Journal of Cell Science 110, 753-763 (1997)
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JCS3499

Cdc6p establishes and maintains a state of replication competence during G_1 phase

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SUMMARY

***CDC6* is essential for the initiation of DNA replication in the budding yeast *Saccharomyces cerevisiae*. Here we examine the timing of Cdc6p expression and function during the cell cycle. Cdc6p is expressed primarily between mitosis and Start. This pattern of expression is due in part to posttranscriptional controls, since it is maintained when *CDC6* is driven by a constitutively induced promoter. Transcriptional repression of *CDC6* or exposure of *cdc6-1^{ts}* cells to the restrictive temperature at mitosis blocks subsequent S phase, demonstrating that the activity of newly synthesized Cdc6p is required each cell cycle for DNA replication. In contrast, similar perturbations imposed on cells arrested in G_1 before Start have moderate or no effects on DNA replication. This suggests that, between mitosis and Start, Cdc6p functions in an early step of initiation, effectively making cells competent for replication. Prolonged exposure of *cdc6-1^{ts}* cells to the restrictive temperature at the pre-Start arrest eventually does cripple S phase, indicating that Cdc6p also functions to maintain this initiation competence during G_1. The requirement for Cdc6p to establish and maintain initiation competence tightly correlates with the requirement for Cdc6p to establish and maintain the pre-replicative complex at a replication origin, strongly suggesting that the pre-replicative complex is an important intermediate for the initiation of DNA replication. Confining assembly of the complex to G_1 by restricting expression of Cdc6p to this period may be one way of ensuring precisely one round of replication per cell cycle.**

Key words: Eukaryotic DNA replication, Initiation, ORC, Origin, Cell cycle, CDC6, *Saccharomyces cerevisiae*, Yeast

INTRODUCTION

The initiation of eukaryotic DNA replication is tightly controlled and coordinated with other events in the life cycle of a cell. Each initiation event is dependent on prior passage of the cell through both mitosis and a point in G_1 called Start, where the cell commits itself to a new round of cell division. Such controls ensure that DNA replication occurs precisely once at the proper time in the cell cycle. The components involved in eukaryotic replication initiation are best characterized in the budding yeast *Saccharomyces cerevisiae* (reviewed by Wang and Li, 1995). Yeast origins are bound at a highly conserved consensus sequence (known as the ACS) by a six-subunit origin recognition complex (ORC) (Bell and Stillman, 1992), whose components are essential for replication initiation (Bell et al., 1993; Foss et al., 1993; Fox et al., 1995; Li and Herskowitz, 1993; Loo et al., 1995; Micklem et al., 1993). This binding cannot be sufficient to induce initiation, however, since ORC binds the origin through much (Diffley et al., 1994), if not all, of the cell cycle. This suggests that other initiator proteins cooperate with ORC to bring about initiation in a cell cycle dependent manner.

Numerous candidates for such initiator proteins have been identified genetically through their role in promoting S phase or in maintaining plasmid stability (reviewed by Wang and Li, 1995). One prominent candidate is encoded by the *CDC6* gene. This protein is required for the initiation of DNA replication, displays genetic interactions with both origins (Hogan and Koshland, 1992) and ORC (Li and Herskowitz, 1993; Liang et al., 1995; Loo et al., 1995), and appears to physically associate with ORC in vitro (Liang et al., 1995). Cdc6p is predicted to be a 58 kDa nucleotide binding protein (Lisziewicz et al., 1988). It shares significant sequence identity with Orc1p (Bell et al., 1995), the largest subunit of ORC, and Cdc18 (Kelly et al., 1993), a homolog of Cdc6p in the fission yeast *Schizosaccharomyces pombe*, which is also required for replication initiation. Transcription of *CDC6* is cell cycle regulated (Piatti et al., 1995); its mRNA normally peaks shortly after mitosis as cells enter G_1 (Piatti et al., 1995; Zwerschke et al., 1994). An additional peak of mRNA, however, can be observed later in cells that are recovering from a temporary G_1 arrest or delay (Bueno and Russell, 1992; Piatti et al., 1995; Zhou and Jong, 1990; Zwerschke et al., 1994) This later peak of mRNA has been shown to coincide with the transient accumulation of Cdc6p (Piatti et al., 1995), suggesting that the protein is unstable and controlled by the cell cycle regulation of its mRNA. Blocking the induction of Cdc6p in G_1 leads to a complete failure to initiate replication (Piatti et al., 1995), indicating that new synthesis of Cdc6p is required each cell cycle for entry into S phase.

Using genomic footprinting one can distinguish at least two types of complexes occupying the origin during the cell cycle: a pre-replicative complex in G_1, and a post-replicative complex in S, G_2, and M phase (Diffley et al., 1994). The footprint of the post-replicative complex resembles that generated by purified ORC in vitro, suggesting that this complex contains ORC. The footprint of the pre-replicative complex, in contrast, is broader and missing a distinctive ORC-induced hypersensitive site, leading to the speculation that other initiator proteins join ORC at the origin. Although the composition of the pre-replicative complex remains to be determined, its appearance in G_1 raises the possibility that it is an intermediate in the initiation of replication and has led to a 2-step model for initiation involving first the formation of the pre-replicative complex in G_1 and second the activation of this complex at the G_1/S boundary (reviewed by Wang and Li, 1995; Zwerschke et al., 1994). Whether the pre-replicative complex is indeed an initiation intermediate remains to be resolved, although the recent finding that Cdc6p is needed to establish and maintain the pre-replicative complex (Cocker et al., 1996) is consistent with such a notion.

To elucidate the role of *CDC6* in the initiation of DNA replication, we have examined the timing of Cdc6p expression during the cell cycle and investigated when Cdc6p functions to promote initiation. We demonstrate that Cdc6p transiently accumulates between mitosis and Start and that this expression pattern is maintained by posttranscriptional as well as transcriptional controls. During this period of peak Cdc6p expression, cells lose their sensitivity to transcriptional repression of the *CDC6* gene and temperature sensitive cdc6-1 cells acquire the ability to enter S phase at the restrictive temperature. This result supports a 2-step model for initiation by suggesting that Cdc6p carries out a function before Start that makes cells competent to trigger initiation at the G_1/S boundary. Because prolonged exposure to the restrictive temperature at a G_1 arrest does eventually disrupt S phase in *cdc6-1* cells, Cdc6p also appears required to maintain the initiation competence of cells until S phase is actually triggered. We further show that the requirement for Cdc6p to establish and maintain initiation competence correlates with the requirement for Cdc6p to establish and maintain the pre-replicative complex. This correlation suggests that the pre-replicative complex is an important part of the competent state and hence a key intermediate in the initiation of DNA replication.

MATERIALS AND METHODS

Yeast media, growth, budding index

YEP medium and synthetic medium lacking methionine (Guthrie and Fink, 1991) were supplemented with 2% dextrose (YEPD; SD-met), 2% raffinose (YEPR; SR-met), or 2% each galactose and raffinose (YEPRG; SRG-met). To shut off the *MET3* promoter (pMET) (Mountain and Korch, 1991), synthetic medium was supplemented with 2 mM methionine (+met medium). Unless otherwise specified cells were grown at 30°C. Alpha factor was used at 50 ng/ml, hydroxyurea at 0.2 M, and nocodazole at 10 μg/ml. For analysis of budding index, cells with bud diameters less than 50% that of the mother cell were scored as small budded cells.

