Activation of S-phase-promoting CDKs in late G₁ defines a “point of no return” after which Cdc6 synthesis cannot promote DNA replication in yeast

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In eukaryotic cells, DNA replication is confined to a discrete period of the cell cycle and does not usually recur until after anaphase. In the budding yeast *Saccharomyces cerevisiae*, assembly of pre-replication complexes (pre-RCs) at future origins as cells exit mitosis (or later during G₁) is necessary for subsequent initiation of DNA replication triggered by activation in late G₁ of Cdc28/Cdk1 kinases associated with B-type cyclins Clb1–Clb6. The absence of pre-RCs during G₂ and M phases could explain why origins of DNA replication fire only once during the cell cycle, even though S-phase-promoting Cdk1s remain active from the beginning of S phase through the end of M phase. Formation of pre-RCs and their maintenance during G₁ depend on the synthesis and activity of an unstable protein encoded by *CDC6*. We find that Cdc6 synthesis can only promote DNA replication in a restricted window of the cell cycle: between destruction of Clbs after anaphase and activation of Clb5/ and Clb6/Cdk1 in late G₁. The latter corresponds to a “point of no return,” after which Cdc6 synthesis can no longer promote DNA replication. Cdc6 protein can be made throughout the cell cycle and, in certain circumstances, can accumulate within the nuclei of G₂ and M phase cells without inducing re-replication. Thus, control over Cdc6 degradation and/or nuclear localization is not crucial for preventing origin re-firing. Our data are consistent with the notion that cells can no longer incorporate de novo synthesized Cdc6 into pre-RCs once Clb/Cdk1 kinases have been activated. We show that Cdc6p associates with Clb/Cdk1 kinases from late G₁ until late anaphase, which might be important for inhibiting pre-RC assembly during S, G₂, and M phases. Inhibition of pre-RC assembly by the same kinases that trigger initiation explains how origins are prevented from re-firing until Clb kinases are destroyed after anaphase.

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There is considerable evidence that chromosome duplication is triggered by the activation in late G₁ of particular cyclin-dependent kinases, known as S-phase-promoting Cdk1s. In the budding yeast *Saccharomyces cerevisiae*, entry into S phase depends on Cdk1s formed by the association of one of six unstable B-type cyclins, Clb1–Clb6, with a single kinase subunit, Cdk1/Cdc28 (Schwob et al. 1994). Cyclin B/Cdk1 kinases are also required for the assembly of a mitotic spindle and for the onset of anaphase in yeast (Surana et al. 1991; Fitch et al. 1992; Schwob and Nasmyth 1993).

All six cyclin B/Cdk1 kinases are inactivated as cells complete anaphase (Amon et al. 1994; Irmiger et al. 1995). Their reactivation during the subsequent G₁ pe-

riod involves periodic transcription of cyclin genes (Koch and Nasmyth 1994), cessation of cyclin B proteolysis (Amon et al. 1994), and, most important, destruction of p40^{SIC1}, which otherwise inhibits cyclin B/Cdk1 kinases through the formation of ternary p40^{SIC1}/cyclinB/Cdk1 complexes (Mendenhall 1993; Schwob et al. 1994). Inactivation of all six B-type cyclins or mutations in genes like *CDC4* and *CDC34*, which are needed for p40^{SIC1} proteolysis, causes cells to arrest in G₁, whereas inactivation of the *SIC1* gene modestly advances S phase (Schwob et al. 1994). The equivalent S-phase-promoting Cdk1s in metazoa appear to be complexes between an S-phase-specific kinase subunit Cdk2 and a similar set of cyclins, known as E-type cyclins.

It is thought that Clb5/ and Clb6/Cdk1 kinases normally trigger S phase in yeast (Epstein and Cross 1992; Schwob and Nasmyth 1993); these are the first B-type cyclins to appear because of transcriptional controls that cause *CLB5* and *CLB6* mRNAs to accumulate in late G₁.

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CLB3 and *CLB4* are transcribed in S and G_2 phases, whereas *CLB1* and *CLB2* are transcribed in G_2 and M phases (Koch and Nasmyth 1994). Deletion of both *CLB5* and *CLB6* is not lethal but causes S phase to be delayed by about 30 min (Schwob and Nasmyth 1993), that is, until the later Clbs have had time to accumulate.

It is now clear that one or another of the six S-phase-promoting cyclinB/Cdk1 kinases are active from the time of p40^{STC1} destruction in late G_1 to the activation of cyclin B proteolysis during anaphase, and yet, origins fire once, and only once, during this wide window of the yeast cell cycle. Why do S-phase-promoting Cdks trigger chromosome duplication in G_1 cells but not in G_2 ? Might the appropriate substrates for S-phase-promoting Cdks only exist in G_1 cells? Proteins that bind to origins of DNA replication might be the key substrates for Cdks, and their availability might vary during the cell cycle. A large multisubunit complex called the origin recognition complex (ORC) binds to and is required for the activity of yeast origins (Bell and Stillman 1992; Micklem et al. 1993; Fox et al. 1995; Liang et al. 1995). ORC is bound to origins at all stages of the cell cycle (Diffley and Cocker 1992), but the DNase I sensitivity of the neighboring chromatin changes during the cycle (Diffley et al. 1994). Sequences adjacent to ORC are protected from DNase I during G_1 but not during G_2 or M phase. The prereplicative protected state of origins arises at the end of mitosis, following the inactivation of Clb/Cdk1 kinases, and lasts until the beginning or middle of S phase (Diffley et al. 1994).

The formation and maintenance of the prereplicative state of origins depend on the synthesis of an unstable protein encoded by the *CDC6* gene (Cocker et al. 1996), which is essential for the initiation of DNA replication (Hartwell 1976; Bueno and Russell 1992; Liang et al. 1995; Piatti et al. 1995). Defects in the replication of minichromosomes in *cdc6* mutants can be alleviated by addition of extra origins, suggesting that Cdc6 acts at origins (Hogan and Koshland 1992). *CDC6* transcription occurs in two bursts during the cycle: at the end of mitosis, coinciding with the switch of origins from postreplicative to prereplicative state, and in late G_1 (Zwerschke et al. 1994; Piatti et al. 1995). Cells that exit from mitosis without *de novo* Cdc6 protein synthesis fail both to establish the prereplicative state at their origins (Cocker et al. 1996) and to initiate DNA replication, despite activating S-phase-promoting Cdks on schedule; such cells proceed with the alignment of unreplicated chromosomes on mitotic spindles and finally undergo a "reductional" anaphase (Piatti et al. 1995).

These data suggest that Cdc6 synthesis is required for the assembly of protein complexes adjacent to ORC (pre-replicative complexes or pre-RCs), and the formation of such pre-RCs at the end of mitosis or in late G_1 (when there is a second burst of Cdc6 synthesis) might be the first of two steps needed for the initiation of DNA replication; the second step being the activation of cyclin B/Cdk1 kinases. According to this notion, the presence of pre-RCs in G_1 , but not in G_2 cells, could explain why S-phase-promoting Cdks trigger only the former to enter

S phase. Thus, the disassembly of pre-RCs upon origin firing and their failure to reform during S, G_2 , and M phases could have an important role in preventing re-replication during the cell cycle.

