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Genetics

cdc18$^+$ regulates initiation of DNA replication in *Schizosaccharomyces pombe*

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ABSTRACT **In the fission yeast *Schizosaccharomyces pombe* the *cdc18*$^+$ gene is required both for initiation of DNA replication and for coupling mitosis to the completion of S phase. Cells lacking Cdc18 fail to enter S phase but still undergo nuclear division. Expression of *cdc18*$^+$ is sufficient to drive a G_1-arrested *cdc10*^{ts} mutant into the S phase of the cell cycle, indicating that *cdc18*$^+$ represents a critical link between passage through START and the initiation of DNA replication. Here we show that Cdc18 is a highly unstable protein that is expressed only once per cell cycle at the boundary between G_1 and S phase. *De novo* synthesis of Cdc18 is required before, but not after, the initiation of DNA replication, indicating that Cdc18 function is not necessary once the initiation event has occurred. Overproduction of the protein results in an accumulation of cells with DNA content of greater than 2C and delays mitosis, suggesting that Cdc18 is sufficient to cause reinitiation of DNA replication within a given cell cycle. Our data indicate that the synthesis of Cdc18 protein is a critical rate-limiting step in the initiation of DNA replication during each cell cycle. The extreme lability of the protein may contribute to the prevention of reinitiation.**

DNA replication in eukaryotic cells is regulated by a complex set of mechanisms that mediate the precise duplication and transmission of the chromosomes during each cell cycle. Although DNA synthesis can be initiated at many different sites along the chromosomal DNA, only a single initiation event can occur at any given site, ensuring that each segment of the genome is duplicated precisely once. In the budding yeast *Saccharomyces cerevisiae* origins of replication are bound by the origin recognition complex (ORC) (1, 2). A number of the constituent proteins of this six-subunit complex are required for the initiation of DNA replication (3–5). Footprinting experiments have indicated that the ORC remains bound to origins not just during S phase but throughout the cell cycle (2). Prior to S phase the ORC footprint is extended by an additional region of protection (6). These findings are consistent with models in which the initiation of DNA replication is a two-step process, the first step being recognition of the origin by ORC, and the second being the precise triggering of initiation at the G_1/S boundary. It is likely that additional factors contribute to this second step and to the prevention of multiple initiations from single origins during a given cell cycle.

In *Schizosaccharomyces pombe* an essential requirement for entry into S phase is the transient activation of a transcription factor containing the products of the *cdc10*$^+$ and *sct1*$^+$ genes (7, 8). Activation of this factor in late G_1 depends on the activity of a cyclin-dependent protein kinase whose catalytic subunit is encoded by the *cdc2*$^+$ gene (9). A key target of the Cdc10/Sct1 transcription factor is the *cdc18*$^+$ gene (10, 11), a member of a recently described family of S phase regulators that includes the budding yeast Cdc6 protein and an *S. pombe* homologue of the large subunit of ORC (12). Overexpression of *cdc18*$^+$ suppresses the G_1 arrest of *cdc10* temperature-sensitive mutants, indicating that *cdc18*$^+$ is limiting for entry into S phase in such mutants (11). Deletion of *cdc18*$^+$ prevents entry into S phase, resulting in the accumulation of cells with a 1C DNA content. The cells then undergo a lethal mitotic event despite having failed to duplicate their DNA. Thus expression of *cdc18*$^+$ is required for initiation of DNA replication and for coordinating the completion of S phase with the onset of mitosis.

To investigate the role of *cdc18*$^+$ in regulating the initiation of DNA replication we have analyzed the timing of Cdc18 protein expression and the consequences of under- and overexpression of Cdc18. We demonstrate that Cdc18 is expressed periodically during progression through the cell cycle and is detectable only during a narrow window at the boundary between G_1 and S phases. Measurement of Cdc18 degradation indicates that the protein is highly labile. Expression of Cdc18 is required before the initiation of DNA replication but not once the initiation event has occurred. Overexpression of Cdc18 results in a mitotic delay and accumulation of cells with DNA content greater than 2C, indicative of reinitiation of DNA replication within the same cell cycle. These results are consistent with Cdc18 acting as a rate-limiting positive regulator of DNA replication. Degradation of Cdc18 after the initiation of S phase may be required to prevent rereplication and to allow mitosis to occur.