Plasmid and strain construction

The plasmid pCD6 (*(HA)3-CDC6*) contains the *Eco*RI-*Eco*RI *CDC6* genomic fragment cloned into the *Eco*RI site of pRS306 (Guthrie and Fink, 1991). The sequence ATGCGCGGCCGC containing the *Not*I restriction site was inserted just upstream of the *CDC6* ORF and into this site was inserted three tandem copies of the hemagglutinin epitope (HA)3 (Tyers et al., 1992). To generate pCD22 (*pMET-CDC6*) the region of pCD6 5′ of the *CDC6* ORF from *Bsg*I to the downstream *Not*I site was replaced with the following sequence: *Bsg*I/*Sal*I blunt end junction TCTAGAG, nt 386-882 of the *MET3* promoter, GATCCATATGA *Not*I site. The underlined ATG provides the translational Start. pCD18 (pMET-(HA)3-CDC6) is identical to pCD22 but contains the (HA)3 tag reinserted at the *Not*I site. The *CDC6* constructs on these plasmids were substituted for the wild-type *CDC6* gene by 2-step gene replacement (Guthrie and Fink, 1991) and the desired replacements confirmed by southern analysis (Ausubel et al., 1989).

Table 1. Yeast strains used in this study

Strain	Genotype	Source
YJL310	*MAT**a** leu2-3,112 ura3-52 trp1-289 bar1::LEU2*	R. Deshaies (California Institute of Technology)
YJL736	*MAT**a** cln1Δ::URA3 cln2Δ::LEU2 cln3Δ::URA3 ura3-52 trp1-289 leu2::[pGAL-CLN3 LEU2]*	R. Deshaies
YJL1470	*MAT**a**(HA)3-CDC6 leu2-3,112 ura3-52 trp1-289 bar1::LEU2*	This study
YJL1471	*MAT**a** pMET-(HA)3-CDC6 leu2-3,112 ura3-52 trp1-289 bar1::LEU2*	This study
YJL1472	*MAT**a** (HA)3-CDC6 cdc15-1^{ts} leu2-3,112 bar1::LEU2*	This study
YJL1473	*MAT**a** pMET-(HA)3-CDC6 cdc15-1^{ts} leu2-3,112*	This study
YJL1474	*MAT**a** cdc6-1^{ts} leu2-3,112 ura3-52 trp1-289 bar1::LEU*	This study
YJL1475	*MAT**a** pMET-(HA)3-CDC6 cln1Δ::URA3 cln2Δ::LEU2 cln3Δ::URA3 ura3-52 trp1-289 leu2::[pGAL-CLN3 LEU2]*	This study
YJL1476	*MAT**a** cdc6-1^{ts} cln1Δ::URA3 cln2Δ::LEU2 cln3Δ::URA3 ura3-52 trp1-289 his3 leu2::[pGAL-CLN3 LEU2]*	This study

Cell growth, arrest, and release

For examining the expression of Cdc6p in cells synchronously released from mitotic arrest (see Fig. 1A), YJL1472 (*cdc15-1 (HA)3-CDC6*) cells were prearrested in YEPD at 23°C with hydroxyurea for 5 hours, and then released into a *cdc15* arrest by changing the medium to fresh YEPD prewarmed to 37°C. After 80 minutes, when most of the cells had arrested, the cultures were shifted back to 23°C to release the cells from the mitotic arrest. The pre-arrest in HU enhanced the recovery from the *cdc15* arrest. To examine the expression of Cdc6p after synchronous release from alpha factor arrest (see Fig. 1B), YJL1470 (*MAT**a** (HA)3-CDC6 bar1*) cells were arrested with alpha factor (Sigma) until 95% were unbudded (90 to 120 minutes), and then released by washing and resuspending in fresh YEPD.

For examining the replication of cells deprived of Cdc6p at the *cdc15* arrest (see Fig. 2A), YJL1473 (*pMET-(HA)3-CDC6 cdc15-1*) cells were arrested at *cdc15* block in SD-met medium using the protocol described for YJL1472 in Fig. 1A. Methionine was added to half the culture and both halves were held at the arrest point for 30 minutes before being released by shifting the cultures to 25°C. For determining the effect of thermal inactivation of Cdc6p in mitosis (see Fig. 2B), YJL1474 cells (*MAT**a** cdc6-1 bar1*) or YJL310 cells (*MAT**a** CDC6 bar1*) were arrested in YEPD at 23°C with nocodazole (Sigma) until greater than 90% were large budded. The cells were then shifted to 37°C for 30 minutes before being released from arrest by filtering and resuspending the cells in YEPD prewarmed to 37°C.

For examining the effect of depleting Cdc6p at an arrest arising from G_1 cyclin depletion (see Fig. 3), YJL1475 (*pMET-(HA)3-CDC6 cln1-3Δ pGAL-CLN3*) cells growing exponentially in SRG-met at 30°C were filtered and resuspended in SR-met. Once the culture had fully arrested (about 4 hours) methionine was added to half the culture. After an additional hour of incubation, both halves were released from arrest by adding galactose to 2% w/v.

To look at the effect of thermally inactivating Cdc6p at an arrest arising from G_1 cyclin depletion (see Fig. 4), YJL1476 (*cdc6-1 cln1-3Δ pGAL-CLN3*) and YJL736 (*CDC6 cln1-3Δ pGAL-CLN3*) cells growing exponentially in YEPRG at 23°C were filtered and resuspended in YEPR. Once cells had fully arrested (about 4 hours), they were preincubated at 37°C for 0, 1, 2, or 3 hours, before being released from the arrest (still at 37°C) by the addition of galactose to 2% (w/v). Genomic footprints were performed on these cells just before their release to determine the fate of the pre-replicative complex (see Fig. 6).

To investigate the effect of thermally inactivating Cdc6p on the formation of the pre-replicative complex (see Fig. 5A), YJL1474 or YJL310 were arrested in mitosis and released as described for Fig. 2B except that alpha factor was added at the time of the release to prevent cells from progressing beyond Start. Cells were harvested at 0, 1, and 2 hours after release for genomic footprinting. To accumulate G_1 arrested cells that had entered the cell cycle in the absence of Cdc6p (see Fig. 5B), YJL1471 cells growing exponentially in SR-met were first prearrested in the preceding cell cycle with alpha factor and released from this arrest into YEPR with methionine. These cells progressed normally through the remainder of that cell cycle, but were incapable of expressing Cdc6p beyond the release. At 105 minutes into the release, the vast majority of cells had escaped from the prearrest but had not yet entered the next cell cycle. Alpha factor was added back at this point to rearrest these cells in the next G_1 phase. Once the cells had accumulated at this second arrest point, Cdc6p was induced by changing the medium to SRG-met + alpha factor. Arrested cells were harvested for genomic footprint analysis before and after Cdc6p induction.

FACS analysis

A 0.5 ml sample of cells at A_{600} 0.5-1.5 were fixed for at least 1 hour in 70% EtOH, washed and resuspended in 1 ml of 50 mM Tris-HCl, pH 7.5, sonicated, and incubated overnight in 1 mg/ml DNase free RNase (Qiagen) at 4°C. Samples were washed in PBS, resonicated, and resuspended in 300 μl of 50 μg/ml propidium iodide (Sigma) in PBS for at least 1 hour. After a 5-fold dilution into PBS, fluorescence was monitored on a Becton Dickson fluorescence activated cell sorter.

Immunoblot analysis

Cells (1×10^8) were lysed by vortexing 3× 90 seconds in 300 μl SDS-Laemmli loading buffer (Ausubel et al., 1989) containing 300 μl of 0.5 mm glass beads (Biospec Products). The resulting whole cell extracts (60 μg) were immunoblotted (Ausubel et al., 1989) using the following primary antibodies: 12CA5 anti-HA ascites (Babco) at 1:2,000; anti-Sec61p antiserum (a gift from Sylvia Sanders) at 1:1,000, or anti-Clb2p antiserum (a gift from Doug Kellogg) at 1:1,000. Goat anti-mouse or donkey anti-rabbit antibodies conjugated to HRP (Bio-Rad) were used as secondary antibodies at a dilution of 1:2,000. Blots were developed with the Amersham ECL system.