To address how budding yeast restricts the formation of pre-RCs to a short period following anaphase, we investigated at what time during the cell cycle Cdc6 protein is capable of forming pre-RCs and promoting DNA replication. We show that Cdc6 is capable of inducing DNA replication only when synthesized during a narrow window of the cell cycle, between the end of anaphase, when B-type cyclins are destroyed, and a point that roughly coincides with the beginning of the subsequent S phase, when Clb5-6/Cdk1 kinases are reactivated. There exists, therefore, a "point of no return" in late G_1 , after which Cdc6 protein loses the ability to promote DNA replication, presumably because it can no longer form pre-RCs. Our data are consistent with the proposal that assembly of pre-RCs is inhibited by the same set of cyclin B/Cdk1 kinases that trigger DNA replication (Dahmann et al. 1995). We find that Cdc6p can accumulate throughout the cell cycle when expressed ectopically, but that it accumulates efficiently in the nucleus only between the end of mitosis and late G_1 . The lower Cdc6 accumulation within nuclei cannot, however, account for its inability to form pre-RCs and promote DNA replication in G_2 , because a mutation (*cdc4-1*) that reduces the rate of Cdc6 proteolysis allows it to accumulate to high levels within G_2 /M phase nuclei without causing them to re-replicate. We find that Cdc6 protein associates with cyclinB/Cdk1 kinases during S, G_2 , and M phases. This interaction could be part of the mechanism by which cyclin B/Cdk1 kinases inhibit the formation of pre-RCs.

Results

Cdc6 synthesis is able to promote formation of pre-RCs in α -factor- but not in nocodazole-arrested cells

We have shown that *de novo* synthesis of Cdc6, either as cells exit from mitosis or, subsequently, in late G_1 , is necessary for DNA replication in budding yeast but not for progress through other aspects of the cell cycle. Cells that fail to synthesize Cdc6 during G_1 proceed with the formation of mitotic spindles and segregate their unreplicated chromosomes at random to mother and daughter cells (Piatti et al. 1995). Inactivation of mitotic Clb/Cdk1 kinases (by elevating the temperature of a strain lacking *CLB1*, *CLB3*, and *CLB4* and carrying a temperature-sensitive allele of *CLB2*), which are required for assembly of mitotic spindles, prevented the abnormal nuclear division of cells deprived of Cdc6 but not, surprisingly, their rapid death; that is, they lost the ability to form colonies when Cdc6 and Clb2 functions were restored (see Fig. 6 in Piatti et al. 1995). We repeated this experiment and measured the DNA content of cells by fluorescence-activated cell sorting (FACS) analysis during the abortive recovery period. We found that cells

were unable to replicate their chromosomes even when Cdc6 synthesis was restored 0.5 hr before shift to the permissive temperature, which restores Clb2 function (data not shown).

These data could be explained if Cdc6 synthesis were capable of promoting DNA replication only during certain stages of the cell cycle; cells deprived of Clb1–Clb4 might arrest at a stage of the cell cycle during which Cdc6 cannot promote the formation of pre-RCs. Therefore, we compared the formation of pre-RCs in response to de novo Cdc6 synthesis in cells arrested by pheromone in a G₁-like state with the formation of pre-RCs in cells arrested by nocodazole in a G₂ or M phase-like state. Because we shut off Cdc6 synthesis prior to arresting the cells, these two states do not correspond to conventional G₁ or G₂/M states. G₁ cells normally possess pre-RCs at replication origins, and nocodazole-arrested cells normally have replicated chromosomes. To perform the experiment, we used a strain whose only functional Cdc6 protein was fused to ubiquitin and was expressed from the *GAL1-10* promoter (*GAL-ubiCDC6*). The strain also contained a *cdc15* mutation that allowed us to arrest cells in late anaphase by incubation at 37°C. Upon a shift down to 25°C in the presence of α factor but in the absence of galactose, cells entered the next G₁ phase synchronously without producing Cdc6 protein. This produced a culture of unbudded cells lacking pre-RCs at the 2 μ origin (Fig. 1). The culture was then split and one-half was incubated for 90 min in medium still lacking galactose, but containing nocodazole, which caused cells to arrest with buds and high levels of Clb/Cdk1 kinases but with unreplicated chromosomes lacking pre-RCs. Reactivation of Cdc6 synthesis by addition of galactose induced formation of pre-RCs (as measured by the protection from DNase I of DNA sequences adjacent to those bound by ORC) in the α -factor arrested culture but not in the nocodazole one. These data suggest that de novo Cdc6 synthesis cannot induce the formation pre-RCs in cells whose state resembles that of a G₂ or M-phase cell, even when a prior lack of Cdc6 synthesis has prevented the replication of their chromosomes.

Delayed Cdc6 synthesis cannot promote DNA replication

We set out next to measure the cell-cycle dependence of Cdc6's capacity to promote DNA replication. We generated synchronized populations of G₁ cells that lack Cdc6 protein and turned on Cdc6 synthesis at will during the subsequent progression of these cells through the cell cycle. We synchronized cells in late anaphase by use of a temperature-sensitive *cdc15* mutation. Figure 2A shows cellular DNA contents measured by FACS analysis in the period following release from a *cdc15* arrest in cells containing an intact *CDC6* gene. Cells started with a 2C DNA content (because of 1C chromosome clusters/nuclei at each end of the budded cell) and most, if not all, cells replicated DNA before they completed cell division, accumulating with 4C DNA contents. To estimate

Figure 1. pre-RCs cannot be formed in G₂/M. *cdc15 GAL-ubiCDC6* (K5033) cells were arrested in either α factor or nocodazole after having been deprived of Cdc6 protein (see Materials and methods). During the arrests the synthesis of Cdc6 protein was reinduced by shift to galactose medium. The triangles indicate increasing concentrations of DNase I. The position of the ORC-induced hypersensitive site is indicated by an asterisk (*).

more accurately the extent to which replication occurs, we use the FACS profiles to calculate the fraction of 2C nuclei by the equation described in Materials and methods. Application of this formula shows that most nuclei have a 1C DNA content during the *cdc15* arrest and duplicate their DNA within 60 min following release (Fig. 2D).

We repeated the experiment with a *cdc15 GAL-ubiCDC6* strain. We first synchronized cells in late anaphase by shifting cells to the restrictive temperature. Cdc6 synthesis was then repressed by removal of galactose from the medium, and 30 min later, cells were released from their mitotic arrest (still in the absence of galactose) by lowering of the temperature. Under these circumstances, cells exited from their late anaphase arrest in the absence of de novo Cdc6 synthesis and, as a consequence, they failed to enter S phase; nuclei re-

Figure 2. Cells deprived of Cdc6 in late mitosis are unable to replicate in the next cell cycle but undergo reductional anaphase. *cdc15* (K1993) and *cdc15* *GAL-ubiCDC6* cells (K5032) growing in YEPRG medium were arrested by a 3-hr incubation at 37°C. Afterwards, cells were filtered, resuspended in YEPR at 37°C and after 30 min (time=0) released into either YEPR (A,B) or YEPRG medium at 25°C (C). In D for the same experiment described in A, B, and C, the fractions of 2C nuclei are shown [i.e., nuclei that have undergone DNA replication] calculated with the equation described in Materials and methods.

tained a 1C DNA content for at least 120 min following release (Fig. 2B), but they subsequently (between 180 and 240 min) underwent a reductional anaphase to produce nuclei with DNA contents less than 1C. Readdition of galactose to the medium when cells were returned to 25°C allowed 85% of the nuclei to replicate with kinetics similar to *CDC6*⁺ cells, which demonstrates that de novo synthesis of Cdc6 from the *GAL* promoter at the time of *cdc15* release is sufficient to drive S phase (Fig. 2C).

We then repeated the above experiment varying the time of *GAL-ubiCDC6* induction; that is, we arrested cells at 37°C, shut off Cdc6 synthesis, and reinduced it at 0, 10, 20, 30, ... min after release from the *cdc15* arrest. Figure 3A shows for each time point the percentage of nuclei that replicated their DNA during the subsequent 120 min. All cells retained the ability to replicate DNA upon addition of galactose until 20 min after the release, some of the cells lost the ability by 30 min, and few, if any, cells retained it by 40 min. We repeated this experiment using a strain in which the synthesis of Cdc6 (this time not fused to ubiquitin) was instead controlled by the methionine-repressible *MET3* promoter. Cells again lost the ability to replicate DNA upon induction of Cdc6 synthesis 40 min after release from the late anaphase *cdc15* arrest (data not shown). These data suggest that there exists a point of no return, between 30 and 40 min after *cdc15* release, after which induction of *CDC6* transcription is ineffective in driving DNA replication. It is interesting that this point is around the time that cells would normally have entered S phase, had the *CDC6* gene been fully functional.