MATERIALS AND METHODS

Strain and Plasmids. Genetic manipulations and yeast transformations were performed as described (13). All strains were grown in Edinburgh minimal medium (EMM) plus the required supplements (13). Strain YMF189, carrying the triple hemagglutinin (HA) epitope-tagged version of *cdc18*$^+$ in an *h*$^-$ *cdc25-22 leu1-32 ura4-D18* background, was constructed as follows: the triple HA epitope sequence (14) was inserted after the first codon of the *cdc18*$^+$ open reading frame by first introducing a *Not* I site after the start codon followed by introduction of the tag as a *Not* I fragment. The tagged *cdc18* gene (*3HA-cdc18*) was excised by *Sac* I digestion and transformed into a haploid *S. pombe* strain carrying a chromosomal disruption of the *cdc18*$^+$ gene (*cdc18*::*ura4*$^+$) and a wild-type copy of *cdc18*$^+$ on the plasmid pREP41 (15). *ura4*$^-$ transformants were selected and screened by PCR for the *3HA-cdc18* gene, and the plasmid was lost by outgrowth on nonselective media. Southern analysis showed that the resulting strain (YMF187) contained one copy of the *3HA-cdc18* gene at the normal chromosomal site under the control of the wild-type *cdc18*$^+$ promoter. YMF189 was obtained by crossing the *cdc25-22* marker into YMF187. The strain YMF31 has the genotype *h*$^-$ *leu1-32 ura4-D18* pMF56. The plasmid pMF56 was constructed by inserting a copy of the *3HA-cdc18* gene between the *Sal* I and *Bam*HI sites of the expression vector pREP3 (16). In this plasmid the *3HA-cdc18* gene is under the

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Abbreviations: ORC, origin recognition complex; HA, hemagglutinin; DAPI, 4′,6-diamidino-2-phenylindole.

control of the *nmt1*$^+$ promoter. YMF15 was generated by replacing the chromosomal *cdc18*$^+$ promoter, from nucleotide −254 to −1, in the strain *h*$^-$ *leu1-32 ura4-D18* with the *leu1*$^+$ gene and the attenuated *nmt1*$^+$ promoter from pREP41 (15). The attenuated promoter is 6.7-fold less active than the wild-type *nmt1*$^+$ promoter. For *cdc18*$^+$ overexpression experiments, the strain *h*$^-$ *leu1-32 ura4-D18*, harboring the plasmid pREP3Xcdc18$^+$ (11), was used. This plasmid contains the complete *cdc18*$^+$ open reading frame and 262 bp of 3′ untranslated region inserted downstream of the *nmt1*$^+$ promoter. Transformants carrying the plasmid were isolated and propagated in the presence of 5 μg of thiamine per ml to repress the *nmt1*$^+$ promoter.

Analysis of Cdc18 in a Synchronous Culture. Synchronization was accomplished by releasing YMF189 from a *cdc25* late G_2 cell cycle block (17). For this purpose, an exponentially growing culture was arrested for 5 h at the nonpermissive temperature of 35.5°C and then shifted to the permissive temperature of 25°C. Samples were taken every 15 min and cell extracts were prepared for immunoblot analysis as described (18). Immunoblots were probed with the monoclonal antibody 12CA5 (19) to detect 3HA-Cdc18 and with the monoclonal antibody TAT-1 (20) to detect tubulin as a loading control. Synchrony was evaluated by measuring the percent of septated cells by dark-field microscopy.

Half-Life Measurement. YMF31 was grown in minimal medium to early logarithmic phase. A sample of cells was taken (time 0) and then 5 μg of thiamine per ml was added to the culture in order to repress the *nmt1*$^+$ promoter. Aliquots were fixed at the indicated time points by addition of trichloroacetic acid and proteins were extracted for immunoblot analysis as above. The level of 3HA-Cdc18 relative to tubulin was determined by immunoblot analysis as above, using an ^{35}S-labeled secondary antibody, followed by PhosphorImager detection and quantitation using IMAGEQUANT software (Molecular Dynamics).