Immunofluorescence

Immunofluorescence was performed essentially as described by Pringle et al. (see Guthrie and Fink, 1991). Rat anti-tubulin antibody (YOL1/34; a gift of J. Kilmartin) was used at 1:250 dilution. The secondary antibody, fluorescein-conjugated goat anti-rat (Sigma), was used at 1:100 dilution.

Genomic footprinting

Genomic footprinting was performed essentially as described (Diffley and Cocker, 1992; Huibregtse and Engelke, 1991).

RESULTS

Cdc6p is expressed periodically between mitosis and Start

Previous work has shown that *CDC6* mRNA levels can be expressed at two points in the cell cycle (Bueno and Russell, 1992; Piatti et al., 1995; Zhou and Jong, 1990; Zwerschke et al., 1994). Normally, *CDC6* mRNA appears transiently shortly after cells exit mitosis, but if G_1 is temporarily delayed before Start, an additional peak of mRNA is observed later in G_1 during the recovery from the delay. In contrast to the numerous studies of *CDC6* mRNA expression, CDC6 protein expression has only been reported for unbudded G_1 daughter cells that were collected by centrifugal elutriation at 4°C and released into the cell cycle at 30°C (Piatti et al., 1995). In these cells, Cdc6p was initially undetectable, appeared in late G_1, declined during entry into S phase, and finally reappeared in G_1 of the next cell cycle. The first appearance of the protein corresponded to the extra peak of *CDC6* mRNA observed after a G_1 delay. The reappearance of Cdc6p in the following cell cycle was presumed to correspond to the normal peak of *CDC6* mRNA observed shortly after mitosis. Neither the duration nor the precise timing of this reappearance was determined, however, limiting the temporal resolution of this analysis. Hence, we reinvestigated the pattern of CDC6 protein expression during the cell cycle, using cells released from either mitotic or G_1 arrest. For these studies, the protein was tagged at its N terminus with three tandem copies of the hemagglutinin epitope ($(HA)_3$-Cdc6p) and detected with anti-HA antibodies. Cells expressing this protein display normal growth rate, morphology, size and progression through the cell cycle (data not shown). We monitored progression through the cell cycle by following FACS analysis of DNA content, accumulation of small budded cells (passage through Start), decline in large budded cells (cytokinesis), and the appearance and disappearance of elongated spindles (anaphase) and Clb2p (mitosis).

As can be seen in Fig. 1A cells released from a block in late anaphase imposed by a *cdc15* mutation exhibited a rapid and sharp increase in Cdc6p abundance. Peak levels were reached as the majority of cells completed anaphase, underwent cell division, and entered the next cell cycle. By the time the entire population had entered G_1 phase, Cdc6p had fallen to low levels (detectable with prolonged exposure to film). This decline began well before bud emergence and the onset of DNA replication, indicating that most of the protein did not persist beyond Start. Quantitative analysis showed that peak and trough levels varied by 10- to 20-fold (data not shown). A similar post-mitotic burst of Cdc6p occurred in the next cell cycle. Although the culture had lost some of its synchrony by then, Cdc6p could be observed to peak during the resolution of anaphase and the onset of cell division. This pattern of protein expression coincides with the previously reported pattern of *CDC6* mRNA expression observed following mitotic release (Piatti et al., 1995; Zwerschke et al., 1994).

To corroborate this analysis, the protein was examined in cells released from a pre-Start G_1 arrest induced by alpha factor pheromone. In cells undergoing such an arrest, Cdc6p transiently appears shortly after mitosis before declining to very low levels at the arrest point (data not shown). As seen in Fig.

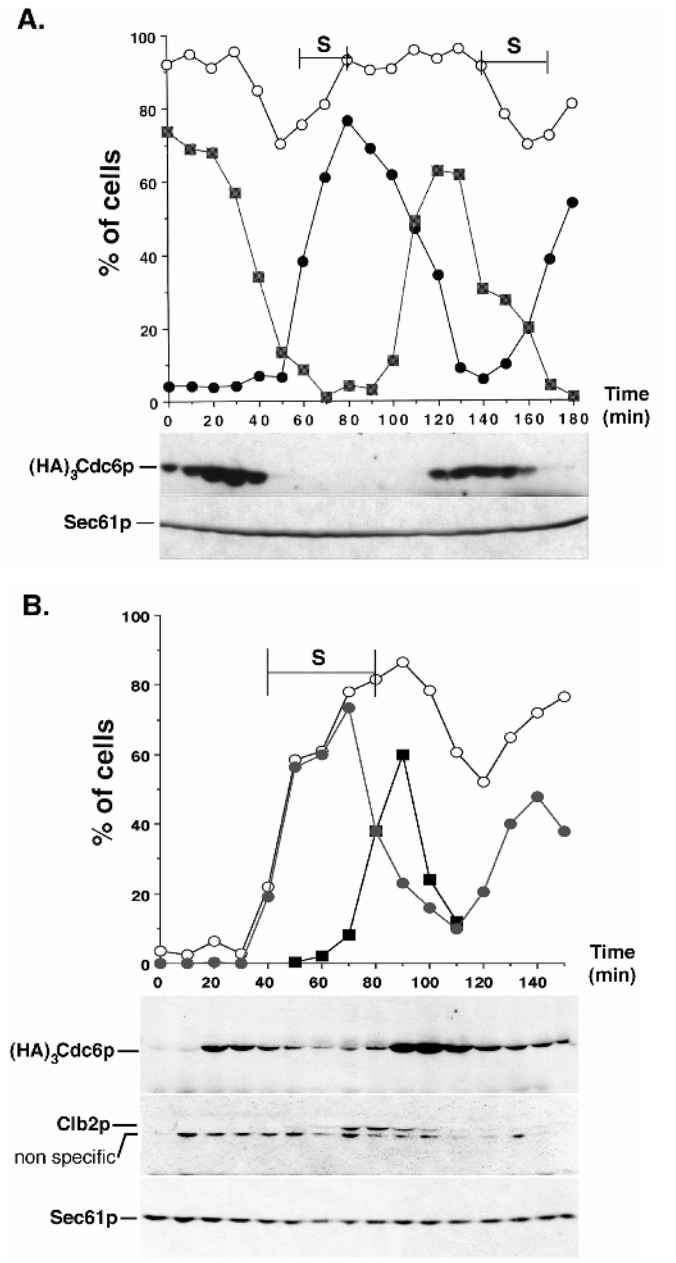

Fig. 1. $(HA)_3$Cdc6p is expressed predominately in late mitosis and early G_1. (A) *cdc15*-1^{ts} *(HA)3-CDC6* cells were arrested in late anaphase by incubation at 37°C and synchronously released from arrest at 25°C. (B) *(HA)3-CDC*6 cells were synchronously released from alpha factor arrest. $(HA)_3$Cdc6p, Sec61p (loading control), and Clb2p (mitotic marker) were detected by immunoblotting. S phase was identified by FACS (period when DNA increases from 1N to 2N). Closed squares, % cells with elongated mitotic spindles (anaphase). Closed circles, % small budded cells. Open circles, % budded cells.