To test whether a cell's failure to induce DNA replication after the point of no return is caused by a simple failure to synthesize sufficient Cdc6 protein, we repeated the previous experiment using a strain whose sole functional *CDC6* gene is an HA-tagged version under the control of the *GAL1-10* promoter (*GAL-HA3CDC6*).

The HA-tagged gene is fully functional because these cells proliferate normally in galactose medium. The epitope tag allowed us to follow accumulation of Cdc6 protein by Western blotting after inducing *GAL-HA3CDC6* expression at various times following *cdc15* release. As shown for *GAL-ubiCDC6* cells, *GAL-HA3CDC6* cells lost the ability to replicate DNA in response to galactose induction 40 min after release (Fig. 3B), even though they retained the ability to accumulate Cdc6 protein throughout the time course (Fig. 3C). In fact, we observed higher levels of HA3Cdc6 accumulation 60 min after *cdc15* release, at which time no cells were capable of replicating, than we did 20 min after release, at which time galactose induced most cells to replicate. Thus, an increased proteolysis of Cdc6 after the point of no return is not responsible for inability of cells that have passed it to replicate DNA in response to de novo Cdc6 synthesis.

When does Cdc6 execute its function?

To address whether Cdc6 is needed for DNA replication subsequent to the formation of pre-RCs, we arrested wild-type and temperature-sensitive *cdc6-1* cells in α factor at the permissive temperature, incubated them at 37°C for 2 hr [under which circumstance pre-RCs disappear (Cocker et al. 1996)], and then released them from the pheromone block at 37°C. Wild-type cells replicated their DNA synchronously, whereas the *cdc6* mutants replicated extremely poorly, if at all (data not shown). Therefore, Cdc6's replication function is also needed after a pheromone-induced G₁ arrest, even though cells had previously formed pre-RCs. We conclude that Cdc6 is necessary for the formation and maintenance of pre-RCs during early G₁, when Cdk1 is inactive, but also for subsequent DNA replication, when Cdk1 is activated in late G₁.

Figure 3. Cells pass a point of no return at the time of S-phase entry, after which Cdc6 synthesis is unable to promote DNA replication. (A) *cdc15* (K1993) and *cdc15 GAL-ubiCDC6* (K5032) cells were treated as in Fig. 2. After release from the *cdc15* arrest in YEPR ($t=0$), aliquots of the *cdc15 GAL-ubiCDC6* culture, withdrawn at 10 min intervals (right), were supplemented with 2% galactose to reinduce Cdc6 synthesis. For each independent culture, cell samples were analyzed by FACS every 30 min for 2 hr. The fractions of 2C nuclei at each time point have been calculated by use of the formula described in Materials and methods. (B) The same experimental procedure used in A has been applied to a *GAL-HA3CDC6* strain (K5763), with the exception that 0.1% galactose has been used to reinduce Cdc6 synthesis. (C) Under the same experimental conditions as in B, expression of HA3Cdc6 at the different time points has been evaluated by Western analysis after 30 min of induction. Swi6p was used as an internal loading control.

The abundance of Cdc6 in the nucleus varies during the cell cycle

We addressed whether a change in cells' ability to accumulate Cdc6 in the nucleus could explain their point of no return. By use of strains where the endogenous Cdc6 was tagged at its carboxyl terminus by 12 c-myc epitopes, we observed that the protein accumulated within nuclei at the end of mitosis (data not shown). This result does not tell us much about the ability of Cdc6 to enter nuclei after the point of no return, how-

ever, because Cdc6 protein levels drop dramatically as cells enter S phase (Piatti et al. 1995). To address this issue, we constructed strains carrying, in addition to the endogenous *CDC6* gene, a version of *CDC6* tagged at its amino-terminus by nine c-myc epitopes expressed from the *GAL1-10* promoter (*GAL-myc9CDC6*). The myc9Cdc6 protein was fully functional, because it rescued strains carrying a *CDC6* deletion and could be detected by in situ immunofluorescence 90 min after induction (not shown). myc9Cdc6's localization depended on cell-cycle position; the protein accumulated to relatively high levels in the nuclei of post-anaphase

and G₁ cells, but was more evenly distributed between nucleus and cytoplasm in cells at other stages of the cell cycle. We investigated this cell-cycle dependence further by analyzing the distribution of myc9Cdc6 in cells released from a *cdc15* arrest (Fig. 4A,C). Myc9Cdc6 protein was present throughout the cell in *cdc15*-arrested cells (it was neither excluded from nor accumulated within nuclei), it was rapidly concentrated within nuclei shortly after release, but around the time of S-phase entry (45 min after release) it lost this nuclear accumulation, becoming redistributed throughout the cell; this distribution lasted until cells re-entered anaphase, when the protein re-accumulated within nuclei. The protein levels did not change dramatically during this period (Fig. 4B), which suggests that the lack of nuclear accumulation during S, G₂, and M phases is caused by a changed distribution and not attributable simply to lower amounts of protein per cell. We obtained very similar results with cells synchronized by centrifugal elutriation (Fig. 4D). The most noticeable change in Cdc6 distribution was a redistribution to the cytoplasm around the beginning of S phase and reaccumulation within nuclei during anaphase. This pattern resembles that of Cdc47/Mcm proteins, which are also required for the initiation of DNA replication (Hennessy and Botstein 1990; Yan et al. 1993; Dalton and Whitbread 1995). We conclude that a reduction in Cdc6's ability to accumulate within nuclei might contribute to its inability to promote DNA replication once cells pass the point of no return, but it is

unlikely to be responsible for this phenomenon because Cdc6 is never excluded from nuclei.

The point of no return depends on activation of S-phase-promoting CDKs

The point of no return corresponds roughly with activation of Cln1,2/ and Clb5,6/Cdk1 kinases in late G₁. Dahmann et al. (1995) have postulated that Clb/Cdk1 kinases have dual roles: they trigger origins that have previously formed pre-RCs to initiate DNA replication while they simultaneously prevent de novo formation of pre-RCs. If S-phase-promoting Clb/Cdks were involved in preventing Cdc6 from inducing replication, then inactivation of *CLB5* and *CLB6* should delay the point of no return, as they are the first B-type cyclin genes to be expressed during the cell cycle (Schwob and Nasmyth 1993). S-phase entry is delayed by ~30 min in *clb5 clb6* double mutants; that is, until the later accumulation of Clb1-4/Cdk1 kinases (Schwob et al. 1994). This delay is also seen during release from a *cdc15* arrest. *CLB5 CLB6 cdc15* cells commence replication about 45 min after their release, whereas *clb5 clb6 cdc15* mutants do not start until 90 min, even though budding and activation of Clb2/Cdk1 kinase occur on schedule (Fig. 5A,B). Deletion of *CLB5* and *CLB6* also extended the length of time, following release from a *cdc15* arrest, during which induction of Cdc6 was capable of promoting DNA replication (cf. Figs. 3A and 5C); 50% of *CLB5 CLB6* cells

Figure 4. The abundance of nuclear Cdc6 varies during the cell cycle. *cdc15 GAL-myc9CDC6* (K5923) cells growing in YEPRG were arrested for 3 hr at 37°C ($t=0$) and then released at 25°C. Cell samples were collected every 15 min for FACS, Western analysis, and in situ immunofluorescence with the 9E10 antibody. (cyc) Cycling cells. (DIC) differential interference contrast. (A) In situ immunofluorescence. (B) Western analysis; C is a protein that cross-reacts with the anti-Cdc28 antibody and was used as internal loading control. (C) FACS analysis; the fraction of 2C nuclei at each time point was calculated as described in Materials and methods. (○) Percent 2C nuclei; (●) percent brightly stained nuclei. (D) *GAL-myc9CDC6* (K5919) cells growing in YEPR at 30°C were induced for 90 min by addition of 2% galactose and then elutriated. Small G₁ cells were grown out in YEPRG at 30°C, and at the indicated times cells were collected for FACS, Western analysis (not shown), and in situ immunofluorescence. (□) Percent budded cells; (○) percent 2C cells; (●) percent brightly stained nuclei.

