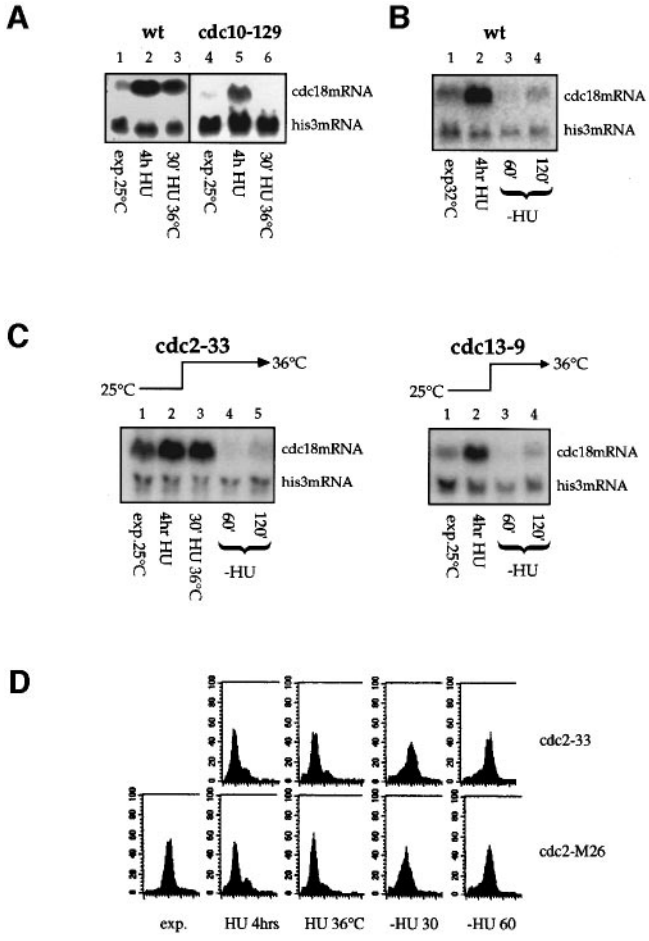

Fig. 4. *cdc2* function is not required for the maintenance of high levels of *cdc10*-dependent transcription in S-phase or for repression of this transcription in G2 cells. (**A**) Wild-type and *cdc10-129* cells were arrested at the onset of S-phase by the addition of 11 mM HU. Cells were then shifted to 36°C for 30 min. (**B**) Wild-type cells were arrested in HU for 4 h then washed, synchronously releasing cells into S-phase and G2. (**C**) *cdc2-33* and *cdc13-9* cells were arrested in HU for 4 h (lanes 1 and 2) and shifted to 36°C for 30 min to inactivate the temperature-sensitive functions (*cdc2-33*, lane 3; data not shown for *cdc13-9*). HU was then washed out at 36.5°C (final lanes) enabling cells to pass through S-phase and into G2 (confirmed by FACS analysis). Samples were taken for Northern analysis and probed for *cdc18* and *his3*. (**D**) *cdc2-33* and *cdc2-M26* strains were subjected to a HU block and release, like that carried out in (C), and samples taken for FACS analysis.

temperature for *cdc10-129* did not (Figure 4A, compare lanes 3 and 6). Thus, continued *cdc10* function is required to maintain *cdc18* transcript levels. In a second experiment (Figure 4B), *cdc18* mRNA levels were monitored in wild-type cells which were first arrested in HU and then washed free of HU, allowing them to pass through S-phase and into G2. As expected, *cdc18* mRNA levels rapidly decreased as cells passed through S-phase, remaining low in G2 cells.

To investigate whether cdc2p plays a role in maintaining *cdc10*-dependent transcription during S-phase, or in switching off this transcription at the end of S-phase, we monitored *cdc18* transcript levels, first in a HU block with and without *cdc2* function, and secondly after release from HU into mutant blocks lacking mitotic CDK activity. We utilized the CDK mutant strains *cdc2-33* and *cdc13-9*, which contain temperature-sensitive mutations in cdc2p and cdc13p (the major cyclin partner in G2 and M cells). Exponentially growing cells were arrested at the beginning of S-phase in HU for 4 h at 25°C (Figure 4C) and then shifted to 36°C to inactivate the mutant proteins. *cdc18* mRNA levels remained high in the absence of *cdc2* function (Figure 4C, lanes 1–3, data not shown for *cdc13-9*). Therefore, active *cdc10*-dependent transcription during S-phase does not require continued CDK activity. After 30 min at the restrictive temperature, cells were washed with pre-warmed media to remove HU; *cdc18* transcript levels rapidly decreased in both *cdc2-33* and *cdc13-9* cells at the restrictive temperature (Figure 4C, *cdc2-33* lanes 4 and 5, and *cdc13-9* lanes 3 and 4). In *cdc2-33* cells released from HU into G2 at the restrictive temperature, cdc2p-associated H1 kinase activity was assessed and found to be only 0.75% of the level seen in wild-type cells in G2. We conclude that the moderate levels of *cdc2* kinase activity found in wild-type G2 cells are not required to switch off *cdc10*-dependent transcription after exit from S-phase. In this experiment, *cdc2-33* and *cdc2-M26* cells released from the HU block were able to enter and complete S-phase (Figure 4D), suggesting that although *cdc2* function is required for the onset of S-phase after cells have accumulated cdc18p, it is not required for the completion of DNA replication once cells are arrested in HU.

Transcriptional complex components and S-phase transcription

Having established that *cdc2* function is unlikely to play a role in activating, maintaining or switching off *cdc10*-dependent transcription, we next assessed the role in periodic S-phase transcription of the components of the transcriptional complex itself. These studies used strains deleted for one of the genes, *cdc10*, *res1*, *res2* or *rep2*, that contribute to the function of the transcriptional complex (Aves *et al.*, 1985; Marks, 1992; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995). The *cdc10* deletion is inviable, so the double mutant *cdc10*Δ *sct1-1* (Marks *et al.*, 1992; Caligiuri and Beach, 1993) was used in which a point mutation in *res1* (*sct1-1*) suppresses the lethality of the *cdc10*Δ. *sct1-1 cdc10*$^{+}$, *res2*Δ*rep2*Δ strains were also studied (Figure 5C).

