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

Buzz Baum, Jérôme Wuarin and Paul Nurse

Imperial Cancer Research Fund, Cell Cycle Laboratory, 44 Lincoln’s Inn Fields, London WC2A 3PX, UK

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 cdc10p, 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 mitotic 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. Keywords: cdc10/CDK/cell cycle/fission yeast/periodic transcription

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, SW14–SW16 and SW16–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 SW16p 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 G1 by the CDK, CDC28–CLN3 (Tyers et al., 1993; Dirick et al., 1995). Transcription from SCB elements is then switched off in G2 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 SW14/SW16–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 G2 (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 cdc10p-dependent transcription are triggered by G1 cdc2p activity (Raymond 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 (McEneny 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 G2 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 G2-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 G1 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 G1, 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 G1 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 ~1 h before the onset of S-phase (Figure 1A). This suggests that cdc18 transcription is activated in small G1 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 cdc2-M26 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 G1 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 G2 (Figure 2). cdc10 function is required for re-replicative DNA synthesis (Moreno and Nurse, 1994). Cells were arrested in G2 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 G2 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

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**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 G1 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 re-entering S-phase from G2 (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. \textit{cdc2} function is not required for the re-activation of \textit{cdc10}-dependent transcription following re-entry into S-phase from G$_2$. \textit{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 \textit{cdc2}-33 (28.5°C or 36.5°C). (A) The Northern blot was probed for \textit{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.

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

We conclude that \textit{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 \textit{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 \textit{cdc2p} activity remained to activate \textit{cdc10}-dependent transcription. However, this seems unlikely given the low levels of \textit{in vitro} H1 kinase activity detectable in the \textit{cdc2}-M strains at the restrictive temperature.

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

Three B-type cyclins act together with \textit{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 \textit{cdc10} function in G$_1$, we used a strain containing the temperature-sensitive \textit{cdc10-V50} mutation in which two of the \textit{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 \textit{cdc10}-dependent transcription and CDK activity independently. As a control strain, \textit{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 \textit{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 \textit{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).
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 cig1Δcig2Δ 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...
arrested at the onset of S-phase by the addition of 11 mM HU. Cells that contribute to the function of the transcriptional complex for FACS analysis. Cells harbouring deletions of components of the transcriptional complex (cdc10Δ and cdc13-9Δ) for cdc2-33Δ the temperature-sensitive functions (cdc10Δ, lane 3; data not shown for cdc10Δ, cdc18Δ and cdc2-M26Δ strains). (D) cdc2-33Δ and cdc2-M26Δ strains were subjected to a HU block and release, like that carried out in (C), and samples taken for FACS analysis.

temperature for cdc10-129Δ 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 cdc2-33Δ and cdc13-9Δ, 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 cdc13-9Δ). 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 cdc2-33Δ and cdc13-9Δ cells at the restrictive temperature (Figure 4C, cdc2-33Δ lanes 4 and 5, and cdc13-9Δ lanes 3 and 4). In cdc2-33Δ 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, cdc2-33Δ and cdc2-M26Δ 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, cdc10Δ, res1Δ, res2Δ or rep2Δ, 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 cdc10Δ sct1-1Δ (Marks et al., 1992; Caligiuri and Beach, 1993) was used in which a point mutation in res1Δ (sct1-1) suppresses the lethality of the cdc10Δ sct1-1Δ cdc18+ rep2Δ rep2Δ cells 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 cdc13-9Δ). 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 cdc2-33Δ and cdc13-9Δ cells at the restrictive temperature (Figure 4C, cdc2-33Δ lanes 4 and 5, and cdc13-9Δ lanes 3 and 4). In cdc2-33Δ 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, cdc2-33Δ and cdc2-M26Δ 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.

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 res1Δ cells, levels of cdc18 transcript were similar to the low levels seen in wild-type cells, exponentially growing rep2Δ and cdc10Δ sct1-1Δ cells exhibited even lower cdc18 transcript levels (Figure 5B, columns 1, 4, 10 and 13), whereas in the res2Δ 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 res1Δ or cdc10Δ sct1-1Δ cells and only a small

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**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 and then released into S-phase and into G2. (C) cdc2-33Δ and cdc13-9Δ cells were arrested in HU for 4 h (lanes 1 and 2) and then shifted to 36°C for 30 min to inactivate the temperature-sensitive functions (cdc2-33Δ, lane 3; data not shown for cdc13-9Δ). 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 his3Δ. (D) cdc2-33Δ and cdc2-M26Δ strains were subjected to a HU block and release, like that carried out in (C), and samples taken for FACS analysis.
Fig. 5. The role of components of the cdc10 complex in the control of periodic transcription in mitotic cells. res1Δ, res2Δ, rep2Δ and sct1-1 cdc10Δ (A and B) and the double mutant res2Δ rep2Δ and sct1-1 cdc10Δ (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 rep2Δ 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 res2Δ 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 1 h of removing HU, as S-phase was completed, cdc18 transcript levels fell dramatically (Figure 5B, column 3). rep2Δ cells and cdc10Δ sct1-1 did not exhibit a reduced cdc18 transcript level after HU was removed, whereas a reduction was observed in rep2Δ cells (Figure 5B, columns 6, 12 and 15). As a control for cdc10Δ 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 cdc10Δ sct1-1 strain.