Figure 5. Deletion of *CLB5* and *CLB6* causes a delay in S-phase entry and a similar delay in the point of no return. *cdc15* (K1993) and *cdc15 clb5 clb6* (K5027) cells growing in YEPD were arrested for 3.5 hr at 37°C and then released at 25°C. At different time points, cell samples were withdrawn for FACS analysis and budding index (A) and to assay Clb2-dependent histone H1 kinase activity (B). (cyc) Cycling cells. (C) *cdc15 clb5 clb6 GAL-ubiCDC6* cells (K5231) were treated as described for K5032 in Fig. 3. After release from *cdc15* arrest into YEPR ($t=0$), aliquots of K5231 culture, withdrawn at 10-min intervals (right), were supplemented with 2% galactose and incubated in YEPRG for 180 min. From each individual culture, cell samples were collected every 30 min for FACS analysis, and the fractions of 2C nuclei were evaluated by use of the equation described in Materials and methods. [○] *cdc15 GAL-ubiCDC6*; [●] *cdc15 clb5 clb6 GAL-ubiCDC6*.

passed the point of no return by 30 min, whereas the equivalent point was not reached until 60 min in *clb5 clb6* double mutants. We obtained very similar results comparing the points of no return of *cdc15 MET-CDC6* and *cdc15 clb5 clb6 MET-CDC6* cells (data not shown). Thus, loss of Clb5 and Clb6 activity delays passage through the point of no return to a degree that is similar to the delay in S-phase entry. This finding implies that Cln/Cdk1 kinases, which are also required for S-phase entry as activators of Clb kinases (Amon et al. 1994; Dirick et al. 1995), are not sufficient to promote passage

through the point of no return, because the kinetics of their activation is not affected by deletion of *CLB5* and *CLB6*. Clb1–Clb4 assume the S-phase-promoting function of Clb 5 and Clb 6 in *clb5 clb6* double mutants. We presume that they also assume from Clb 5 and Clb 6 the function of promoting passage through the point of no return, but for technical reasons, we have not been able to test this (see below). Nevertheless, this presumption is consistent with the finding that inhibition of all six Clb/Cdk1 kinases in nocodazole-blocked cells is sufficient to induce pre-RCs (Dahmann et al. 1995).

Passage through the point of no return does not require Cdc7

The point of no return and its dependence on Cdks that promote DNA replication can be explained by the proposal that, in addition to triggering replication from origins that have previously formed pre-RCs, cyclin B/Cdk1 kinases also inhibit de novo assembly of pre-RCs (Dahmann et al. 1995). An alternative explanation is that some other event occurring at the time of S-phase entry shuts off Cdc6's ability to promote DNA replication. Might the inhibition of Cdc6 function in late G₁ be caused by the activation of S-phase-promoting factors other than Clb/Cdks? Note, however, that their activation would have to depend on Clb5 and Clb6. One candidate is the Dbf4/Cdc7 protein kinase (Jackson et al. 1993; Yoon et al. 1993). *cdc7* mutants arrest in G₁ with pre-RCs still present at origins (Diffley et al. 1994) despite having activated Clb/Cdk1 kinases (Amon et al. 1992; Schwob et al. 1994). To test whether the Cdc7 protein kinase is needed for passage through the point of no return, we analyzed whether de novo Cdc6 synthesis could induce DNA replication upon release of cells arrested by a temperature-sensitive *cdc7* mutation.

We arrested temperature-sensitive *cdc7-1* WT-*CDC6* and *cdc7-1* *GAL-ubiCDC6* cells with nocodazole at 25°C in the presence of galactose (*GAL-ubiCDC6* on) and then shifted them to medium lacking both nocodazole and galactose (*GAL-ubiCDC6* off) at 37°C, which caused cells to exit from mitosis and arrest in the next cycle as budded cells with a 1C DNA content. Under these circumstances, WT-*CDC6* but not *GAL-ubiCDC6* cells will have formed pre-RCs (*GAL-ubiCDC6* *CDC7* cells were unable to replicate after release from nocodazole in the absence of galactose at 37°C (Fig. 6A), confirming that *GAL-ubiCDC6* cells failed to produce Cdc6 protein under these circumstances). Both WT-*CDC6* *cdc7* and *GAL-ubiCDC6* *cdc7* cultures were split and galactose added to one-half to reinduce Cdc6 synthesis. Finally, 15 min later, all four cultures were shifted to 25°C to restore Cdc7 function. *GAL-ubiCDC6* cells largely failed to undergo DNA replication whether or not galactose was present, despite efficient induction of *CDC6* transcripts in its presence (data not shown). WT-*CDC6* cells, in contrast, underwent a complete round of replication in both media (Fig. 6A). This result suggests that *cdc7*-arrested cells have already passed through the point of no return. Cdc7 is therefore not required for this transition, even though it is normally required for the conversion of origins from a pre-replicative state containing pre-RCs to a postreplicative state lacking them (Diffley et al. 1994). The failure of *GAL-ubiCDC6* induction to promote DNA replication in cells previously arrested by a *cdc7* mutation contrasts with its ability to do so in cells arrested by α -factor, which blocks cells in G₁ with neither Cln/Cdk1 nor Clb/Cdk1 kinases (Fig. 6B).

An important prediction of the hypothesis that Clb/Cdk1 kinases have dual roles is that Cdc6 should be able to promote DNA replication when synthesized de novo

in cells lacking Clb/Cdk1 kinases even if they possess fully active Cln1,2/Cdk1 kinases. Therefore, we performed experiments similar to the ones described above using *cdc4* and *cdc34* mutant cells, which, upon shift to 37°C, arrest in G₁ with multiple buds, with active Cln1,2 kinases, but with inactive Clb/Cdk1 kinases due to accumulation of the Clb-specific CDK inhibitor p40^{SIC1} (Schwob et al. 1994). The results of these experiments were inconclusive because *cdc4* and *cdc34* cells could never be efficiently depleted of Cdc6 protein prior to their arrest at 37°C (see below).

Cdc6 degradation in G₂ depends on CDC4

CDC4 and *CDC34* are required for proteolysis of p40^{SIC1} (Schwob et al. 1994). Our failure to deplete Cdc6 rapidly from *cdc4* or *cdc34* mutant cells when *GAL-* or *MET-CDC6* gene fusions are repressed (see above) suggests that Cdc4 and Cdc34 may also be involved in degrading Cdc6. To test this, we compared the stability of myc9Cdc6 protein expressed from the *GAL* promoter in nocodazole-arrested *CDC4*⁺ and *cdc4-1* cells. Cells grown in raffinose medium at 25°C were arrested in G₂/M by nocodazole and then treated with galactose for 30 min to induce myc9Cdc6 synthesis. Both cultures were then incubated in glucose medium to repress *GAL-myc9CDC6* transcription and shifted to 35°C to inactivate Cdc4 in *cdc4-1* cells. myc9Cdc6 protein disappeared within 30 min in *CDC4*⁺ cells, but persisted for at least 120 min in *cdc4-1* cells (Fig. 7). FACS analysis showed that both cultures remained arrested by nocodazole with a 2C DNA content throughout the experiment (data not shown). Therefore, we conclude that Cdc4 is necessary for myc9Cdc6 proteolysis, at least during G₂ and M phases.