Nuclear Staining and Flow Cytometry. Cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described (13). Stained cells were visualized using an Axioskop fluorescence microscope (Zeiss). For flow cytometry, cells were fixed in 70% ethanol, digested with RNase A, and stained with propidium iodide as described (21). Flow cytometry was performed using a Becton Dickinson FACScan and data was analyzed using CELLQUEST software.

RESULTS

Cdc18 Is Expressed Periodically During Cell Cycle Progression. To examine the expression of Cdc18 protein during the cell cycle, we introduced a triple HA epitope (14) after the first codon of the chromosomal copy of the *cdc18*$^+$ gene (11). A synchronous culture of this strain was prepared by releasing cells from a *cdc25*^{ts} block (17) (Fig. 1). Synchrony and cell cycle position were monitored by measuring septation (Fig. 1*A*), which peaks at the G_1/S boundary. Cdc18 protein levels were followed by immunoblot analysis (Fig. 1*B*). Accumulation of Cdc18 protein was strikingly periodic when compared to levels of the loading control tubulin. The protein was absent from G_2 cells, rose to a maximum just before the peak of septation at the G_1/S boundary, and declined rapidly thereafter. In other experiments we have shown that the protein accumulates between the time of Cdc10 activation in late G_1 and the hydroxyurea block point in early S phase (data not shown).

Cdc18 Is Extremely Unstable. The accumulation of Cdc18 protein mirrored the previously observed periodicity of *cdc18* mRNA (11), suggesting that Cdc18 might be an unstable protein. To examine this possibility directly, we generated the strain YMF31 in which expression of the epitope-tagged Cdc18 was under the control of the thiamine-repressible *nmt1*$^+$ promoter (16). An exponentially growing culture of this strain was incubated in medium containing 5 μg of thiamine per ml to repress transcription of the gene, and the fate of the tagged Cdc18 protein was followed by immunoblotting. Under these conditions, the apparent half-life of the protein was <5 min (Fig. 2). This value probably underestimates the lability of the protein since some time is required for the establishment of repression and the decay of preexisting messages. A similar half-life for Cdc18 was observed in cells arrested at the beginning of S phase by hydroxyurea (data not shown). These experiments indicate that the observed fluctuation of Cdc18 levels during cell cycle progression is largely due to the periodic