Cells harbouring deletions of components of the transcriptional machinery were grown at 30°C, where they are viable, subjected to a HU-induced arrest followed by release, and analysed by Northern blotting to assess *cdc18* message levels. FACS analysis (data not shown) confirmed that, in all cases, at least 90% of cells were arrested with a G1 DNA content 4 h after the addition of HU to cultures, and that >95% of cells were in G2 an hour after the removal of HU.

During log-phase growth of *res1*Δ cells, levels of *cdc18* transcript were similar to the low levels seen in wild-type cells, exponentially growing *rep2*Δ and *cdc10*Δ *sct1-1* cells exhibited even lower *cdc18* transcript levels (Figure 5B, columns 1, 4, 10 and 13), whereas in the *res2*Δ strain, *cdc18* transcript levels were elevated (Figure 5B, column 7). After treatment with HU for 4 h, cells arrested in early S-phase. In the wild-type, *cdc18* transcript levels were elevated during S-phase. However, no significant elevation was seen in *res1*Δ or *cdc10*Δ *sct1-1* cells and only a small

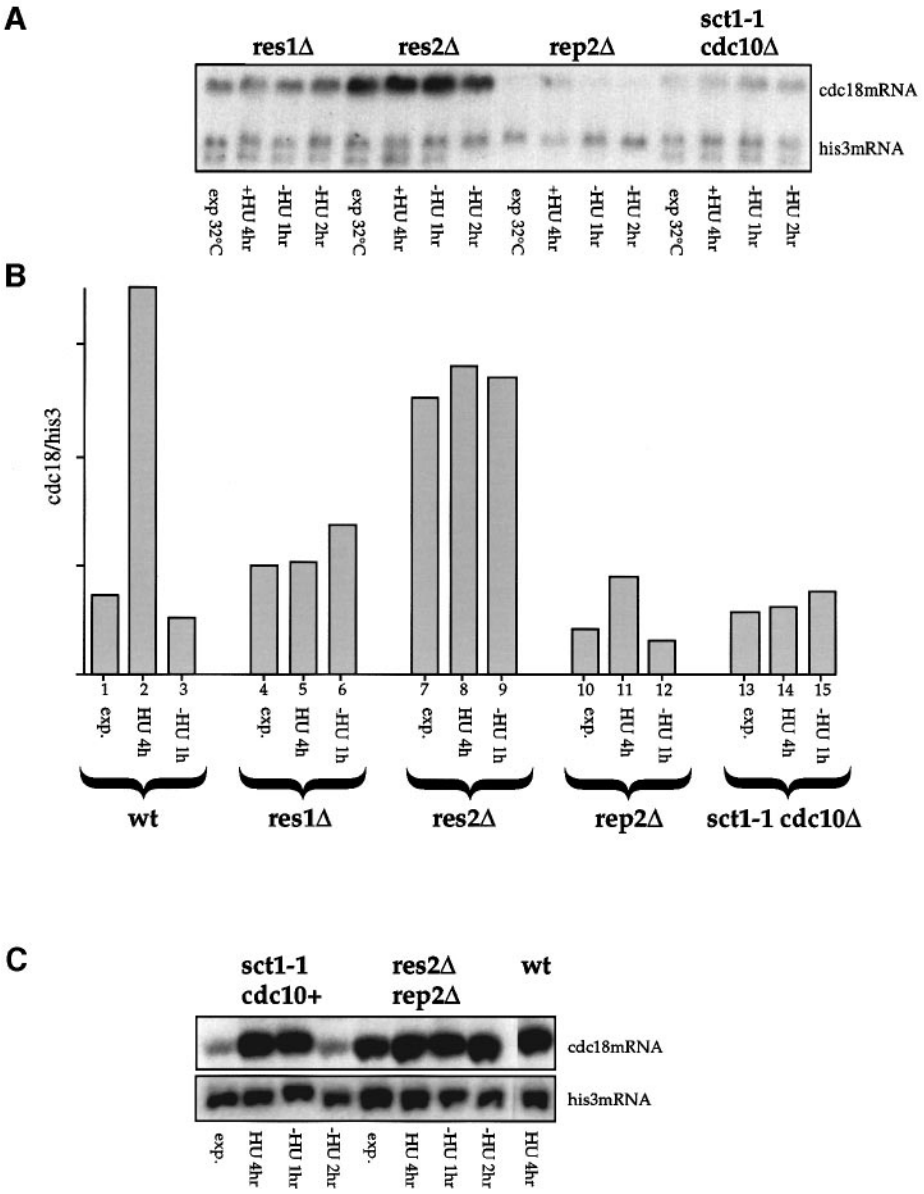

Fig. 5. The role of components of the *cdc10* complex in the control of periodic transcription in mitotic cells. *res1*Δ, *res2*Δ, *rep2*Δ and *sct1-1 cdc10*Δ (**A** and **B**) and the double mutant *res2*Δ *rep2*Δ and *sct1-1 cdc10*$^{+}$ (**C**) strains were grown at 30°C, where they are all viable, and then arrested at S-phase by the addition of 11 mM HU for 4 h. HU was then washed out, enabling cells to pass through S-phase and into G2 (confirmed by FACS analysis, see text). Samples were taken for Northern analysis (A and C) and probed for *cdc18* mRNA, *his3* serving as a loading control. (B) The Northern blot containing samples from experiments shown in (A) and in Figure 4B was quantified by phosphorimager analysis (using an arbitrary scale).

elevation was seen in *rep2*Δ cells, suggesting that these gene products are required for the elevated levels of *cdc18* transcription (Figure 5B, columns 2, 5, 11 and 14). In the *res2*Δ strain, *cdc18* transcript levels were high, both during and after release from the HU block (Figure 5B, columns 8 and 9), and were close to the peak level seen in S-phase wild-type cells. In wild-type cells, within l h of removing HU, as S-phase was completed, *cdc18* transcript levels fell dramatically (Figure 5B, column 3). *res1*Δ and *cdc10*Δ *sct1-1* did not exhibit a reduced *cdc18* transcript level after HU was removed, whereas a reduction was observed in *rep2*Δ cells (Figure 5B, columns 6, 12 and 15). As a control for *cdc10*Δ *sct1-1*, the single mutant *sct1-1* was also monitored. Periodic transcription of *cdc18* was retained in the *sct1-1* strain (Figure 5C), although there was some delay in the down-regulation of *cdc18* message as cells left the HU block, suggesting that the *sct1-1* mutation may cause a partial deregulation of *res1* function. However, the experiment confirms that the *sct1-1* mutation is not responsible for the aperiodic behaviour of *cdc18* transcription in the *cdc10*Δ *sct1-1* strain.