Cdc6 proteolysis and the block to re-replication

Massive overexpression of Cdc18, Cdc6's homolog in *Schizosaccharomyces pombe*, causes *S. pombe* cells to re-replicate DNA in the absence of nuclear division (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). It has, therefore, been proposed that rapid proteolysis of ScCdc6/SpCdc18 protein might have an important role in preventing origins from firing more than once during the cell cycle. Our discovery that Cdc4 is needed for Cdc6 proteolysis allowed us to test this notion. When expressed from the *GAL* promoter, myc9Cdc6 accumulated to high levels within nuclei at most stages of the cell cycle in *cdc4-1* cells growing at 25°C (Fig. 8). The protein accumulated to much higher levels within nuclei of large budded pre-anaphase cells in *cdc4-1* mutants than it did in *CDC4*⁺ cells (where it was distributed more evenly between nucleus and cytoplasm). The same was true for cells arrested by nocodazole (Fig. 8). It is interesting that the *cdc4-1* mutation seemed to affect only the levels of myc9Cdc6 within nuclei. This observation is consistent with the previous finding that Cdc4 protein is localized in the nucleus (Choi et al. 1990) and suggests that Cdc4-mediated proteolysis of Cdc6 might

Figure 6. *CDC7* is not required for passage through the point of no return. (A) *cdc7* WT-CDC6 (K2032) and *cdc7* GAL-ubiCDC6 (K5029) cells, growing in YEPRG medium at 25°C, were arrested in G_2 by incubation with nocodazole for 2.5 hr at 25°C. Cells were then released into prewarmed YEPR (GAL-ubiCDC6 off) at 37°C for 3 hr. At this point, cultures were split into two, and 2% galactose was added to one-half, followed by an additional 15 min incubation at 37°C. Cells were then released ($t=0$) from the *cdc7* arrest into either YEPR (GAL-ubiCDC6 off) or YEPRG (GAL-ubiCDC6 on) at 25°C and analyzed by FACS every 30 min. GAL-ubiCDC6 (K4675) cells released from nocodazole into YEPR (GAL-ubiCDC6 off) at 37°C are shown as a control for the efficiency of Cdc6 depletion. (B) wild-type (WT-CDC6, K699) and GAL-ubiCDC6 (K4675) cells growing in YEPRG were synchronized by nocodazole treatment as in A. Cells were then released into YEPR (GAL-ubiCDC6 off) + α -factor at 25°C for 3 hr. Then, the culture was split in two, and one-half was incubated in the presence of galactose (2%) for 15 min. Cells were then released from the pheromone arrest ($t=0$) into either YEPRG or YEPR medium at 25°C for 2 hr. Every 30 min, cell samples were analyzed by FACS. Similar results were obtained by arresting cells in α factor at 37°C instead of 25°C. Arrows indicate the beginning of S-phase. [cyc] Cycling cells; [noc] nocodazole arrest; [R] raffinose; [G] galactose.

be confined to the nucleus. The *cdc4-1* mutation did not, however, greatly enhance the nuclear accumulation of myc9Cdc6 in cells with small buds (i.e., in S-phase cells). *cdc4-1* cells expressing myc9Cdc6 proliferate normally at 25°C and do not re-replicate their DNA before completing nuclear division (data not shown). We conclude that neither Cdc6's exclusion from nuclei nor its instability can explain its failure to promote DNA replication in G_2 .

Cdc6p interacts in vivo with *Clb/Cdk1* kinases

A histone H1 kinase activity co-immunoprecipitates with a version of Cdc6 protein tagged at its carboxyl terminus with the HA3 epitope and expressed from its own promoter (Fig. 9A). We assayed the Cdc6-associated

Figure 7. Cdc6 instability in G_2 /M depends on Cdc4. Wild-type (WT) and *cdc4-1* cells carrying the GAL-myc9CDC6 gene (K5919 and K5955) were arrested by nocodazole for 2.5 hr in YEPR at 25°C. Afterwards, cells were incubated 30 min in the presence of 2% galactose and nocodazole and then shifted by filtration into YEPD + nocodazole at 35°C. At the indicated times, cells were collected for Western analysis of the myc9Cdc6 protein. C, used as internal loading control, is a protein that cross-reacts with the anti-Cdc28 antibody. [Raf] raffinose.

Figure 8. Cdc6 is present at high levels in the G_2 and M nuclei of *cdc4* cells. Wild-type (WT) and *cdc4-1* cells carrying the *GAL-myc9CDC6* gene [K5919 and K5955] were grown in YEPR at 25°C and induced by the addition of 2% galactose for 4 hr or shifted to YEPRG + nocodazole at 25°C for 2.5 hr. (cyc) Cycling cells; (noc) nocodazole arrest; (DIC) differential interference contrast.

kinase in extracts derived from cells of various *cdc* mutants incubated in either permissive or restrictive conditions and found active kinase in all extracts except those prepared from *cdc28-13* and *cdc4-1* mutant cells incubated at the non-permissive temperature (Fig. 9B). Western analysis showed that Cdc6p was present at similar levels in these extracts (Fig. 9B). Several data suggest that the activity associated with Cdc6 corresponds to Clb/Cdk1 kinases. First, these kinases are inactive in *cdc4-1* cells caused by accumulation of p40^{SIC1} protein (Schwob et al. 1994) and deletion of *SIC1* restored Cdc6-associated kinase activity (Fig. 9B). Second, we found little or no kinase activity associated with HA tagged Cdc6 expressed from the *GAL* promoter (*GAL-HA3CDC6*) in *cdc28-4* cycling cultures (Fig. 9C). Third, the kinase was inhibited by the addition of purified p40^{SIC1} (Fig. 9D). Fourth, Cdc28 protein coimmunoprecipitated with Cdc6p in extracts from *GAL-HA3CDC6* cells (Fig. 9E). These data suggest that Cdc6p made in S, G_2 or M phases associates with Clb/Cdk1 kinases. This association might be instrumental in preventing Cdc6 from forming pre-RCs at origins during these stages of the cell cycle. The point of no return might, therefore, correspond to the point in the cell cycle at which Cdc6 protein, if present or produced, associates with Clb/Cdk1 kinases. We do not know whether Cdc6p is a target for these kinases in vivo; it can, however, be phosphorylated by

Clb/ but not by Cln/Cdc28 kinases in vitro (data not shown).

Discussion

Cdc6's ability to promote formation of pre-RCs and DNA replication is restricted to the G_1 phase of the cell cycle

De novo Cdc6 protein synthesis is required for a change in the chromatin structure of origins of DNA replication, which normally occurs as cells exit from mitosis and enter G_1 (Cocker et al. 1996). The pattern of DNase I sensitivity surrounding origins suggests that the ORC binds to origins throughout the cell cycle but that another factor, possibly Cdc6 itself, joins it in the interval between exit from mitosis and initiation of DNA replication in the subsequent cycle (Diffley et al. 1994). Cdc6 protein has been shown to interact with ORC in vitro (Liang et al. 1995). Cdc6 is required for the initiation of DNA replication but it is not required for other aspects of cell-cycle progression such as the formation of mitotic spindles or even the movement on these spindles of unreplicated chromosomes to opposite poles of the cell (Piatti et al. 1995). These findings suggest that a change in the state of replication origins mediated by Cdc6 synthesis at the end of mitosis, or later during G_1 , is essential for the subsequent initiation of DNA replication triggered by activation of Clb/Cdk1 kinases in late G_1 . The initiation of DNA replication in yeast can, therefore, be viewed as a two-step process: formation at origins of pre-RCs, driven by Cdc6 synthesis, followed by activation of Clb/Cdk1 kinases caused by destruction of the Clb-specific Cdk inhibitor p40^{SIC1} (Schwob et al. 1994). This paper addresses whether these two processes must occur in the correct order. Clb/Cdk1 kinases are activated on schedule even when Cdc6 protein is not made during G_1 . What happens then, when Cdc6 synthesis is restored to cells that have already activated Clb/Cdk1 kinases? Is this effective in promoting DNA replication? We investigated the effectiveness of Cdc6 synthesis at different cell-cycle stages in promoting DNA replication and discovered a point of no return after which de novo Cdc6 synthesis fails to drive DNA replication. This point of no return is delayed by inactivation of Clb5 and Clb6, which are normally the first Clbs to appear during the cell cycle. Passage through the point of no return did not, however, depend on activity of the Cdc7 protein kinase, which is also necessary for initiation of DNA replication and for the switch of origins from a prereplicative to a postreplicative state. Our data suggest that the point of no return corresponds to a point in the cell cycle after which Cdc6 protein is no longer able to drive the formation of pre-RCs at origins of DNA replication and that the crucial event responsible for this switch in cell-cycle state is the activation of Clb/Cdk1 kinases. The point of no return not only coincides with Clb/Cdk1 activation, but is also delayed when their activation is delayed.