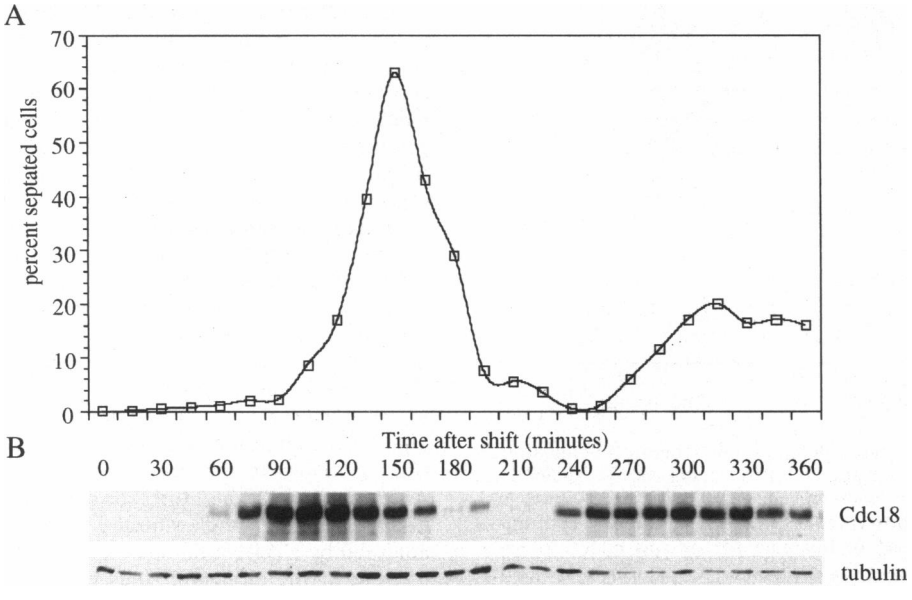

FIG. 1. Cdc18 protein appears transiently at the G_1/S phase boundary. A *S. pombe* strain carrying an epitope-tagged version of *cdc18*$^+$ in a *cdc25*^{ts} background was synchronized by shifting a mid-exponential phase culture to 36°C, incubating for 5 h, and shifting back to 25°C. Samples were taken every 15 min and cell extracts were prepared for immunoblot analysis. (*A*) Percent of septated cells determined by dark-field microscopy at indicated times following release from the *cdc25* block. (*B*) Immunoblot analysis of extracts prepared from the synchronous culture at the indicated times, probed to detect 3HA-Cdc18 and to detect tubulin as a loading control.

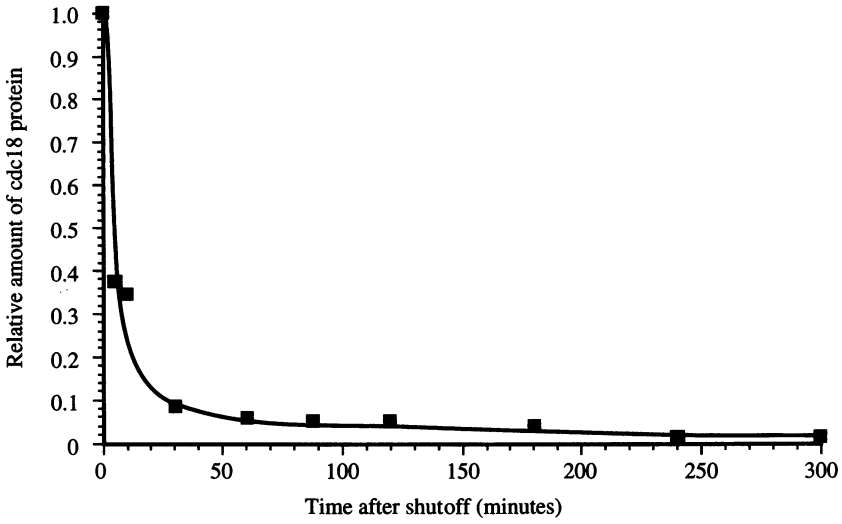

FIG. 2. Cdc18 is an unstable protein. A logarithmically growing culture of a *S. pombe* strain carrying the HA epitope-tagged *cdc18*$^+$ gene under the control of the *nmt1*$^+$ promoter was transferred to medium containing thiamine to repress 3HA-Cdc18 synthesis. Samples were collected at intervals following promoter shutoff and the level of 3HA-Cdc18 relative to the level of tubulin was determined.

transcription of *cdc18*$^+$ and indicate that Cdc18 is synthesized *de novo* each cell cycle.

Requirement for *de Novo* Synthesis of Cdc18. The experiments described above demonstrate that Cdc18 is a highly labile protein that is synthesized anew each cell. To investigate the functional significance of the periodic synthesis of Cdc18 during the cell cycle we examined the progression of a synchronous population of cells in the presence and absence of Cdc18 expression (Fig. 3*A*). Cells expressing *cdc18*$^+$ under the control of an attenuated *nmt1*$^+$ promoter were arrested in early S phase by incubation in the presence of hydroxyurea. At 4 h, half of the culture was shifted to medium containing thiamine to eliminate Cdc18 expression ("promoter off") and the other half was left in medium lacking thiamine ("promoter on"). A similar protocol with the strain YMF31, which carries the *3HA-cdc18* gene under control of a thiamine-repressible promoter, demonstrated loss of detectable Cdc18 by 5 min after the addition of thiamine (not shown). At 5 h, both cultures were released from the hydroxyurea block. Both cultures completed DNA replication by 7 h (DNA content increased from 1C to 2C) and then underwent cell division, with the peak of septation occurring at ≈7.5 h (Fig. 3*B*), demonstrating that Cdc18 expression is not required for cell cycle progression once cells have reached the hydroxyurea arrest point in early S phase. Following the first division, at 8 h, the cells lacking Cdc18 accumulated with a 1C DNA content, indicating that they were unable to initiate DNA synthesis in the second cycle. These cells then underwent an abnormal mitosis and cell division, resulting in daughter cells with DNA content of less than 1C. Despite the complete lack of an S phase in the second cell cycle, the timing of cell division was not significantly advanced, as judged by the peak in septation index. These data indicate that *de novo* synthesis of Cdc18 is required prior to S phase during each cell cycle. The protein does not appear to be required after DNA synthesis has initiated, as these cells are capable of completing DNA replication and mitosis in the absence of Cdc18.