These results show that *cdc18* transcript levels are reduced and constant during the HU block and release in *res1*Δ and *cdc10*Δ*sct1-1* strains. *cdc18* transcription is elevated and constant in *res2*Δ cells, and is reduced but periodic in *rep2*Δ cells. In the double mutant *rep2*Δ*res2*Δ, transcript levels are high (Figure 5C), confirming that rep2p has no role in the absence of res2p (Nakashima *et al.*, 1995). We also confirmed previous work (data not shown) suggesting that *rep1*, a *rep2* homologue, has no

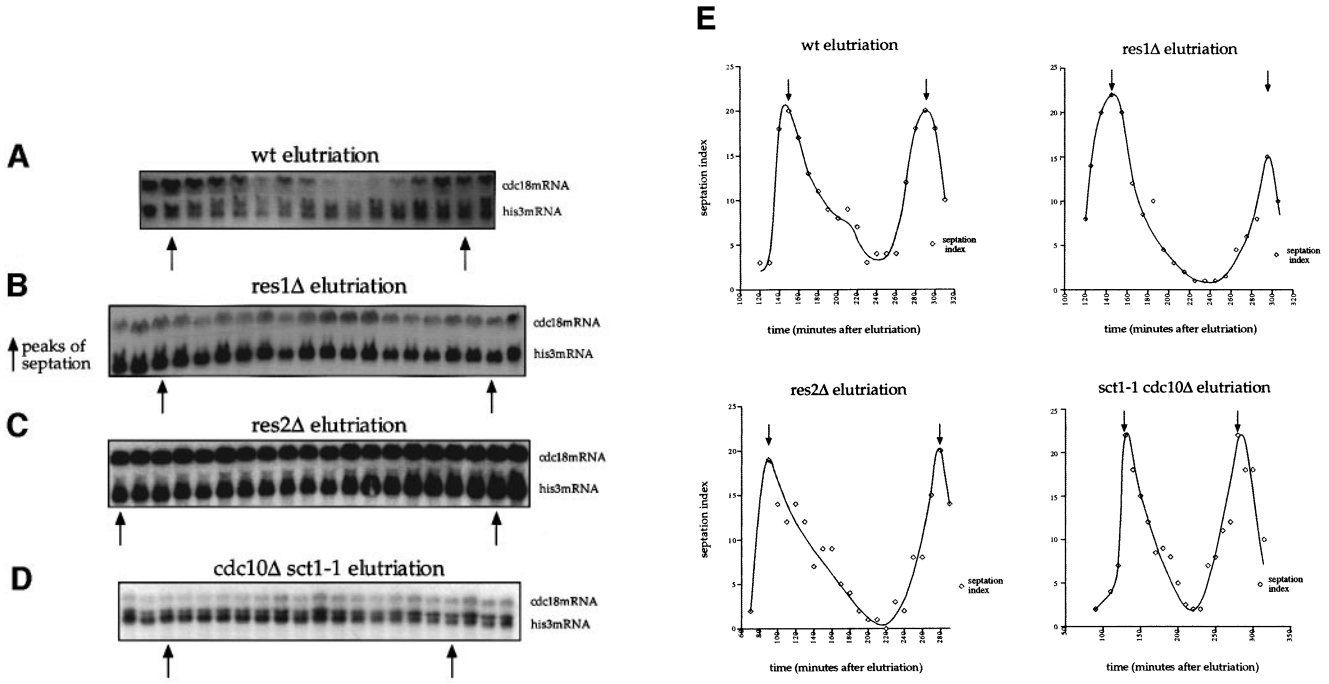

Fig. 6. The role of components of the *cdc10* complex in the control of periodic transcription through a synchronous cell cycle. Cultures of wild-type (**A**), *res1*Δ (**B**), *res2*Δ (**C**) and *sct1-1cdc10*Δ (**D**) cells were elutriated at 30°C. Cells were then followed through two synchronous rounds of cell division. The level of synchrony for each culture is indicated by the septation index shown in (**E**). Cell number and FACS data confirmed the cell synchrony (data not shown). Northern blots were probed for *cdc18*, *his3* mRNA serving as a control, and message levels quantified by phosphorimager analysis to confirm the results (data not shown). (Arrows denote the peaks of septation.)

role in S-phase transcription during the mitotic cell cycle (Sugiyama *et al.*, 1994).

The role of the various components of the transcriptional complex in controlling the periodicity of S-phase transcription was investigated further using synchronous cultures of wild-type fission yeast and strains deleted for *cdc10*, *res1* and *res2* (Figure 6). The level of synchrony of each culture is indicated by the septation index (shown in Figure 6E). Similar results were obtained using *cdc18*, *cdc22*, *cdt1* and *cig2* mRNA levels as a measure of *cdc10*-dependent transcription (data not shown). In wild-type cells synchronized by elutriation, the level of *cdc18* transcript is periodic during the cell cycle, being maximal around the peak in septation (Kelly *et al.*, 1993) (Figure 6A). In the *res1*Δ strain (Figure 6B), *cdc18* transcripts were present at low levels throughout the cell cycle. Thus, res1p is required to activate *cdc10*-dependent transcription during the cell cycle and, in its absence, no periodicity is observed and the absolute level of transcription is reduced. In the *res2*Δ strain, no significant periodicity in the level of *cdc18* message was observed in the synchronous culture (Figure 6C). We conclude that res2p is required to repress transcription periodically during the cell cycle; in its absence, no periodicity is observed and absolute levels are increased. In the *cdc10*Δ *sct1-1* strain (Figure 6D), *cdc18* transcript levels were constant but very low throughout the cell cycle. We conclude that although the *cdc10*Δ *sct1-1* strain can activate transcription of *cdc10* targets to a low level, cdc10p is absolutely required for the periodicity of this transcription. Finally, we confirmed our previous observation that in a *rep2*Δ strain, *cdc10*-dependent transcription was still periodic, although at a reduced absolute level during the cell cycle (data not shown).