Dahmann et al. (1995) showed that inactivation of Clb/Cdk1 kinases in cells arrested in nocodazole by

Figure 9. Clb/Cdc28 kinase associates with Cdc6p in vivo during S, G₂ and M phases. (A) Cell extracts from wild-type cells containing an untagged (K699) or a HA3-tagged version of *CDC6* (*CDC6HA3*, K4528) were incubated in either the presence (+ Ab), or in the absence (– Ab) of mAb 12CA5. After immunoprecipitation, histone H1 kinase was assayed. (B) K4528 (wild-type), K5275 (*cdc28-13*), K5082(*cdc34-2*), K5272(*cdc7-1*), K5074(*cdc34-2, sic1*), and K5279(*cdc15-2*) cells, all containing the *CDC6HA3* gene, were grown in YEPD at 25°C and arrested for 3.5 hr at 37°C. K4528 cells were arrested with 80 mM hydroxyurea (HU) at 25°C for 3.5 hr. Cell samples were collected for Western blot with the mAb 12CA5 (bottom), kinase assays, and FACS analysis (not shown). (C) The loading control is a protein cross-reacting with mAb 12CA5. For kinase assays, Cdc6p was immunoprecipitated with mAb 12CA5 from 0.5 mg of protein extracts. (C) Wild-type (WT, K4527) and *cdc28-4* (K5472) cells, both containing five copies of *GAL-HA3CDC6*, were grown in YEPR and induced for 4 hr in YEPRG at 25°C; K5472 cells were also arrested for 3 hr in YEPR at 37°C or incubated for 1 hr in YEPRG at 25°C and then arrested for 3 hr at 37°C. Cdc6 associated kinase activity was measured from 0.2 mg of protein extract. (R) Raffinose; (G) galactose. (D) One milligram of protein extract from K4527, grown in YEPR at 25°C or induced for 4 hr in YEPRG, was immunoprecipitated with mAb 12CA5 and before the last washing step, split into four aliquots. Kinase activity was measured in the absence or in the presence of the indicated amounts of purified p40^{SIC1}. (R) Raffinose; (G) galactose. The addition of p40^{SIC1} causes the appearance of two additional signals probably attributable to phosphorylation of p40^{SIC1}. (E) Cdc6p was immunoprecipitated with mAb 12CA5 from protein extracts (5 mg) of K4527 cells grown at 25°C in YEPR and induced for 4 hr in YEPRG. Protein A–Sephacel beads were boiled in SDS buffer and analyzed by Western blot using Cdc28 antibody (1:500). (R) Raffinose; (G) galactose.

overproduction of the p40^{SIC1} Cdk inhibitor is sufficient to induce the formation of pre-RCs at origins. As a result, they proposed that Clb/Cdk1 kinases have two opposing roles in the initiation of S-phase: to inhibit the de novo formation of pre-RCs and to trigger initiation only from those origins that have previously formed pre-RCs. The re-replication of *S. pombe* cells either defective for Cdc2 or the B-type cyclin Cdc13, or overproducing the Cdc13/Cdc2 kinase inhibitor Rum1, is consistent with this hypothesis (Broek et al. 1991; Hayles et al. 1994; Moreno and Nurse 1994). According to this model, activation of Clb/Cdk1 kinases prior to the formation of pre-RCs (i.e., prior to the de novo synthesis of Cdc6 protein) would finesse the system and prevent initiation of DNA replication. Our results show that the timing of Cdc6 syn-

thesis relative to Clb/Cdk1 activation is indeed crucial. The point of no return may correspond to the point in the cell cycle when Clb/Cdk1 kinases become sufficiently active to inhibit the formation of pre-RCs at origins.

Our experiments concentrated on the consequences of delaying Cdc6 synthesis relative to Clb/Cdk1 activation. However, the hypothesis that Clb/Cdk1 kinases inhibit the formation of pre-RCs predicts disaster not only when Cdc6 synthesis is delayed but also when Clb/Cdk1 kinases are activated prematurely. E. Schwob and K. Nasmyth (pers. comm.) have analyzed the effects on replication of deleting the *SIC1* gene, which causes the premature activation of Clb/Cdk1 kinases. *sic1* mutants initiate DNA replication somewhat earlier than wild-

type cells but proceed through S phase more slowly. Furthermore, chromosomes and minichromosomes are highly unstable in *sic1* mutants and this defect is suppressed either by deletion of *CLB5* and *CLB6* or by the addition of extra origins to the chromosomes. Thus, premature activation of Clb/Cdk1 kinases has a similar effect to delaying Cdc6 synthesis: It also reduces the efficiency with which origins fire.

What prevents the formation of pre-RCs during G₂?

The lack of DNA replication in cells that express *GAL-CDC6* only after they have passed the point of no return is not caused by their failure to accumulate sufficient Cdc6 protein; induction of *GAL-CDC6* causes more Cdc6p to accumulate in cells that have long passed this point than in those that have not (Fig. 3C). Cdc6p is an unstable protein and it normally disappears from cells during S and G₂ phases, but this is primarily attributable to control of *CDC6* transcription. Under conditions in which Cdc6 was synthesized continuously and protein levels remained constant throughout the cycle (because of expression from the *GAL* promoter), we noticed that more Cdc6 protein accumulated within nuclei of post anaphase and early G₁ cells than during S, G₂, and M phases. Rapid proteolysis of Cdc6 seems partly responsible for this pattern because Cdc6 protein accumulated to high levels within nuclei at most stages of the cell cycle in cycling cultures of *cdc4* mutants, which are defective in Cdc6 degradation. Changes in the rate of Cdc4-mediated Cdc6 proteolysis within nuclei could be responsible for the Cdc6's greater accumulation within nuclei in early G₁. For example, phosphorylation of Cdc6 by the Clb/Cdk1 kinases that are present during G₂ and M phases might stimulate proteolysis.

The cell-cycle control of Cdc6's cellular distribution resembles that of Cdc46/Mcm proteins, which are also needed for the firing of origins and which also accumulate in the cytoplasm during G₂. We doubt, however, that Cdc6's failure to function in cells past the point of no return is because of its insufficient accumulation within nuclei. Cdc6 protein is never actually excluded from nuclei in *CDC4* cells, and it accumulates to very high levels within G₂ nuclei of *cdc4* mutant cells growing at 25°C without bypassing the block that prevents re-replication until cells have undergone anaphase. Our results raise the question as to why Cdc6 is synthesized only during G₁ and is rapidly degraded in wild-type cells. This seems to be a conserved feature of the fungal cell cycle, because Cdc18p levels in *S. pombe* are regulated in a similar manner (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). We have shown that the block to re-replication does not depend on Cdc6's absence from the nucleus during G₂. It might nevertheless contribute to the fidelity with which such cells prevent re-replication.

Cdc6's inability to promote DNA replication in cells that have passed the point of no return could be caused by its association with inhibitory factors or its inhibition by modification. Our finding that Cdc6 associates with Clb/Cdk1 kinases could be important in this re-

gard. Phosphorylation of Cdc6 by these kinases might prevent it from forming pre-RCs. Alternatively, Clb/Cdk1 kinases associated with Cdc6 might inhibit, through phosphorylation, other Cdc6-interacting proteins needed for pre-RC formation (e.g., members of ORC).