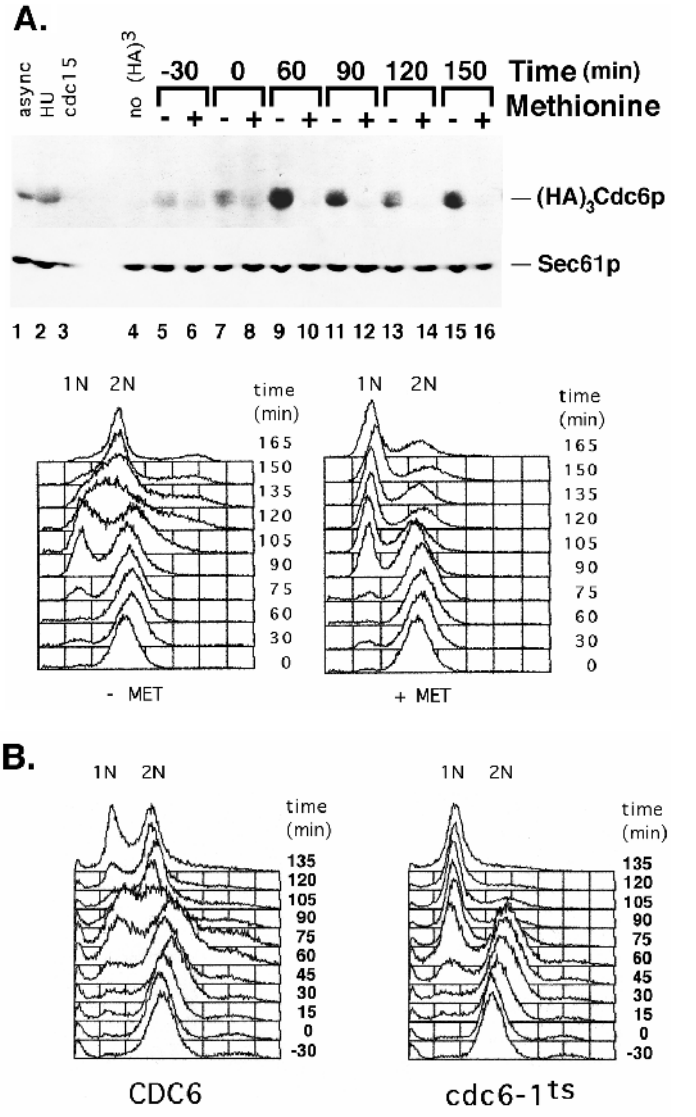

Fig 2. *CDC6* is required after mitosis for DNA replication. (A) A *pMET-(HA)3-CDC6* strain was arrested at a *cdc15* block in late anaphase (–30 minutes), treated with methionine for 30 minutes to repress *CDC6* transcription, then released from arrest in the presence of methionine (+Met) at time 0. A parallel culture was untreated with methionine (–Met). DNA content was monitored by FACS analysis and $(HA)_3$Cdc6p levels were examined by immunoblotting. Asynch, asynchronous cells; HU, hydroxyurea arrested S phase cells; *cdc15, cdc15* arrested cells; no (HA)3, untagged control cells. (B) FACS analysis of *cdc6-*1^{ts} cells arrested in mitosis with nocodazole at 25°C, shifted to the restrictive temperature of 37°C for 30 minutes, then released from nocodazole arrest at 37°C.

1B, on release from this arrest, an additional peak of Cdc6p appeared in G_1 before the onset of budding and DNA replication. In the next cell cycle, Cdc6p reverted back to its regular appearance shortly after mitosis, reaching peak levels as cells completed anaphase and divided. A virtually identical pattern of Cdc6p expression was observed in cells synchronously released from a pre-Start G_1 arrest induced by depletion of the G_1 cyclins, CLN1-3 (data not shown). Both protein expression profiles mirrored the previously reported mRNA expression profiles that were observed after release from alpha factor arrest (Bueno and Russell, 1992; Piatti et al., 1995; Zhou and Jong, 1990; Zwerschke et al., 1994). Taken together, these results indicate that Cdc6p is an unstable protein that normally accumulates between mitosis and Start. Our data also confirm the previous report that a peak of protein is induced in G_1 following recovery from a pre-Start G_1 delay (Piatti et al., 1995).

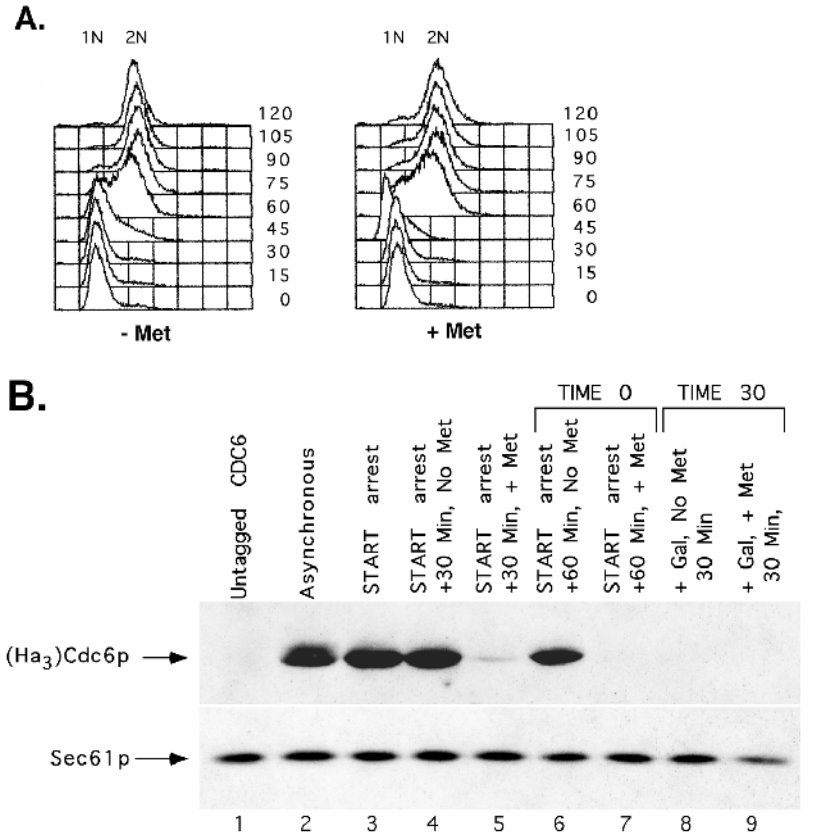

Fig. 3. Depletion of Cdc6p at Start does not disrupt replication competence in G_1 phase. A *pMET-(HA)3-CDC6* strain was arrested before Start by depletion of G_1 cyclins. The culture was split in half at –60 minutes and methionine was added to one half. After incubation for 1 hour both cultures were released from Start arrest at time 0 by induction of *CLN3*. (A) DNA content monitored by FACS following release from arrest in the absence (–MET) or presence (+MET) of methionine. (B) (HA)3Cdc6p was detected by immunoblot at the indicated times relative to the arrest release. Sec61p was monitored as a loading control.

Cells become resistant to perturbation of *CDC6* function during the transition from mitosis to Start

The periodic expression of Cdc6p in early G_1 suggested that the activity of newly synthesized Cdc6p is required at the beginning of each cell cycle for entry into S phase. To test this we constructed a series of strains in which the endogenous *CDC6* gene was placed under the control of the methionine repressible MET3 promoter (*pMET-CDC6*). Similar strains have been constructed by others (Piatti et al., 1995). In the absence of methionine, these strains are comparable to wild-type strains in size, growth rate, and distribution of bud morphology and DNA content across the cell cycle. Addition of methionine to an asynchronous population of these strains causes them to rapidly cease proliferating because of an inability to enter S phase (data not shown). Related strains expressing an HA tagged version of Cdc6p (*pMET-(HA)3-CDC6*) were constructed to allow us to follow Cdc6p levels.

pMET-(HA)3-CDC6 cells were arrested in anaphase using a temperature sensitive *cdc15* mutation, shifted to medium containing methionine for 30 minutes, then released back into the cell cycle at the permissive temperature. Under these conditions Cdc6p was barely detectable during the release (Fig. 2A). Although these cells were able to complete mitosis, undergo cell division, and rebud, they accumulated with a 1N DNA content. (At later timepoints cells with a DNA content <1N eventually appeared, echoing previous observations made in both *S. cerevisiae* and *S. pombe* that in the complete absence of*CDC6* (Piatti et al., 1995) or its *S. pombe* homolog *CDC18* (Kelly et al., 1993) cells attempt mitosis despite failing to enter S phase.) In contrast, control cells not exposed to methionine proceeded into S phase during this period. Identical results were observed with the untagged *pMET-CDC6* strain (data not shown). These findings confirm previous observations that de novo synthesis of Cdc6p after mitosis is required for S phase entry (Piatti et al., 1995, 1996).