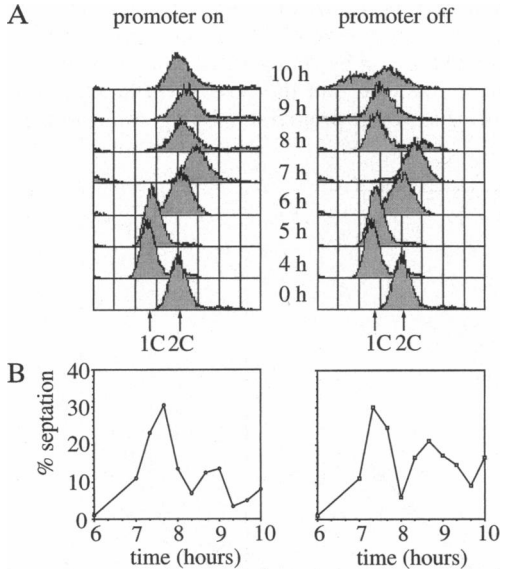

FIG. 3. *De novo* synthesis of Cdc18 is required before, but not after, the initiation of DNA replication. Cells carrying *cdc18*$^+$ under the control of the *nmt1*$^+$ promoter were arrested in early S phase by incubation in 25 mM hydroxyurea for 4 h. The culture was split into two equal parts, and 5 μg of thiamine per ml was added to one (promoter off). Incubation was continued for 1 h, at which time both cultures were harvested and resuspended in minimal medium plus thiamine (promoter off) or minimal medium (promoter on). Samples were removed every hour. (*A*) DNA content of cells released from arrest in the absence ("promoter on"; *Left*) or presence ("promoter off"; *Right*) of thiamine, analyzed by flow cytometry. (*B*) Septation index.

Overexpression of *cdc18*$^+$. Since Cdc18 is clearly a positive effector of initiation of DNA replication, we examined the effects of overproduction of the protein. For this purpose the *cdc18*$^+$ gene, under the control of the strong *nmt1* promoter, was introduced into wild-type *S. pombe* on a multicopy plasmid. The cells were grown to mid-exponential phase in medium containing thiamine, washed extensively, and transferred to fresh media without thiamine (promoter on) to induce high-level expression of Cdc18. As a control, a similar culture was incubated in the presence of thiamine (promoter off). Approximately 25% of the cells overexpressing Cdc18 became highly elongated and exhibited strikingly large nuclei when analyzed by DAPI staining (Fig. 4 *A* and *B*). When the DNA content of the elongated cells was compared to that of the normal size cells by flow cytometry, the latter exhibited the typical 2C peak, while the elongated cells had DNA contents greater than 2C (Fig. 4*C*). These observations indicate that overproduction of Cdc18 delays mitosis and leads to more than one round of DNA synthesis. This phenotype is reminiscent of