The failure of cells past the point of no return to form pre-RCs and to promote replication in response to de novo Cdc6 synthesis could also stem from the inactivity in these cells of other proteins needed to form pre-RCs or to initiate DNA replication from them. For example, proteins of the Cdc46/Mcm class, which are essential for origin firing, are found in the cytoplasm during G₂. Thus, a drop in the concentration of Mcm proteins within nuclei or a reduction in their ability to bind chromatin might be responsible for the point of no return.

Passage through the point of no return does not depend on Cdc7

Our finding that Cdc7 is not required for passage past the point of no return is particularly revealing. The coincidence of the point of no return with the point at which cells normally initiate S phase suggests that the point of no return might correspond to the point in the cell cycle at which cells attempt to initiate DNA replication but fail to do so because of the lack of Cdc6. However, *cdc7* mutants do not attempt to initiate DNA replication even in the presence of pre-RCs. A potential to fire origins is therefore not required for cells to lose the ability to utilize Cdc6. Despite their failure to initiate DNA replication, *cdc7* mutants proceed with the activation of Clb/Cdk1 kinases, and we propose that this may be the reason why they cannot utilize Cdc6 protein when it is synthesized for the first time during their arrest.

Is the eukaryotic replication cycle driven by a reciprocating engine?

A key requirement for the mechanism that drives chromosome duplication is that it should permit replication origins to fire once, and only once, between succeeding rounds of sister chromatid segregation. Bacteria must also restrict replication to once every mass doubling, but the mechanism by which they do this is different because of their possession of only a single origin of DNA replication. Imperfections in the bacterial firing mechanism are not disastrous because unscheduled initiation from a single origin leads to replication of the entire genome; that is, it does not lead to over- or under-representation of genes. The downside of this simple device is that replication can take longer than mass doubling, and this problem can only be solved by the reinitiation of replication before sister chromatids from the previous round have been segregated. The mechanism used by eukaryotes whose genomes are carried on multiple chromosomes, each of which is replicated from multiple origins, must, therefore, be much more efficient than that used by bacteria. There has been much speculation as to

how eukaryotic cells might achieve an efficient once-only firing device, but few hard facts.

Our current knowledge about replication in *S. cerevisiae* is sufficient to assemble, possibly for the first time, the crude outlines of the device actually used (see Fig 10; Dahmann et al. 1995). Initiation is from defined sites and depends on two types of factors: those like ORC and, presumably, Cdc6 and Cdc47/Mcm proteins, that bind to origins or the sequences that surround them, and S-phase-promoting factors like Clb/Cdk1 and Cdc7 kinases, whose activation in late G₁ actually triggers the initiation of DNA replication. Origins and their surrounding chromatin exist in two states: a post-replicative one, in which they are bound by ORC alone and a pre-replicative one, in which they are bound by ORC and possibly also by Cdc6 and Mcm proteins (Diffley et al. 1994; Cocker et al. 1996). We refer to the latter as the pre-replication or pre-initiation complex. Only origins in a pre-replicative state can be triggered to initiate DNA replication by Clb/Cdk1 kinases. Key aspects of this once-only replication device are first the mechanisms that govern the transitions between the two states of origins, and second, the mechanisms that generate sharp fluctuations in the activity of Clb/Cdk1 kinases. We propose that pre-RCs are destroyed either by initiation itself or by replication through them and that the formation of new pre-RCs is inhibited by the very same set of Clb/Cdk1 kinases that trigger initiation. According to this scheme, the replication cycle is driven by a Clb/Cdk1 cycle. It starts with a period of low Clb/Cdk1 activity (G₁), which permits formation of pre-RCs. Activation of Clb/Cdk1 kinases triggers replication from pre-RCs that had formed during the previous period of low Clb/Cdk1 activity and simultaneously blocks formation of any new pre-RCs (S phase). The subsequent period of high Clb/Cdk1 kinase activity (G₂/M phases) maintains the block to the formation of pre-RCs. The cycle is completed by the final inactivation of Clb/Cdk1 kinases and re-entry into the low kinase state, which is mediated, at least in part, by the same complex (the APC) that promotes the segregation of sister chromatids (Irniger et al. 1995). The antagonistic effects of Clb/Cdk1 kinases on initiation ensure that there is no stage during this cycle in which cells can both form pre-RCs and trigger initia-

tion of DNA replication from them. Involvement of the APC in both cyclin destruction and in promoting sister chromatid separation may be key to linking re-replication with chromosome disjunction.

The momentum of our device, that is, successive rounds of DNA replication, can be maintained only by fluctuations in the activity of Clb/Cdk1 kinases. It is a reciprocating device in which motion (i.e., rounds of replication) is driven by the alternate inactivation and reactivation of Clb/Cdk1 kinases. In this sense, it is deeply analogous to the reciprocating steam engine, whose steam corresponds to kinase activity and whose piston corresponds to origins. Steam, that is, kinase, can only do work by driving the piston upwards (i.e., drive replication) if it (i.e., origins) has previously returned to the down state (i.e., contains pre-RCs). Passage from the up state (an origin that has initiated replication) to the down state depends on evacuation of the steam (i.e., inactivation of kinases) from the chamber containing the piston (i.e., the cell). The essence of the reciprocating steam engine is that entry of steam into the chamber only performs work if the piston has been returned to the down state by previous evacuation of steam from the chamber. Likewise, Clb/Cdk1 kinases only drive replication when their prior inactivation has permitted the formation of pre-RCs. Just as the piston can only move once during a cycle of expansion and contraction, so too can origins fire only once during a cycle of kinase activation and inactivation. If there exists a cell-cycle engine (Murray and Hunt 1993), then this surely could be a key part of it.

Timing is crucial to the operation of the reciprocating steam engine. The implication of our finding that Cdc6 must be synthesized before Clb/Cdk1 kinases are reactivated, suggests that timing is also crucial to the reciprocating replication cycle in *S. cerevisiae*.

Materials and methods

Strains, media, and reagents

All yeast strains were derivatives of, or were backcrossed at least three times to W303 (*HMLa*, *HMRa*, *ho*, *ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11,15*, *ura3*, *ssd1*). Cells were grown in YEP medium (1% yeast extract, 2% Bactopeptone, 50 mg/liter of adenine) supplemented, as indicated, with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose + 2% galactose (YEPRG), except for the experiment described in Figure 3, B and C, where 0.1% galactose was added to YEPR medium. The synthetic medium lacking methionine (–Met medium) is yeast nitrogen base (0.8%) supplemented with amino acids, adenine, uracil, and 2% glucose.

Plasmid constructions and genetic manipulations

To generate the *GAL-ubiCDC6* construct (C2835), the ATG of *CDC6* was replaced by a *Bam*HI site by PCR. Afterwards, a 2-kb *Bam*HI–*Hind*III fragment of *CDC6* has been used to replace the *Bam*HI–*Hind*III fragment of a *GAL-ubiR-MCM1* fusion in YIplac211 (Althöfer et al. 1995). The resulting plasmid was cut with *Apa*I for integration in single copy at the *URA3* locus of W303. This strain was then crossed to K4055 (*cdc6::hisG*, *trp1::TRP1 MET-CDC6*; Piatti et al. 1995) to generate, after

Figure 10. The pre-RC/Cdk1 cycle in yeast.

sporulation, strain K4675 [*cdc6::hisG*, *ura3::URA3 GAL-ubiCDC6*]. K4675 was crossed to a *cdc15-2* strain (K1994) or to a *cdc7-1* strain (K2033) to generate strains K5032 (*MATa*, *cdc15-2*, *cdc6::hisG*, *ura3::URA3 GAL-ubiCDC6*), K5033 (*MATa*, *cdc15-2*, *cdc6::hisG*, *ura3::URA3 GAL-ubiCDC6*), and K5029 (*MATa*, *cdc7-1*, *cdc6::hisG*, *ura3::URA3 GAL-ubiCDC6*).