To explore further the role of *resl*, *res2* and *cdc10* in the control of periodic transcription, we investigated the effects of ectopic expression of these factors on periodic S-phase transcription (Figure 7). *cdc25-22* strains were transformed with multicopy plasmids containing either *res1*, *res2* or *cdc10* under control of the thiamine-repressible *nmt* promoter (Maundrell, 1993). Ectopic expression of *res1* and *res2* was driven by the full strength *nmt* promoter while the medium strength promoter was used to drive *cdc10* expression (the cells became sick on high-level overexpression of *cdc10*). The culture was split in two, and expression from the *nmt* promoter was induced in half the cells by growth in the absence of thiamine, for 20 h at 25°C. Both induced and uninduced cultures subsequently were incubated at 36°C for 4 h to inactivate the *cdc25*^{ts} function, thereby arresting cells in G2, where *cdc18* transcript levels are normally low. Cells were then released at 25°C into mitosis and a subsequent cell cycle.

In the presence of thiamine, which prevents ectopic expression of *res1*, *res2* or *cdc10* (marked OFF in Figure 7), *cdc18* transcript levels were low in G2, increased to peak levels on release into mitosis and decreased after entry into S-phase. However, in G2-arrested cells expressing *res1* from the *nmt* promoter (marked ON in Figure 7), *cdc18* transcription was activated to maximal levels (other *cdc10* targets, *cdc22*, *cdt1* and *cig2*, were similarly affected, data not shown). Following release of the *res1*-expressing cells into mitosis and the subsequent cell cycle, *cdc18* transcription was maintained at high levels. These results are in agreement with previously published data showing that overexpression of res1p can drive expression of *cdc10* targets (Ayte *et al.*, 1995). However, our results differ from those of Ayte *et al.*, in that we observed no significant G1 arrest after release from the *cdc25* block. This difference may be explained

by the short time-course of induction in our experiments. Thus, we were able to separate direct effects on transcription from blocks in cell cycle progression. Identical effects were observed on the other *cdc10* target transcripts, *cdc22*, *cdt1* and *cig2* (data not shown). We conclude that *res1* plays an important role in activating periodic transcription during the cell cycle. Ectopic expression of *cdc10* (Ayte *et al.*, 1995; McInerny *et al.*, 1995) and *res2* had no strong activating or repressing effect on the periodic transcription of *cdc10* targets, although transcription may be slightly repressed in both cases. It is possible that if expressed at higher levels, *res2* and *cdc10* could significantly affect transcription.

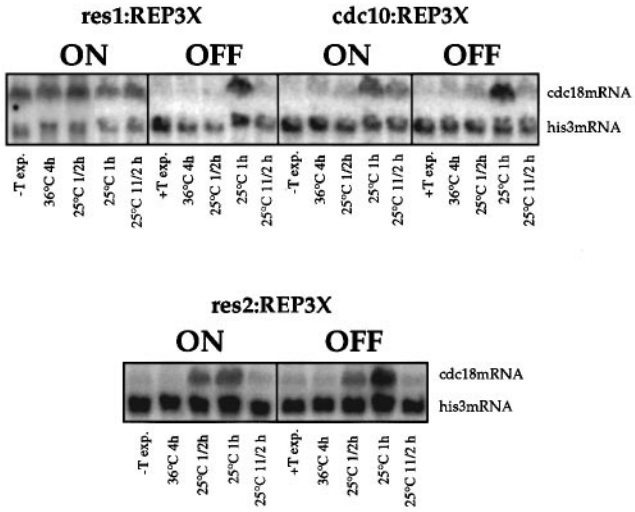

Fig. 7. The effects of overexpression of components of the *cdc10* complex in cells synchronized in G2 and released into the cell cycle. *cdc25-22* cells growing exponentially in the presence of thiamine, containing *res1*, *res2* or *cdc10* behind the *nmt* promoter, were washed four times to induce *nmt*-driven gene expression and, after growth for 20 h at 25°C, were shifted to the restrictive temperature for 4 h. Cells were then cooled rapidly to 25°C, allowing synchronous entry into the mitotic cycle (confirmed by the septation index, data not shown). The Northern blot was probed for *cdc18* and *his3* message.

Analysis of the composition and cell cycle behaviour of DSC1

To provide a biochemical correlate for our analysis of *cdc10*-dependent transcription, we investigated the behaviour of DSC1, the bandshift activity which binds to MCB-containing promoters and contains cdc10p and res1p (Lowndes *et al.*, 1992b; Ayte *et al.*, 1995). The DSC1 bandshift was obtained by incubating cell extracts with a radiolabelled fragment of the *cdc18* promoter containing both putative palindromic MCB repeats. The bandshift was shown to contain cdc10p (Figure 8A) and to be sensitive to cold competitor DNA (data not shown). It is therefore likely to represent the same complex previously identified as DSC1.

To determine which gene functions are required to generate the DSC1 bandshift activity, its presence was monitored in extracts made from wild-type, *res1*Δ, *res2*Δ, *cdc10*Δ*sct1-1* and *rep2*Δ cells (Figure 8b). Previous work