A very different result was obtained in cells that were arrested in G_1 before Start. Cells arrested by depletion of the G_1 cyclins were shifted to medium containing methionine for an hour to shut off transcription of *CDC6*, then released from arrest (in the continued presence of methionine) by reinducing *CLN3* synthesis. Cdc6p protein levels dropped rapidly after methionine addition to levels well below those normally induced after mitosis, demonstrating that the transcriptional shut off had a marked effect on protein abundance (Fig. 3B). Despite this decline, cells underwent S phase with identical kinetics to control cells that were unexposed to methionine (Fig. 3A). Thus, during progression from mitosis to Start, the cell's ability to initiate replication loses its sensitivity to transcriptional repression of *CDC6*. This result suggests that during the post-mitotic induction of Cdc6p, the protein performs an essential early step in the initiation of DNA replication. Once the protein performs this step, the cell becomes competent to enter S phase in the absence of further induction or elevated levels of Cdc6p.

Additional evidence suggesting that Cdc6p executes a function before Start that is required for initiation was obtained by comparing the sensitivity of *cdc6-1^{ts}* cells to the restrictive temperature at a mitostic arrest versus a pre-Start G_1 arrest. *cdc6-1^{ts}* cells arrested in mitosis with nocodazole at 25°C were shifted to the restrictive temperature of 37°C for 30 minutes

(identical results were obtained without this preincubation), then released from arrest still at the restrictive temperature (Fig. 2B). As was observed following transcriptional shut off of *CDC6*, these cells divided and budded with normal kinetics (data not shown), but accumulated a 1N DNA content (Fig. 2B). Meanwhile, wild-type *CDC6* cells progressed through S phase and began to enter the next cell cycle. This result further supports the conclusion that Cdc6p must function after mitosis for entry into S phase and illustrates that this function is highly sensitive to the restrictive temperature in *cdc6-1* cells.

In contrast, *cdc6-1* cells arrested in G_1 before Start were much less sensitive to the restrictive temperature of 37°C. CDC6 and *cdc6-1* strains growing at 25°C were arrested by depletion of G_1 cyclins (see Materials and Methods), then released from the arrest by reinducing *CLN3* at 37°C (Fig. 4). The wild-type *Cdc6* strain commenced replication approximately 30 minutes after release, replicated at least half of its DNA by 45 minutes, and had virtually completed replication by 60 minutes. Because cells released from a pre-Start G_1 arrest delay separation from their daughter cells after the first cell cycle, mother and daughter cells enter S phase of the next cell cycle still attached to one another, resulting in the transient appearance of a 4N FACS peak. (This delayed cell separation has been observed in three different strain backgrounds (data not shown) and in cells released from *cdc15* arrest (Piatti et al., 1996).) *cdc6-1* cells subjected to the same treatment progressed through S phase with similar kinetics (Fig. 4A); nuclear division and rebudding also proceeded on time (data not shown), indicating that S phase was normal enough to avoid triggering any mitotic checkpoint controls. Only in the next cell cycle was replication disrupted, as manifested by the absence of the 4N peak in the FACS profile. Even after preincubation at the arrest point for 1 hour at 37°C, *cdc6-1* cells still entered S phase on time and replicated nearly half their DNA by 45 minutes (Fig. 4B). Although this S phase was not entirely normal (see below), the significant replication that did occur contrasts sharply with the absolute block to DNA replication observed when *cdc6-1* cells were shifted to 37°C at mitosis (Fig. 2B). This result is consistent with Cdc6p performing an early initiation step which is highly thermosensitive in *cdc6-1* cells; presumably execution of this step during the post-mitotic induction of Cdc6p accounts for the decrease in sensitivity of *cdc6-1* cells to the restrictive temperature.

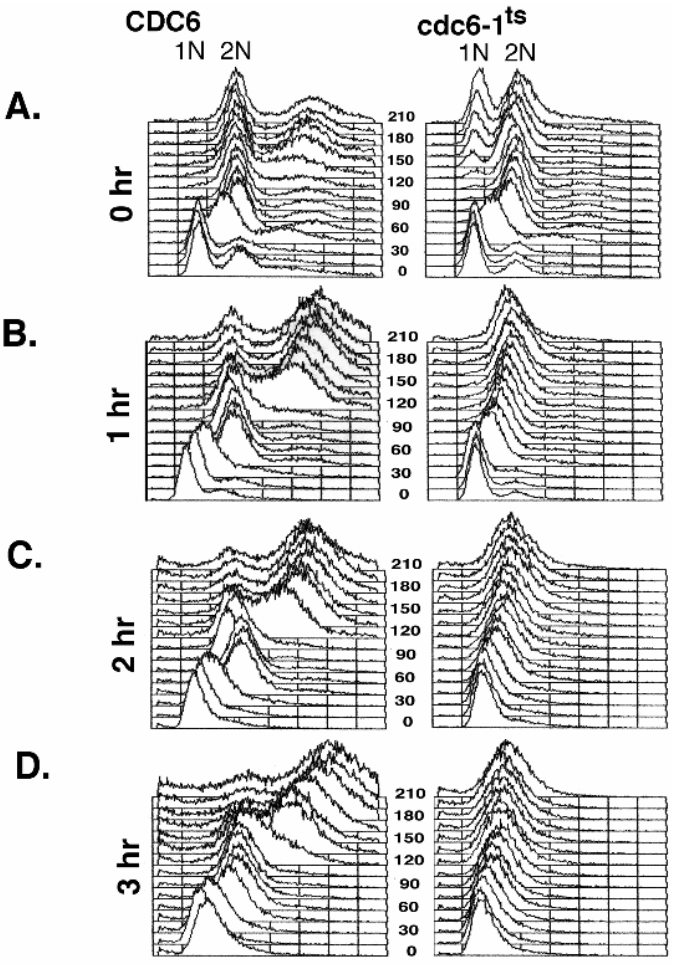

Fig. 4. *CDC6* is required to maintain replication competence in G_1 phase. *CDC6*$^+$ and *cdc6-1* cells growing at 23°C were arrested at Start G_1 cyclin depletion then shifted to 37°C for 0, 1, 2 or 3 hours (A-D, respectively) before being released from the arrest at 37°C. Cells were harvested every 15 minutes following release at time 0 and analyzed for DNA content by FACS.

CDC6 function is required to maintain a state of initiation competence

Despite the considerable resistance of *cdc6-1* cells to the restrictive temperature at a pre-Start G_1 arrest, prolonged exposure of these cells to this temperature before their release eventually did disrupt DNA replication. The initially rapid replication that occurred after a 1 hour preincubation was followed by a slower phase of DNA synthesis (Fig. 4B). Both nuclear (data not shown) and cell division (Fig. 4B, see persistence of 2N peak) were blocked suggesting that replication was incomplete and had induced a mitotic checkpoint arrest. Longer preincubation for 2 or 3 hours resulted in an even slower accumulation of DNA (Fig. 4C and D); the burst of DNA synthesis normally observed between the 30 and 45 minute time points was absent. Although crippled, this accumulation of DNA represented true S phase synthesis, as it was both sensitive to hydroxyurea and dependent on passage through Start (data not shown). Such a disruption could formally be attributed to either defective initiation, in which insufficient origins fire to fully replicate the genome, or to defective fork elongation; we favor the former notion because *cdc6-1* cells are not affected by a shift to the restrictive temperature at a hydroxyurea block (Hartwell, 1976), where S phase has already initiated. Hence, these data suggest that Cdc6p is still required at or after the pre-Start arrest point for initiation to occur. The simplest interpretation of this finding is that, after establishing a state of initiation competence, Cdc6p is needed to maintain this state until S phase is triggered.