The carboxyl-terminal HA3-tagged version of *CDC6* used here is similar to the one described before (Piatti et al. 1995) with the exception that it does not contain His tags (plasmid C2838). C2838 was integrated in single copy at the *CDC6* locus of W303 to generate a full-length tagged version of *CDC6* flanked by a truncated untagged version of the gene (strain K4528). K4528 was crossed to various *cdc* mutants to generate, after sporulation of the corresponding diploids, the strains used in Figure 9B.

The *GAL-HA3CDC6* (C2837) construct was made by insertion of the triple HA *NotI* cassette (Tyers et al. 1992) after the ATG of *CDC6*, where a *NotI* site was introduced by PCR. The tagged gene (ending at the *NdeI* site of *CDC6*) was then cloned in Yiplac211 downstream of a *GAL1-10* promoter containing 70 bp of *CLB5* leader sequence. Subsequently, the plasmid was cut with either *BclI* for integration at the *CDC6* locus (in one copy, strain K5095, or in five copies, strain K4527), or *StuI* for integration at *URA3* (strain K5761). K5761 was crossed to K4143 [*cdc15-2*, *cdc6::hisG*, *trp1::TRP1 MET-CDC6*] to generate strain K5763 [*cdc15-2*, *cdc6::hisG*, *ura3::URA3 GAL-HA3CDC6*].

The myc12-tagged version of *CDC6* (C3336) was obtained by replacement of the *NotI* HA3 cassette of C2838 by two copies of a *NotI* myc6 cassette, whereas the *GAL-myc9CDC6* construct (C3292) was generated by replacing the *NotI* HA3 cassette of C2837 by a *NotI* myc9 cassette (the *NotI* myc6 and myc9 cassettes were obtained by PCR of a *SHE3-myc6* fusion (Jansen et al. 1996). C3336 was linearized with *BclI* to direct its integration at the *CDC6* locus, to generate a full-length myc-tagged version and a truncated, untagged version of *CDC6* (strain K5920); C3292 was cut with *StuI* for integration at the *URA3* locus (strain K5919).

Cell synchronization techniques

To perform *cdc15-2* block/release experiments, cells were grown to exponential phase, filtered and inoculated into prewarmed medium. Cells were arrested at 37°C for 3 hr. Cells were then filtered again and released at 25°C.

During a *cdc15* block cells are arrested in late anaphase with 2C DNA contents (because of two separated 1C nuclear masses). Upon release, we always failed to see a transient accumulation of cells with 1C DNA content (as would be expected if cells divided before replicating), but saw instead a transient accumulation of cells with 4C DNA content, suggesting that most cells started replicating before cell division. To use the FACS profiles to estimate the DNA contents of nuclei during a *cdc15* release, we also measured the fraction of cells at each time point with single or double chromosome clusters/nuclei; that is, uninucleate and binucleate cells (as determined by fluorescence microscopy of propidium iodide stained cells). If we assume that cells with a 4C DNA content are binucleate and that all 1C cells are uninucleate cells, then the percentage of nuclei that have replicated (%2C) during the first cycle following release can be calculated by use of the formula: $\%2C = 100[2C_4 + U - C_1]/(U + 2B)$, where C_1 , C_2 , and C_4 are the fractions of cells at each point with 1C, 2C, or 4C DNA contents, and U and B the fractions of uni- and binucleated cells, respectively. We can assume that the result of this equation reflects reliably the fraction of nuclei that have replicated dur-

ing the S phase immediately after *cdc15* release, but its application during later stages of the cell cycle has some limitations. After completing S phase, a fraction of cells undergoes mitosis before having completed the previous cell separation, producing cells with more than 2 nuclei. Because these cells have been scored as binucleates (for simplicity), the percentage of 2C nuclei will be overestimated when these types of cells appear (some of the cells with a 4C DNA content have four 1C nuclei rather than two 2C nuclei). Another limitation arises from the feature that *Cdc6*-deprived cells undergo anaphase in the absence of DNA replication. In this case, the assumption that 1C cells must be uninucleate no longer holds true. For these cells, calculations are also complicated by the later appearance of cells with <1C DNA contents.

After *cdc15* arrest, in cases where *CDC6* synthesis was controlled by the *GAL1-10* promoter (*GAL-ubiCDC6* or *GAL-HA3CDC6*), cells were harvested by filtration and incubated in prewarmed YEPR medium at 37°C for 30 min (*GAL* promoter off) before release in YEPR at 25°C. For induction, 2% galactose (or 0.1% in the experiment described in Fig. 3B,C) was added to aliquots of cells at the different time points. In cases where *CDC6* expression was driven by the *MET3* promoter (*MET-CDC6*), cells were treated as above with the exception that the permissive and nonpermissive conditions for *CDC6* synthesis were obtained by the use of -Met medium and YEPD + 2% methionine, respectively.

For nocodazole arrest/release experiments described in Figure 6, A and B, cells growing in YEPRG were incubated with 5 μ g/ml of nocodazole (and a final concentration of 1% DMSO) at 25°C for 2.5 hr. Cells were then filtered, washed with 3 volumes of YEPR + 1% DMSO and incubated in either YEPR + 2 μ g/ml of α -factor at 25°C (Fig. 6A) or YEPR at 37°C (Fig. 6B) for 3 hr. Afterwards, cultures were split in two, and one-half was incubated in the presence of 2% galactose for 15 min. Subsequently, cells were released from the block by filtration into either YEPRG or YEPR medium at 25°C for 2 hr.

In vivo footprinting

K5033 cells (*MATa*, *cdc15-2*, *cdc6::hisG*, *ura3::URA3 GAL-ubiCDC6*) exponentially growing in YEPRG, were arrested by 3 hr incubation at 37°C, and then released into YEPD + α -factor (10 μ g/ml) for 90 min. An aliquot of the culture was then transferred into YEPRG + α factor, whereas another was released from α -factor into YEPD + nocodazole (20 μ g/ml). After 90 min, cell samples were withdrawn for in vivo footprinting, and part of the culture containing nocodazole was transferred to YEPRG + nocodazole for 90 min. From each condition, footprinting experiments on the 2 μ origin were performed according to Diffley et al. (1994) on 50 ml of culture (2×10^7 cells/ml).

Northern and Western blot analysis

The methods described by Cross and Tinkelenberg (1991) and Price et al. (1991) were used for RNA isolation and Northern blot analysis respectively.

For Western blot analysis, protein extracts were prepared either as described in Surana et al. (1993) (Figs. 2C and 4B), or by TCA precipitation (Foiani et al. 1994) (Fig. 9B). Protein (50–150 μ g) was transferred to Immobilon P membranes (Millipore). HA-tagged *Cdc6* was detected with 12CA5 MAb (1:100), whereas myc-tagged *Cdc6* was detected by mAb 9E10 (1:200); the signal was amplified as described previously (Piatti et al. 1995). Anti-Swi6 antibodies were used at 1:100,000 and anti-*Cdc28* antibody at 1:1000 dilution. Secondary antibodies were purchased

from Amersham and proteins were detected by an enhanced chemiluminescence system according to the manufacturer.

Immunoprecipitation and kinase assays

Protein extracts were prepared as in Schwob et al. (1994). For Clb2 kinase assays, 100 μ g of total proteins were immunoprecipitated with anti-Clb2 Ab (1:30, Amon et al. 1992). To assay Cdc6-associated kinase, HA3Cdc6 or Cdc6HA3 was immunoprecipitated with 12CA5 antibody (1:10) from the amounts of protein extracts indicated in the legend to Figure 9. Histone H1 kinase activity was measured as described previously (Schwob et al. 1994).

Other techniques

Flow cytometric DNA quantitation was determined according to Epstein and Cross (1992) on a Becton-Dickinson FACScan. In situ immunofluorescence and photomicroscopy were performed according to Nasmyth et al. (1990). To detect immunostaining of myc9Cdc6, 9E10 mAb was used at a 1:5 dilution, and the signal was detected by indirect immunofluorescence with CY3-conjugated anti-mouse antibody (1:200, Sigma).

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Activation of S-phase-promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast.

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