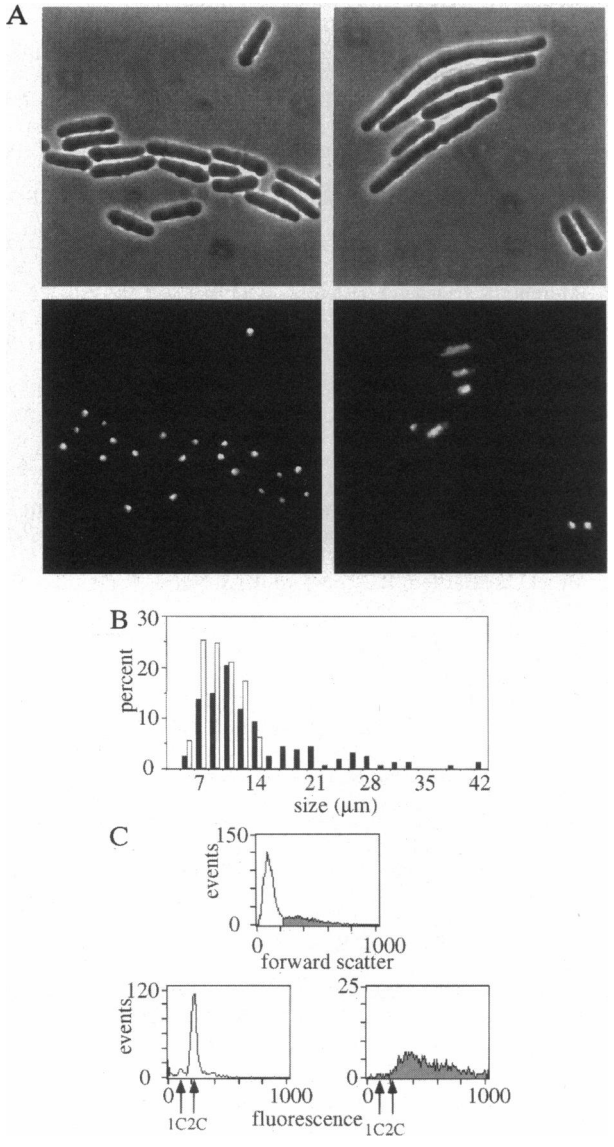

FIG. 4. Overexpression of Cdc18. *S. pombe* cells carrying the multicopy plasmid pREP3Xcdc18$^+$ were incubated in the presence (promoter off) or absence (promoter on) of 5 μg of thiamine per ml for 24 h. (*A*) Phase-contrast microscopy of cells with promoter off (*Upper Left*) or promoter on (*Upper Right*). Fluorescence microscopy of DAPI-stained cells with promoter off (*Lower Left*) or promoter on (*Lower Right*). (*B*) Frequency distribution of cell sizes determined by measuring photographed cells: promoter off, open bars; promoter on, closed bars. (*C*) DNA content of elongated vs. normal-sized cells in the culture with the promoter on. (*Upper*) Frequency distribution of cell size as assessed by forward scatter in the flow cytometer. (*Lower*) DNA content of cells exhibiting increased forward scattering (elongated cells; shaded area) and of cells exhibiting normal forward scattering (normal-sized cells; unshaded area). A gate was set so that the DNA contents of elongated and normal-sized cells could be determined independently.

the phenotypes observed with *rum1*$^+$ overexpression and with certain *cdc13* and *cdc2* mutants (22–25), although Cdc18-induced rereplication does not seem to occur in integral genome increments as was observed in these other cases. It is also consistent with the recent observations of Nishitani and Nurse (26) who found that massive overexpression of Cdc18 from multiply integrated copies of *cdc18*$^+$ driven by the *nmt1*$^+$ promoter leads to rereplication. The fraction of cells not undergoing rereplication may represent those cells carrying the plasmid at reduced copy number. The rereplication phenotype is almost certainly lethal, resulting in a strong selection for cells which do not overexpress Cdc18.