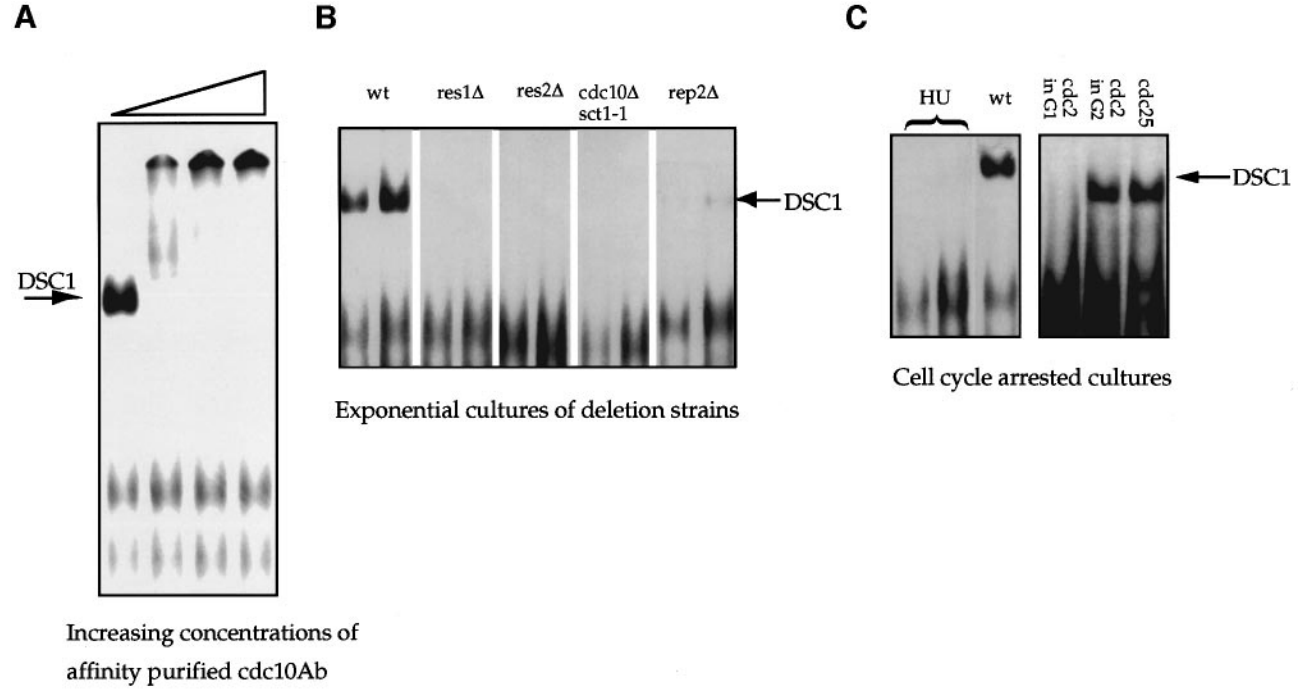

Fig. 8. Components of the *cdc10* complex required for the formation of DSC1 and the periodic appearance of DSC1 throughout the cell cycle. Bandshifts were carried out on cell extracts using the radiolabelled MCB-containing element from the *cdc18* promoter as a probe. In (**A**), wild-type cell extracts were incubated with increasing concentrations of affinity-purified, polyclonal rabbit cdc10Ab. (**B**) Gel-shift assays were performed with cell extracts from exponential cultures, grown at 30°C from wild-type cells and *res1*Δ, *res2*Δ, *cdc10*Δ in a *sct1-1* background and *rep2*Δ mutants. Samples were prepared in duplicate at two different concentrations of cell extract (20 and 40 μg) and loaded in adjacent lanes. (**C**) Gel-shift assays were carried out for cells arrested at various points in the cell cycle. Extracts were taken from exponentially growing wild-type cells and cells arrested at the onset of S-phase by the addition of 11 mM HU for 4 h and loaded on the same gel. Two bandshift assays were conducted using 20 and 40 μg of the HU extract. On a second gel, bandshifts were carried out using 40 μg of cell extracts from *cdc2-M26* cells arrested in G1, 3 h after release from nitrogen starvation (from the experiment shown in Figure 1) and from cells arrested in G2 using mutations in *cdc2* and *cdc25* in which *cdc10*-dependent transcription is inactive (RNA data not shown).

has shown the presence of *cdc10*, *res1* and *res2* in DSC1 (Zhu *et al.*, 1997; Lowndes *et al.*, 1992b; Ayte *et al.*, 1995). All four genes were required to generate the DSC1 bandshift activity, although a very faint bandshift, of equivalent mobility to DSC1, was seen in *rep2*Δ cells, suggesting that in the absence of *rep2*Δ, DSC1 may form inefficiently. It is noteworthy that DSC1 is absent in *res2*Δ cells, in which S-phase transcription is high. DSC1 is also absent in the *cdc10-C4* mutant (Reymond and Simanis, 1993; McInerny *et al.*, 1995), although the effect of this truncation on *cdc10* function is unclear. Thus, the presence of DSC1 does not correlate with the activity of *cdc10*-dependent transcription.

To investigate the behaviour of DSC1 through the cell cycle, we performed gel-shift experiments with extracts from cells arrested in the mitotic cycle; in G1 where *cdc10*-dependent transcription is active, and in G2 where it is inactive. Arrest at the G1–S boundary was achieved using either HU or a *cdc2*^{ts} allele. In the latter case, *cdc2-M26* cells (from the experiment shown in Figure 1B, C and D) were arrested in G1 by nitrogen starvation and then released at the restrictive temperature and sampled after 3 h. Arrest at the G2–M boundary was achieved by shifting *cdc25-22* and *cdc2-33* cells to the restrictive temperature for 4 h (*cdc2-33* cells were first shifted to 34°C for 2 h, enabling cells arrested in G1 to leak through into S-phase, and then to 36.5°C for an additional 2 h leading to a complete cell cycle arrest in G2).

DSC1 was detectable in exponentially growing wild-type cells and in cells arrested in G2 at the *cdc25* or *cdc2* block points, but was consistently undetectable in cells arrested at the G1–S boundary with high levels of *cdc10*-dependent transcription (Figure 8C). These data are consistent with published work on the cell cycle timing of the appearance of DSC1 (Reymond *et al.*, 1993) but indicate that, contrary to previous conclusions, the presence of the DSC1 bandshift activity correlates with inactive *cdc10*-dependent transcription. These data suggest that DSC1 may represent a form of the complex which represses transcription in G2 of the cell cycle. This notion is supported further by the observations that res2p is both present in DSC1 and necessary for its formation, and that res2p is required for the repression of S-phase transcription in G2 cells.