The ability of cells to initiate replication correlates with the presence of the pre-replicative complex. The experiments indicating existence of a pre-Start step in the initiation of DNA replication do not provide any information about the molecular nature of this step. Given the similarity in timing, an obvious possibility is that this step involves the transformation of the post-replicative complex (post-RC) to the pre-replicative complex (pre-RC) at replication origins. Previous reports

(Cocker et al., 1996) have shown that Cdc6p is required to establish and maintain the pre-RC. We sought to determine whether there was a correlation in our experiments between the ability of cells to replicate and the presence of the pre-RC at replication origins. The state of the replicative complex was monitored at the 2μ origin of replication using the DNase I genomic footprint assay developed by Diffley and coworkers (Diffley and Cocker, 1992; Huibregtse and Engelke, 1991). In this assay the most striking feature distinguishing the post- and pre-RC footprints is the presence and absence, respectively, of an ORC-induced hypersensitive site at the origin.

To examine the ability of *cdc6-1* and *CDC6* strains to form the pre-RC in G_1 following release from nocodazole arrest at the restrictive temperature, alpha factor was added during the release to prevent the strains from progressing beyond Start. The genomic footprint of the 2 μm origin of replication was then examined 0, 1 and 2 hours after release (Fig. 5A). As expected, both strains displayed the post-replicative footprint at the mitotic arrest point (0 hours). Upon release from this arrest, the ORC-induced hypersensitive site disappeared over a two hour period in wild-type cells, reflecting the assembly of the pre-RC. In contrast, the hypersensitive site persisted in *cdc6-1* cells, indicating that these cells failed to form the pre-RC just as they failed to initiate DNA replication. This finding supports previous observations that depletion of Cdc6p in cells released from *cdc15* arrest leads to an inability to replicate (Piatti et al., 1996) and an inability to form the pre-RC (Cocker et al., 1996).

We note that the wild-type *Cdc6* cells took 2 hours to complete the transition from post- to pre-RC. We presume that this slow transition was due in part to the delayed and slightly asynchronous recovery of cells from nocodazole arrest; approximately 90 minutes elapsed before the entire population completed mitosis (as estimated from the appearance of cells with a 1N DNA content in Fig. 2B). To examine how rapidly the pre-RC can be established in response to Cdc6p expression, we induced Cdc6p in cells that were arrested before Start but had not experienced the normal post-mitotic induction of the protein (see Materials and Methods). Genomic footprinting showed that the post-RC was still present on the 2μ origin of replication in these cells (Fig. 5B), confirming the necessity of Cdc6p for formation of the pre-RC. Within 30 minutes of a shift to methionine-free medium, the pre-RC appeared (Fig. 5B). Such prompt appearance is consistent with Cdc6p having a direct role in establishing the pre-RC and is similar to observations made by Nasmyth and coworkers using a ubiquitin-Cdc6 fusion construct (Piatti et al., 1996). Cells released from the arrest after Cdc6p induction completed a normal S phase, indicating that the cells had also acquired the competence to initiate DNA replication (data not shown). Thus, the establishment of the pre-RC correlates with and is likely to be important for the establishment of initiation competence.

We have also analyzed the genomic footprint at the 2 μm origin in *cdc6-1* cells that were subjected to progressively longer (0, 1, 2, or 3 hours) 37°C incubation while arrested in G_1 by depletion of G_1 cyclins (Fig. 6); these footprints correspond to the 0 minutes time point in each of the panels in Fig. 4. The presence of the pre-RC is apparent in the absence of any preincubation. After one hour at 37°C, the hypersensitive site appeared at approximately half its maximal level, indicating a partial loss of the pre-replicative complex and corresponding

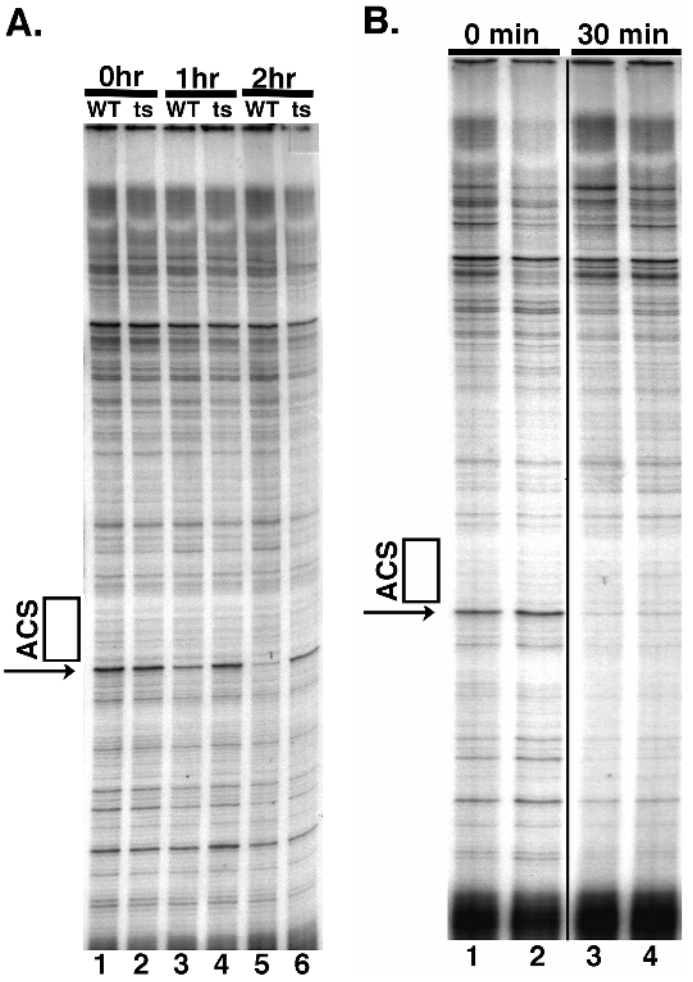

Fig. 5. *CDC6* is required for formation of the pre-replicative complex in G_1 phase. (A) *CDC6*$^+$ (WT) and *cdc6-1* (ts) cells were arrested in mitosis with nocodazole, then released from arrest at 37°C in the presence of alpha factor to prevent cells from progressing beyond Start. At 0, 1, and 2 hours after release, cells were harvested for genomic footprint analysis at the 2 μm origin of replication. (B) *pMET-CDC6* cells were released from alpha factor arrest in the presence of methionine to ensure that they entered the next cell cycle with *CDC6* transcriptionally repressed. After the release alpha factor was added back to the cells to rearrest them in that next cell cycle. Half the cells were harvested at this point for genomic footprint analysis at the 2 μm origin (lanes 1 and 2). The remaining cells were switched to methionine free medium to allow *CDC6* transcription and harvested 30 minutes later for genomic footprint analysis (lanes 3 and 4). Arrow marks the position of the hypersensitive site generated by the post-replicative complex. ACS marks the position of the ARS consensus sequence.

to the partial loss of replication ability seen in Fig. 4B. By two or three hours, the loss of the pre-RC was virtually complete, corresponding to the more severe replication defect seen in Fig. 4C and D. In wild-type control cells, on the other hand, the pre-replicative complex was retained throughout the incubation. These results confirm the previous report that Cdc6p is required to maintain the pre-replicative complex (Cocker et al., 1996). More importantly, the correlation between the gradual loss of the pre-replicative complex and the increasing severity of the replication defect strongly suggests that the competence