DISCUSSION

Previous work has shown that the Cdc18 is required for entry into S phase of the cell cycle (11). The experiments presented here demonstrate that Cdc18 protein is synthesized *de novo* during each cell cycle and accumulates only during a narrow window at the G_1/S boundary. Our data indicate that the half-life of the protein is a few minutes at most. Thus, the observed fluctuation in protein level can be accounted for, at least in part, by the previously documented fluctuation in *cdc18*$^+$ transcript level, although we cannot rule out the possibility that the stability of the protein is also regulated during the cell cycle. The observed periodicity of Cdc18 expression contrasts with other replication proteins that have been carefully studied. Although the mRNAs encoding such proteins are often periodically expressed, the proteins themselves are generally stable and their levels do not fluctuate significantly during the cell cycle (for example, see ref. 18). One notable exception is the budding yeast Cdc6 protein, a homologue of Cdc18 which also appears to be required for cellular DNA replication. The abundance of Cdc6 protein fluctuates during the cell cycle (27) and it is presumably a labile protein, although this has not been addressed directly. It is possible that instability of these positive regulators of S phase is a highly conserved feature of the regulatory circuit that triggers precise initiation of DNA replication once and only once each cell cycle.

Our analysis of cell cycle progression in the absence of Cdc18 expression supports the view that *de novo* synthesis of Cdc18 is an essential prerequisite for entry into S phase but is not required after initiation of DNA replication has occurred. Thus, expression of Cdc18 represents a major link between activation of the Cdc10/Sct1 transcription factor at START and the initiation of DNA synthesis. The precise biochemical activity of Cdc18 is not yet known. However, it seems likely that the protein plays some direct role in the initiation of DNA synthesis at origins. Consistent with this possibility, Cdc6, the budding yeast homologue of Cdc18, has been shown to coprecipitate with budding yeast ORC (5). Interestingly, both Cdc18 and Cdc6 exhibit significant amino acid sequence homology with Orc1, the largest subunit of ORC (12).

We have observed that overexpression of Cdc18 protein causes reinitiation of DNA replication without an intervening mitosis, leading to the accumulation of cells with DNA content in excess of 2C. One interpretation of this observation is that overexpression saturates the machinery that normally degrades Cdc18, resulting in accumulation of significant amounts of the protein after S phase. We suggest that this inappropriate accumulation of Cdc18 leads to reinitiation of DNA replication prior to mitosis. This model is consistent with the previous finding that Cdc18 is a positive effector of DNA replication that is sufficient to induce DNA replication in cdc10^{ts} cells blocked at START (11). We further suggest that the continued initiation of DNA synthesis that occurs in the presence of excess Cdc18 also triggers a checkpoint mechanism that normally acts to prevent mitosis while DNA synthesis is occurring. The resulting mitotic delay leads to the generation of large cells with increased DNA content. In this scenario Cdc18 clearly plays a role as a major rate-limiting initiation factor. An alternative, but somewhat more complex, model is that Cdc18 is an inhibitor of mitotic Cdc2 kinase activity in addition to playing a role in DNA replication. We raise this possibility because several recent studies indicate that rereplication can occur when Cdc2 kinase is either destroyed or inhibited. For example, overexpression of Rum1, a direct inhibitor of the kinase activity of Cdc2-cyclin B, or deletion of the *cdc13*$^+$ gene, which encodes cyclin B, lead to accumulation of cells with

increased DNA. In the case of Rum1 overexpression, however, we have recently shown that Cdc18 protein is induced (P. Jallepalli and T.J.K., unpublished data). Presumably, the re-replication observed in other cases is dependent upon Cdc18, but this remains to be determined.

A number of lines of evidence have suggested that initiation of DNA replication is a two-step process (6, 10, 28–33). During G_1 the cell is rendered competent for DNA replication, probably by formation of prereplication complexes at origins of DNA replication. At the beginning of S phase, competent prereplication complexes are triggered to initiate DNA synthesis by an unknown mechanism. Reinitiation of DNA replication may be prevented by the simultaneous inactivation of one or more factors required for establishment of the competent state. We suggest that Cdc18 is required to establish competent prereplication complexes and may be an actual component of these complexes. The rapid disappearance of the protein may be one factor that prevents reinitiation of DNA replication during a given cell cycle.