Discussion

We have investigated the role of cdc2p and of various components of the S-phase transcriptional machinery in regulating the periodic expression of genes required for S-phase during the fission yeast cell cycle. Cdc2p protein kinase activity does not appear to be required for the activation of *cdc10*-dependent transcription in G1, for the maintenance of this transcription during S-phase or for its repression after S-phase. This behaviour contrasts with the situation in budding yeast, in which *CDC28* has been implicated in both activating and repressing S-phase transcription (Tyers *et al.*, 1993; Dirick *et al.*, 1995; Koch *et al.*, 1996) and in which temperature-sensitive mutations in *CDC28* dramatically reduce the activity of S-phase transcription mediated by *SW14/SW16–MBP1* (Peterson, 1985; Breeden and Nasmyth, 1987). In the parallel experiment in *S.pombe*, where temperature-sensitive alleles of *cdc2* had no effect on *cdc10*-dependent transcription, we cannot rule out the possibility that some residual *cdc2* activity, insufficient for entry into S-phase and undetectable in an *in vitro* protein kinase assay, was still present. In support of our conclusion, similar results were obtained in G1 cells deleted for the three B-type cyclins cig1p, cig2p and cdc13p, in which the H1 kinase activity was reduced to 1.5% of that seen in control cells expressing cdc13p. However, although this latter experiment is not subject to the concern about residual kinase activity of *cdc2*^{ts}, other cyclins may complex with cdc2p and activate *cdc10*-dependent transcription in this strain. In fact, a CLN type cyclin, known as puc1p, has been isolated from *S.pombe*. By analogy with CLNs in *S.cerevisiae*, puc1p could play a role in promoting the passage of cells through G1 into the mitotic cycle, in part by activating S-phase transcription. In contrast to the CLNs in *S.cerevisiae*, however, no role has been established for puc1p in the mitotic cell cycle, in which puc1p is barely detectable by Western blotting. *puc1* expression is induced on cell cycle exit, and its major function may be in controlling entry into meiosis (Forsburg and Nurse, 1994). In additional experiments, we have found that *cdc18* transcripts oscillate normally in *puc1*Δ and *puc1*Δ*cig2*Δ strains subjected to a HU block and release (data not shown), thus ruling out a situation analogous to that in *S.cerevisiae* in which *CLN3* deletions have a profound effect on the activity of *SWI4/SWI6–MBP1* (Tyers *et al.*, 1993; Dirick *et al.*, 1995). In further support of our interpretation, we observed high levels of *cdc18* mRNA in *res2*Δ*cdc2-33* cells after 4 h at the restrictive temperature (data not shown), suggesting that *cdc2* is not required for the elevated transcription seen in *res2*Δ cells. Thus, we conclude that CDKs are not universally responsible for regulating the periodic expression of genes required for S-phase.

In several situations, we observed active *cdc10*-dependent transcription in cells that were effectively pre-START (Hartwell, 1974; Nurse, 1975; Nurse and Bissett, 1981). Firstly, in small G1 cells re-fed after nitrogen starvation, *cdc10*-dependent transcription was activated at least an hour before the onset of S-phase and the observed increase in *cdc2*-associated H1 kinase. Secondly, *cdc10*-dependent transcription was activated in cells arrested pre-START at the G1 *cdc2*^{ts} block and is active in cells arrested by pheromone (Stern and Nurse, 1997). These observations suggest that the activation of *cdc10*-dependent transcription and the accumulation of its major target, cdc18p, are not rate-limiting for the onset of S-phase. We also demonstrated that cdc2p protein kinase activity is required at a late stage in G1 and peaks close to the onset of S-phase. Therefore, the passage of cells beyond START may be driven by the action of G1 CDK activity rather than by the activation of *cdc10*-dependent transcription, and *cdc10*-dependent transcription in early G1 may provide the necessary gene products for a mitotic or meiotic S-phase, depending on the subsequent decision of cells to pass START (see also Miyamoto *et al.*, 1994).

Our studies have uncovered the roles of some of the components of the transcriptional complex in conferring the periodicity of S-phase transcription. We identified an unexpected role for res2p in the periodic repression of S-phase transcription during the mitotic cell cycle. It was thought previously that res2p acted primarily during the

meiotic cell cycle where it is essential (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). We have shown that res2p acts to inactivate transcription at the end of S-phase and to keep it low throughout the G2 phase of the mitotic cell cycle. When res2p is absent, transcription is high and constant throughout the cell cycle. Res2p is also required to form the DSC1 bandshift activity, which appears in G2 and correlates with inactive transcription. Thus, it is possible that res2p brings about repression of transcription in part through the action of the DSC1 complex.

The constitutive expression of *cdc10* targets seen throughout the cell cycle in *res2*Δ cells does not prevent them from dividing relatively normally. Thus periodic control is either redundant or important in other settings. For example, in meiosis, cells must carry out two rounds of nuclear division in the absence of an intervening S-phase. In this case, switching off *cdc10*-dependent transcription at an appropriate time may be critical. Alternatively, res2p may serve a different function in mitotic and meiotic cells; its role in periodic transcriptional control in mitotic cells may be of selective advantage simply because it ensures the efficient use of resources.

In contrast to res2p, res1p has an activating role in regulating periodic transcription. Res1p is required to maintain transcription in S-phase; in its absence, transcription is low and constant throughout the cell cycle. Importantly, high level ectopic expression of res1p during G2 is sufficient to activate transcription to a high level (see also Ayte *et al.*, 1995). This suggests that res1p is a key component in activating transcription periodically during the cell cycle. The cdc10p component is also critical for active transcription and is therefore essential for entry into S-phase (Nurse and Bissett, 1981). However, a mutation in the *res1* gene known as *sct1-1* (Marks, *et al.*, 1992; Caligiuri and Beach, 1993) enables the *cdc10*Δ strain to survive. Transcription in *sct1-1 cdc10*Δ cells is low and constant throughout the cell cycle, whereas it is more or less wild-type in the *sct1-1 cdc10*$^{+}$ strain, suggesting that cdc10p is also essential for the periodicity of S-phase transcription. Ectopically overexpressed cdc10p does not affect the periodicity or level of transcription, an observation supported by previous work (Ayte *et al.*, 1995). However, a truncated version of cdc10p encoded by *cdc10-C4* has been shown to activate transcription in G2 cells in the absence of wild-type cdc10p (McInerny *et al.*, 1995), suggesting an additional role for cdc10p in repressing transcription in G2 cells. Thus, it seems likely that res1p and cdc10p act together to promote activation of periodic S-phase transcription and that an altered complex containing cdc10p and res2p represses this transcription in G2 cells.

The rep2p component does not appear to contribute to the periodicity of S-phase transcription but is required to elevate the absolute level of activity. In the absence of rep2p, transcription is still periodic, but is much reduced in magnitude. Such a general activating role is consistent with its having a strong transcription activation domain (Nakashima *et al.*, 1995; P.Stacey, personal communication). Rep2p is not required to activate transcription when the res2p repressor is absent, suggesting that the role of rep2p may be to counteract the res2p repressor. These results support previous genetic data showing that *rep2* probably acts through *res2* (Nakashima *et al.*, 1995).