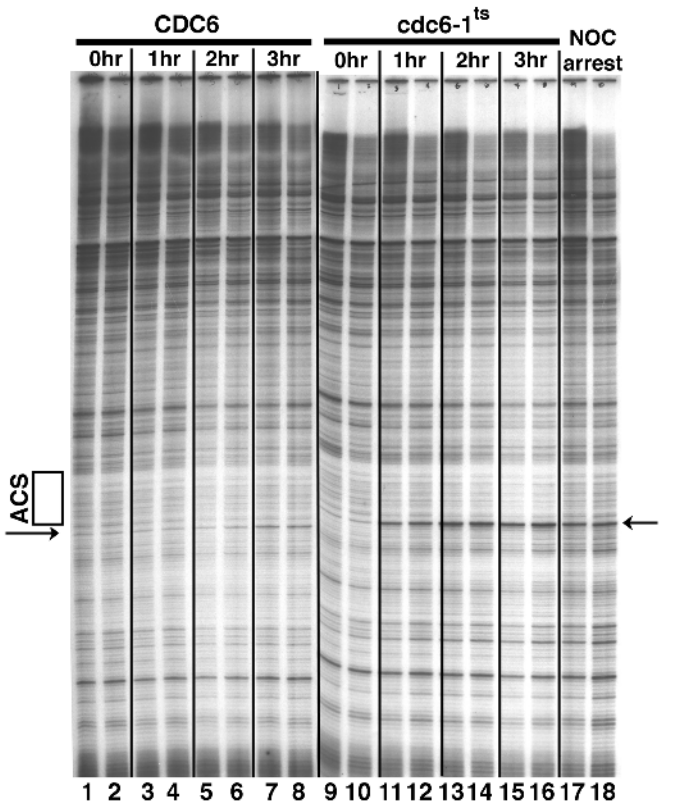

Fig. 6. *CDC6* is required to maintain the pre-replicative footprint in G_1 phase. *CDC6*$^+$ and cdc 6-1 cells growing at 23°C were arrested by depletion of G_1 cyclins (see Materials and Methods) and shifted to the restrictive temperature of 37°C at time 0. Cells were harvested at 0, 1, 2, and 3 hours after the shift for genomic footprint analysis at the 2 μm origin of replication. Arrow marks the position of the hypersensitive site characteristic of the post-replicative footprint. ACS marks the position of the ARS consensus sequence.

of G_1 cells to enter S phase is dependent on the continual presence of the pre-replicative complex. Consistent with this is our observation that the transcriptional shut off of Cdc6p at a pre-Start G_1 arrest, which does not perturb the ability of cells to execute S phase (Fig. 2A), does not disrupt maintenance of the pre-replicative complex (data not shown). Taken together, our results argue that the pre-replicative complex is an important determinant of initiation competence and thus likely to be a critical intermediate in the initiation of DNA replication.

Cdc6p is subjected to posttranscriptional regulation during the cell cycle

Previous work has shown that *CDC6* mRNA levels are determined in large part by the cell cycle regulated transcription of the gene (Piatti et al., 1995). Thus the observation made here and in a previous report (Piatti et al., 1995) that Cdc6p levels closely mirror mRNA levels during the cell cycle suggests that the protein is unstable. Removal of the transcriptional regulation, however, reveals an additional layer of control. Cdc6p levels still fluctuated after *CDC6* was placed under the control of the MET3 promoter, which expresses *CDC6* mRNA levels at constant levels in the absence of methionine (Piatti et al., 1995). Under these conditions the protein was poorly expressed at a cdc15 mitotic arrest, accumulated to maximal levels after release, and declined around the time cells began to enter S phase (Fig. 2A, –met). Moreover, at a pre-Start G_1 arrest, Cdc6p was maintained at high levels but rapidly became undetectable soon after release and well before S phase began (Fig. 3B, –met). Thus, posttranscriptional controls in addition to transcriptional controls ensure that the bulk of Cdc6p expression is confined to a period between mitosis and Start. Massive overexpression of Cdc6p under the *GAL1-10* promoter apparently overwhelms both these regulatory controls; under this promoter Cdc6p is expressed constitutively throughout the cell cycle at levels nearly 50- to 100-fold over those obtained under the *CDC6* or *MET3* promoter (data not shown). Further experiments will be needed to determine whether the post-transcriptional regulation is at the level of protein synthesis or degradation.

DISCUSSION

Cdc6p is an essential protein that is required for the initiation of DNA replication. To understand this role in initiation, we have investigated when the protein is expressed and when it functions during the cell cycle. These studies have led us to define a role for Cdc6p in an early step of initiation, consistent with a two-step model for initiation. They also suggest that the regulation of its expression plays an important role in the regulation of its function.

Regulation of Cdc6p abundance during the cell cycle

We have demonstrated that Cdc6p accumulates transiently shortly after mitosis and is mostly gone before Start (Fig. 1A and B). We have also confirmed an earlier report (Piatti et al., 1995) that, during the recovery from a pre-Start delay in G_1, the protein reappears later in G_1 (Fig. 1B). Both normal and delayed patterns of Cdc6p expression coincide with well documented patterns of *CDC6* mRNA expression, indicating that the protein is inherently unstable. Recent results from Nasmyth and coworkers suggest that this inherent instability may be mediated by cdc4 and cdc34 (Piatti et al., 1996), which target proteins for degradation by ubiquitination (Goebl et al., 1988; R. Deshaies, personal communication). We note, however, that this finding is based on studies of *CDC6* under the *GAL1-10* promoter (Piatti et al., 1996), which significantly overexpresses the protein (C. Detweiler, unpublished observations).

An additional posttranscriptional layer of Cdc6p regulation is revealed by expressing the gene constitutively under the *MET3* promoter, which expresses Cdc6p at nearly endogenous levels; the accumulation of Cdc6p is still confined to between mitosis and Start (Figs 2A and 3B). One possibility is that Cdc6p becomes more unstable after passage through Start and activation of CDC28 kinase. *SIC1* and *CLN2* (Schneider et al., 1996; Schwob et al., 1994) provide examples of proteins believed to be marked for ubiquitination and subsequent destruction as a consequence of such phosphorylation (R. Deshaies, personal communication). Recent observations that Cdc6p is a substrate of CLB-dependent CDC28 kinases (Elsasser et al., 1996) and associates with the kinase in vivo (Elsasser et al., 1996; Piatti et al., 1996) suggests that Cdc6p might be similarly targeted. Consistent with this notion is our observation that Cdc6p levels under

the *MET3* promoter are low when Clbp/Cdc28p kinase activity is high (S, G_2, and M). A similar inverse relationship has been observed in *S. pombe* between the activity of the CDK cyclinB/cdc2$^+$ and the accumulation of the Cdc6p homolog, Cdc18 (Jallepalli and Kelly, 1996). Interestingly, when all five of CDC28 phosphorylation consensus sites ((S/T)PX(K/R)) of Cdc6p are mutated, the protein is expressed at constant levels throughout the *S. cerevisiae* cell cycle (N. Libina and W. Gilbert, unpublished data).

Establishment and maintenance of initiation competence

The experiments presented here strongly suggest that Cdc6p acts before Start to carry out an early step in the initiation of DNA replication. This conclusion is based on the different consequences of transcriptionally repressing or thermally inactivating Cdc6p function in mitotic versus pre-Start arrested G_1 cells. Imposing either treatment at mitosis completely blocks subsequent DNA replication (Fig. 2), confirming that the activity of newly synthesized Cdc6p is required in each cell cycle for S phase to occur (Piatti et al., 1995). In contrast, imposing these treatments at a pre-Start G_1 arrest has either no (Fig. 3) or much reduced effect (Fig. 4) on S phase pro-gression. The simplest interpretation of these results is that, during the transition from mitosis to Start, cells complete an early initiation step(s) that requires induced synthesis of Cdc6p and is highly thermal sensitive in *cdc6-1* cells. Once this step is completed these cells become competent to initiate DNA replication under conditions that would prevent further execution of this step. Thus, we suggest that Cdc6p is required for the establishment of a state of initiation competence in early G_1.