These results show that cdc18 transcript levels are reduced and constant during the HU block and release in res1Δ and cdc10Δ sct1-1 strains. cdc18 transcription is elevated and constant in res2Δ cells, and is reduced but periodic in rep2Δ cells. In the double mutant rep2Δ res2Δ, 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
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 res1Δ 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 res2Δ 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 cdc10Δ sct1-1 strain (Figure 6D), cdc18 transcript levels were constant but very low through-out the cell cycle. We conclude that although the cdc10Δ 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 rep2Δ 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 res1, 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 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...
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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.

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 complex in cells synchronized in G2 and released into the cell cycle. 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, res1Δ, res2Δ, cdc10Δsct1-1 and rep2Δ cells (Figure 8b). Previous work

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.

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 res1Δ, res2Δ, cdc10Δ in a sct1-1 background and rep2Δ 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 (Zha 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 rep2Δ cells, suggesting that in the absence of rep2Δ, DSC1 may form inefficiently. It is noteworthy that DSC1 is absent in res2Δ 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 cdc2Δ 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—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 puc1Δ and puc1Δcig2Δ 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 SW14/SW16-MBP1 (Tyers et al., 1993; Dirick et al., 1995). In further support of our interpretation, we observed high levels of cdc18 mRNA in res2Δcdc2-33 cells after 4 h at the restrictive temperature (data not shown), suggesting that cdc2 is not required for the elevated transcription seen in res2Δ 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 cdc2Δ 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).
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 res2Δ 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 cdc10Δ strain to survive. Transcription in sct1-1 cdc10Δ cells is low and constant throughout the cell cycle, whereas it is more or less wild-type in the sct1-1 cdc10Δ 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 rep2a probably acts through res2 (Nakashima et al., 1995).

![Diagram](https://example.com/diagram.png)

**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.
of the active complex (Reymond et al., 1993). In budding yeast, there is also evidence for a role for SWI/SWI6–MBP1 in both the activation and repression of target gene transcription (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; Broderick 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 media and re-fed by the addition of 5 g/l NH4Cl. 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 ofcdc13 cells always remain in G2. 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 2×106 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. Synchrony was evaluated further by FACScan 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 NH4OAc 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-32P]dATP using a Prime-It Kit (Stratagene). The template DNA for the probes were: an NdeI–BamHI cdc18 fragment from REP1–cdc18 cDNA, an NdeI–EcoRV cdc2 fragment from a genomic cdc2 clone in pAL-SK (Sergio Moreno); a SalI–KpnI his3 fragment from a pKS his3 plasmid; a HindIII ura4 fragment from Rep4; a cdc17 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 antisemur (H.Nishitani); cdc13p polyclonal rabbit antisemur (lab); cdc2p polyclonal rabbit antisemur raised against His 6TAG-cdc10 purified from Escherichia coli and rabbit polyclonal cdc2p antibody, C2 (lab).

Western blot analysis

Cells were boiled for 5 min prior to storage. Following gel band 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) and rabbit polyclonal cdc2p antibody, C2 (lab).

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/ml calf thymus histone H1 (Sigma No. 382150), 200 μM ATP and ATP 40 μCi/ml [γ-32P]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

Table I. Schizosaccharomyces pombe strains used in this study

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<th>Strain</th>
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<td>h–</td>
<td>972</td>
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<tr>
<td>h– cdc2-33</td>
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<tr>
<td>h– cdc13Δ::ura4+ cig1Δ::ura4+ cig2A::ura4+ cdc10-T50 pREP41::cdc13int LEU2</td>
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<tr>
<td>h– cdc13Δ::ura4+ cdc10-T50 pREP41::cdc13int LEU2</td>
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<tr>
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<tr>
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<tr>
<td>h– res2</td>
<td>1359</td>
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<tr>
<td>h– cdc2-22</td>
<td>1405</td>
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<tr>
<td>h– cdc25 leu1-32</td>
<td>836</td>
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<tr>
<td>h– cdc25-22 rep1X::ura4*</td>
<td>1405</td>
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<tr>
<td>or res2</td>
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or res2 behind the full strength nmt promoter in Rep3X, or with cdc10 behind the medium strength nmt promoter in Rep4X, in the presence of 5 μg/ml 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.

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of the active complex (Reymond et al., 1993). In budding yeast, there is also evidence for a role for SWI/SWI6–MBP1 in both the activation and repression of target gene transcription (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; Broderick 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.

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run on a 12% SDS-polyacrylamide gel (Laemmli, 1970). In experiments using temperature-sensitive alleles, immunoprecipitates from control and cycD2 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 with T4 polynucleotide kinase (BioLabs) with [γ-32P]ATP (Amersham) and gel purified on a 4% polyacrylamide gel. Gel band 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 MgCl2 with 0.1 μg/mL 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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