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1. Bell, S. P. & Stillman, B. (1992) *Nature (London)* **357,** 128–134.
2. Diffley, J. F. X. & Cocker, J. H. (1992) *Nature (London)* **357,** 169–172.
3. Li, J. J. & Herskowitz, I. (1993) *Science* **262,** 1870–1874.
4. Micklem, G., Rowley, A., Harwood, J. & Diffley, J. F. X. (1993) *Nature (London)* **366,** 87–89.
5. Liang, C., Weinrich, M. & Stillman, B. (1995) *Cell* **81,** 667–676.
6. Diffley, J. F., Cocker, J. H., Dowell, S. J. & Rowley, A. (1994) *Cell* **78,** 303–316.
7. Lowndes, N. F., McInerny, C. J., Johnson, A. L., Fantes, P. A. & Johnston, L. H. (1992) *Nature (London)* **355,** 449–453.
8. Caligiuri, M. & Beach, D. (1993) *Cell* **72,** 607–619.
9. Reymond, A., Marks, J. & Simanis, V. (1993) *EMBO J.* **12,** 4325–4334.
10. Kelly, T. J., Nurse, P. & Forsburg, S. L. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58,** 637–644.
11. Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A. & Nurse, P. (1993) *Cell* **74,** 371–382.
12. Muzi-Falconi, M. & Kelly, T. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 12475–12479.
13. Moreno, S., Klar, A. & Nurse, P. (1991) *Methods Enzymol.* **194,** 795–823.
14. Tyers, M., Tokiwa, G., Nash, R. & Futcher, B. (1992) *EMBO J.* **11,** 1773–1784.
15. Basi, G., Schmid, E. & Maundrell, K. (1993) *Gene* **123,** 131–136.
16. Maundrell, K. (1993) *Gene* **123,** 127–130.
17. Moreno, S., Hayles, J. & Nurse, P. (1989) *Cell* **58,** 361–372.
18. Muzi-Falconi, M. M., Piseri, A., Ferrari, M., Lucchini, G., Plevani, P. & Foiani, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10519–10523.
19. Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M. & Lerner, R. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4949–4953.
20. Woods, A., Sherwin, T., Sasse, R., MacRae, T. H., Baines, A. J. & Gull, K. (1989) *J. Cell Sci.* **93,** 491–500.
21. Sazer, S. & Sherwood, S. W. (1990) *J. Cell Sci.* **97,** 509–516.
22. Hayles, J., Fisher, D., Woollard, A. & Nurse, P. (1994) *Cell* **78,** 813–822.
23. Broek, D., Bartlett, R., Crawford, K. & Nurse, P. (1991) *Nature (London)* **349,** 388–393.
24. Moreno, S. & Nurse, P. (1994) *Nature (London)* **367,** 236–242.
25. Moreno, S., Labib, K., Correa, J. & Nurse, P. (1994) *J. Cell Sci. Suppl.* **18,** 63–68.
26. Nishitani, H. & Nurse, P. (1995) *Cell* **83,** 397–405.
27. Piatti, S., Lengauer, C. & Nasmyth, K. (1995) *EMBO J.* **14,** 3788–3799.
28. Rao, P. N. & Johnson, R. T. (1970) *Nature (London)* **225,** 159–164.
29. Blow, J. J. & Laskey, R. A. (1988) *Nature (London)* **332,** 546–548.
30. Erdile, L. F., Collins, K. L., Russo, A., Simancek, P., Small, D., Umbricht, C., Virshup, D., Cheng, L., Randall, S., Weinberg, D., Moarefi, I., Fanning, E. & Kelly, T. J. (1991) *Cold Spring Harbor Symp. Quant. Biol.* **56,** 303–313.
31. Coverley, D. & Laskey, R. A. (1994) *Annu. Rev. Biochem.* **63,** 745–776.
32. Su, T. T., Follette, P. J., O'Farrell, P. H., Tyers, M., Tokiwa, G., Nash, R. & Futcher, B. (1995) *Cell* **81,** 825–828.
33. Wang, T. A. & Li, J. J. (1995) *Curr. Opin. Cell Biol.* **7,** 414–420.