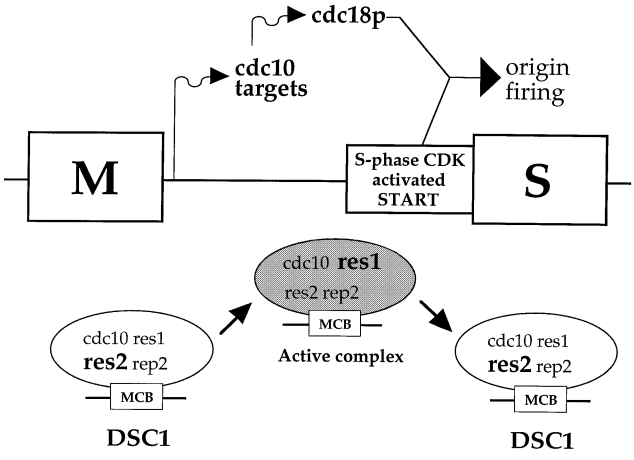

Fig. 9. Model for the control of *cdc10*-dependent transcription. We present a model for the way in which *cdc10*-dependent transcription may be controlled. We propose that *cdc10*-dependent transcription becomes active early in G1, independently of cdc2p activity, and that transcription persists until some time in S-phase. Cdc2p acts late in G1, after the accumulation of *cdc10* targets, to bring about the onset of S-phase. We suggest that cdc10p, res1p, res2p and rep2p are present in large complexes bound to MCB elements in target genes throughout the cell cycle. The periodicity of transcription may be brought about by a switch between an active and an inactive complex. Res1p may be the critical activator component and res2p may inactivate the complex in G2 (bold print shows dominant factor). These changes in activity may be brought about by alterations in the stoichiometry of res1p and res2p in the complex, or by post-translational modification of components with passage through the cell cycle.

Finally, we confirmed earlier reports that rep1p does not play a role in the mitotic cell cycle in the S-phase transcriptional control (Sugiyama *et al.*, 1994).

The above experiments allow us to identify the roles of the S-phase transcriptional components in cell cycle regulation and to propose a model for the way in which they interact to promote periodic transcription (Figure 9). Cdc10p may provide a scaffold on which the regulation of transcription is imposed by the binding of res1p and res2p. Deleting either *res2* or *res1* eliminates cell cycle periodicity, indicating that both are necessary for the switch between active and inactive transcription. Normally, res1p may inactivate the res2p inhibitor, activating transcription. When res1p is overexpressed during G2, inactivation of res2p would occur inappropriately, allowing the complex at the promoter to activate transcription. Ectopic expression of res2p cannot block active transcription, so its presence alone may not define the difference between the active and inactive complexes. However, the stoichiometry of res1p and res2p in the complex may be altered with passage through the cell cycle and define its activity as a repressor or activator of transcription. The role of the *rep2* factor may be to increase the efficiency of the whole complex in promoting transcription in its active state, perhaps by masking the presence of res2p in the active complex.

We have shown that *cdc10*, *res1*, *res2* and *rep2* are all required to form the DSC1 bandshift activity and that the presence of the bandshift correlates with repressed transcription. These observations suggest that all of these components are part of an *in vivo* complex related to DSC1, which binds to MCB sites in G2 cells, but which is unable to activate transcription. In contrast, previous studies proposed DSC1 as a good biochemical correlate

Table I. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype
1	*h⁻ 972*
19	*h⁻ cdc2-33*
1416	*h⁻ cdc13Δ::ura4+ cig1Δ::ura4+ cig2Δ::ura4+ cdc10-V50 pREP41::cdc13int LEU2*
1320	*h⁻ cdc13Δ::ura4+ cdc10-V50 pREP41::cdc13int LEU2*
17	*h⁻ cdc10-129*
275	*h⁻ cdc2-M26*
1435	*h⁻ cdc13-9 leu1-32*
1359	*h⁻ res1Δ::ura4 $^{+}$ ura4-D18*
B50	*h⁻ res2Δ::ura4 $^{+}$ ura4-D18*
1404	*h⁻ rep2Δ::ura4 $^{+}$ ura4 D18*
1365	*h⁻ sct1-1 cdc10Δ:: ura4^{+} ura4-D18*
1366	*h⁻ sct1-1 cdc10+ ura4-D18*
836	*h⁻ cdc25 leu1-32*
1405	*h⁻ res2Δ::ura4/ rep2Δ::ura4^{+} ura4-D18*
B51	*h⁻ cdc25-22 rep1Δ::ura4^{+}*

of the active complex (Reymond *et al.*, 1993). In budding yeast, there is also evidence for a role for *SWI4/SWI6–MBP1* in both the activation and repression of target gene expression (Dirick *et al.*, 1992; Lowndes *et al.*, 1992a; Koch *et al.*, 1996). In mammalian cells, the periodicity of transcription required for S-phase is also controlled in part by the conversion of the active E2F–DP1 transcription factor into a repressor by the binding of Rb early in G1 (Zamanian and La, 1993; Adnane *et al.*, 1995; Bremner *et al.*, 1995). Thus control of periodic expression of S-phase genes by both transcriptional activation and repression may be conserved, although our observation that transcriptional periodicity in fission yeast occurs independently of CDK activity indicates that similar transcriptional mechanisms may be regulated in different ways in different organisms.

Materials and methods

Fission yeast strains and methods

All strains used were constructed using standard procedures and are shown in Table I. Strains were grown in complete media (YES) and minimal media (EMM) as previously described (Moreno *et al.*, 1991). For nitrogen starvation experiments, cells were washed four times and resuspended in minimal-N_2 media and re-fed by the addition of 5 g/l NH_4Cl. In the experiment in Figure 1, cells were nitrogen starved for 15 h and then re-fed at 25 or 36°C. Upon starvation, a small fraction of *cdc2*^{ts} mutant cells always remain in G2. In the *cdc2-33* re-replication experiment (Figure 2), cells were arrested at G2–M at 36°C for 4 h in the absence of nitrogen (a small population of cells arrest at the *cdc2*^{ts} block in G1) and induced to re-enter S-phase by heat shock (Broek *et al.*, 1991). On re-feeding with nitrogen at 28.5°C, all the cells re-entered S-phase (at 25°C the recovery takes longer), but at 36.5°C cells were unable to duplicate their DNA. To enable *cdc10* and CDK activity to be manipulated independently in G1 cells, *cdc13::nmt cdc10-V50 cig1Δ cig2Δ* and *cdc13::nmt cdc10-V50 cig1*$^{+}$*cig2*$^{+}$ strains were constructed (Figure 3). They were arrested in G1 by shifting to 36°C, which inactivates the *cdc10* gene function and, after 3 h at 36°C, *cdc13* was switched off in half the cells by the addition of 5 μg/μl thiamine (Maundrell, 1993). After an additional 1 h at 36°C, cells were shifted back to 25°C, reactivating *cdc10*. In the strain expressing cdc13p but lacking cig1p and cig2p, some cells underwent premature mitosis after entering S-phase, probably as a result of the elevated levels of *cdc13* expressed from the Rep41 promoter. For the HU block and release experiments (Figures 4 and 5), 11 mM HU was added for 4 h to block cells at the G1–S boundary, and removed by two washes with pre-warmed media. To ectopically express *cdc10* components in the *cdc25* block and release experiment (Figure 7), *cdc25-22 leu1-32* cells were transformed with a multicopy plasmid (Maundrell, 1993) containing *res1* or *res2* behind the full strength *nmt* promoter in Rep3X, or with *cdc10* behind the medium strength *nmt* promoter in Rep41X, in the presence of 5 μg/μl thiamine. Cells were then washed to remove thiamine, grown for 20 h at 25°C to induce *nmt*-driven expression and shifted to the restrictive temperature for *cdc25-22* for 4 h. Cooling to 25°C then allowed cells to enter the mitotic cycle in synchrony. In the bandshift experiment shown in Figure 8C, *cdc2-33* cells were shifted to 34°C for 2 h to enable cells arrested in G1 to leak into G2 and then to 36.5°C for another 2 h to complete the cell cycle arrest. The *cdc25-22* cells were blocked at G2–M for 4 h at 36°C.

Flow cytometric analysis

A total of 2×10^6 cells were fixed in 70% ethanol, washed in 3 ml of 50 mM sodium citrate, resuspended in 1 ml of 50 mM sodium citrate, 0.1 mg of RNase, 2 μg/ml of propidium iodide, and incubated for 2 h at 37°C. We followed the previously published protocol for flow cytometry (Sazer and Sherwood, 1990), using a Becton-Dickinson FACScan.

Cultures synchronized by elutriation

Elutriation in all cases was carried out using a Beckman J6 centrifuge and elutriator rotor. The septation index was counted and plotted. Synchronicity was evaluated further by FACS analysis and cell number determination (using a Sysmex Microcellcounter F-800, on the white cell channel) of fixed cells. In the case of mutants grown at 30°C, the elutriator was pre-warmed at this temperature.

RNA preparation and Northern blot analysis

Cultures were washed in STOP buffer, frozen on dry ice and then kept at –70°C. Subsequently, RNA was prepared using glass bead lysis (Sigma No. G9268) in 0.1 M EDTA, 0.1 M NaCl, 0.05 M 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 vols of EtOH. Ten μg of sample RNA was 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 template DNA for the probes were: an *Nde*I–*Bam*H1 *cdc18*$^{+}$ fragment from REP1-*cdc18* cDNA; an *Nde*I–*Eco*RV *cig2* fragment from a genomic *cig2* clone in pAL-SK (Sergio Moreno); a *Sal*I–*Kpn*I *his3* fragment from a pKS *his3* plasmid; a *Hin*dIII *ura4* fragment from Rep4; a *cdt1* fragment from a PCR-derived cDNA clone (H.Nishitani) and a *cdc22* PCR fragment (primers from N.Lowndes). The membrane was hybridized overnight in 1% SDS, 10% dextran sulfate and 1 M NaCl, and washed in 1% SDS, 2× SSC.

Antibodies

The following antibodies were used: cdc18p polyclonal rabbit antiserum (H.Nishitani); cdc13p polyclonal rabbit antiserum (lab); cdc10p polyclonal rabbit antiserum raised against His_6TAG-cdc10 purified from *Escherichia coli* (J.Wuarin); an α-tubulin monoclonal antibody (Sigma); and rabbit polyclonal cdc2p antibody, C2 (lab).

Western blot analysis

Cells were boiled for 6 min prior to storage. Following glass bead lysis in HB buffer (Moreno *et al.*, 1991), protein concentration was determined and cell extracts were then re-boiled in 5× sample buffer. Then 50 μg of protein from each sample were run on an 8% SDS–polyacrylamide gel (Laemmli, 1970). For Western blots, the protein was blotted to Immobilon™-P membrane (Millipore) and detected using ECL (Amersham). Dilutions of the antibodies were 1:1000 for all polyclonal antibodies and 1:50 000 for the anti-α-tubulin monoclonal antibody (Sigma T5168).

H1 kinase assays

Extracts were prepared from frozen cells using glass bead lysis in HB buffer (Moreno *et al.*, 1991). Then 5 μl of rabbit polyclonal cdc2 antibody C2 (or pre-immune serum) was added to 500 μg of protein and incubated on ice for 45 min. Pre-equilibrated protein A–Sepharose beads (Pharmacia Biotech.) were added and the mixture agitated for 30 min at 4°C. Beads were washed three times and then resuspended in 15 μl of reaction buffer, containing 1 μg/μl calf thymus histone H1 (Sigma No. 382150), 200 μM ATP and 40 μCi/ml [γ-^{32}P]ATP (Amersham). Extracts were then incubated at 30°C for 20 min, stopped by boiling for 5 min after the addition of 5× SDS sample buffer and

run on a 12% SDS–polyacrylamide gel (Laemmli, 1970). In experiments using temperature-sensitive alleles, immunoprecipitates from control and *cdc2*^{ts} extracts were pre-incubated at 36.5°C for 5 min prior to the addition of reaction buffer (also at 36.5°C) and incubated for a further 20 min. Pre-immune sera gave no detectable signal.

Bandshift analysis

A double-stranded DNA probe made from the *cdc18* promoter containing both MCB repeats was amplified by PCR (Zhu *et al.*, 1994) and labelled by T4 polynucleotide kinase (BioLabs) with [γ-^{32}P]dATP (Amersham) and gel purified on a 4% polyacrylamide gel. Glass bead lysis was carried out in 25 mM HEPES pH 7.6, 0.1 mM EDTA, 150 mM KCl, 0.1% Triton X-100, 25% glycerol and 1 M urea in the presence of 1 mM dithiothreitol and protease inhibitors. Forty µg of soluble cell extract was pre-incubated for 10 min in gel-shift buffer: 25 mM HEPES pH 7.6, 34 mM KCl, 5 mM $MgCl_2$ with 0.1 µg/µl poly(dI–dC) and sonicated salmon sperm DNA, prior to the addition of excess radiolabelled probe. The gel-shift reaction was incubated for a further 15 min at room temperature then run on a native 4% acrylamide gel in 1× TBE for 3 h. DSC1 disappears upon the addition of cold-specific competitor DNA.

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