Our data also indicate that Cdc6p is required to maintain the competent state once it has been established. Although *cdc6-1* cells at Start are much less sensitive to the restrictive temperature than mitotic cells, increasingly longer exposure of Start-arrested cells to 37°C eventually leads to progressively worse DNA synthesis (Fig. 4). This is consistent with a gradual decay in the competent state arising from a slow loss of Cdc6p function. Thus, both establishment and maintenance of initiation competence are dependent on Cdc6p.

Diffley and coworkers recently showed that Cdc6p is required to establish and maintain the pre-replicative complex at replication origins (Cocker et al., 1996). In our experiments demonstrating that Cdc6p is required to establish and maintain the ability to initiate replication, we have also shown that having this ability correlates with the presence of the pre-RC at replication origins (Figs 4 and 6). Some of these correlations can also be made by comparing the results obtained by Cocker et al. (1996) with those obtained by Piatti et al. (1995, 1996). These results strongly suggest that the pre-RC is indeed a critical initiation intermediate and that its presence is a defining molecular feature of the competent state.

Possible role for Cdc6p at the replication origin

Fig. 7 incorporates the findings from this report and previous studies (Cocker et al., 1996; Diffley et al., 1994; Liang et al., 1995; Piatti et al., 1995, 1996) into a model for the role of Cdc6p in the initiation of DNA replication. In this model the accumulation of high levels of Cdc6p is presumed to be required for the establishment of initiation competence and, in particular, the efficient assembly of the pre-RC (Cocker et al., 1996). Moreover, the rapid decline of Cdc6p around Start helps to confine the establishment of competence to a window in early G_1. Presumably the residual population of Cdc6p observed after this decline is responsible for maintaining the pre-RC and the state of initiation competence. It is tempting to speculate that this subpopulation has become incorporated into the pre-RC and is thus protected from the degradation affecting the bulk of Cdc6p. Assigning Cdc6p to the complex is also consistent with reports of physical association between ORC and Cdc6 (Liang et al., 1995) or their homologs in *S. pombe* (Leatherwood et al., 1996) and recent observations of Cdc6 localization in *Xenopus* nuclei (Coleman et al., 1996) Further studies, however, will be needed to determine the exact composition of the pre-RC and to address whether Cdc6p (as well as ORC) is a component.

As shown in Fig. 7, activation of the pre-replicative complex at the G_1/S boundary is thought to trigger initiation, leading to the generation of two daughter origins, each bound by a post-RC (Diffley et al., 1994). This second step of initiation is con-

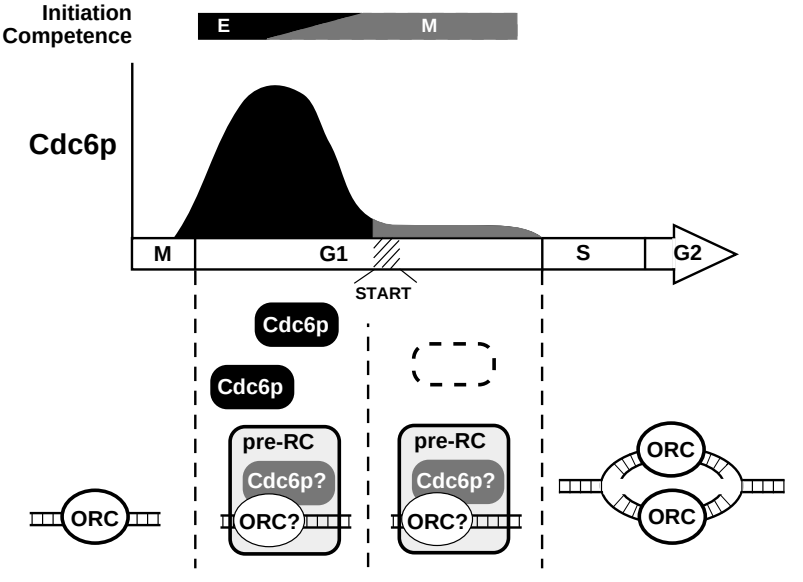

Fig. 7. Model for the role of *CDC6* in DNA replication. See text for discussion. (E) Period when Cdc6p helps to establish initiation competence (and the pre-replicative complex). (M) Period when Cdc6p is still required to maintain initiation competence (and the pre-replicative complex).

tingent on execution of the first step and passage through Start. The Clbp/Cdc28p kinases (in particularly Clb5p/Cdc28p) and the Cdc7p/Dbf4p kinase, which are only active after Start and are required in late G_1 for replication (Jackson et al., 1993; Schwob et al., 1994), have been implicated in this step. The proteins encoded by the *MCM* family of genes (Tye, 1994), which enter the nucleus shortly after mitosis and remain there until replication commences, could be involved in either or both step of initiation. Finally, we note that although our experiments implicate Cdc6p in the establishment (and maintenance) of initiation competence, they do not rule out a direct role for Cdc6p in the second step of triggering initiation.

Involvement of Cdc6p in the cell cycle control of initiation

Re-initiation of replication requires the reestablishment of initiation competence after the onset of S phase. By confining the establishment of competence to the period before Start, the mechanisms that inhibit Cdc6p accumulation after Start can effectively prevent a second round of initiation. These mechanisms presumably do not disrupt the ability of cells to initiate replication once competence is established, thereby ensuring that they do not interfere with the first round of initiation. This presumption is supported by our observation that once Cdc6p is expressed upon exit from mitosis, subsequent reduction of Cdc6p abundance at a pre-Start G_1 arrest (via transcriptional repression of pMet-CDC6) has virtually no affect on S phase progression (Fig. 3).

Previous studies have demonstrated that B-type cyclin dependent kinases (CDK) in *S. cerevisiae* (Dahmann et al., 1995) and *S. pombe* (Broek et al., 1991; Hayles et al., 1994) play a central role in limiting S phase to one round per cell cycle. One way in which these kinases could do this is by preventing the establishment of initiation competence, possibly by interfering with the function of Cdc6p. Piatti et al. (1996) have defined a window of time between the M/G_1 boundary and the onset of CLB kinase activity in late G_1 in which *CDC6* must be transcriptionally active for cells to enter S phase. The onset of CLB kinase activity, which rapidly follows the passage through Start, marks a 'point of no return' beyond which *CDC6* induction will not promote replication. This has led to the suggestion that the kinases antagonize *CDC6* function. The work presented in this report raises the possibility that one way in which these kinases could interfere with *CDC6* function is by posttranscriptionally restricting the accumulation of Cdc6p; a similar notion has been suggested for the regulation of Cdc18 activity in *S. pombe* (Jallepalli and Kelly, 1996).

Although the regulation of Cdc6p abundance is sufficient to account for the block to reinitiation, it is apparently not necessary, since constitutive overexpression of Cdc6p does not lead to re-initiation or even re-establishment of the pre-RC (C. Detweiller, unpublished data; Bueno and Russell, 1992; Piatti et al., 1996). In addition, in at least one of the 'point of no return' experiments, it is clear that Cdc6p levels are not limiting when induced late in G_1, even though replication is hampered (Piatti et al., 1996). This suggests that redundant mechanisms may be employed by CDKs in *S. cerevisiae* to prevent initiation. For example, the activity of Cdc6p may be regulated independently of its abundance, or the function of other proteins required to establish initiation competence (e.g. the Mcm proteins) may be inhibited by CDKs. A contrasting view of replication control has arisen from the observation that overproduction of Cdc18 in *S. pombe* does lead to re-initiation of replication (Muzi-Falconi et al., 1996; Nishitani and Nurse, 1995). This finding has led to the suggestion that regulation of Cdc18 abundance is the primary if not sole means of preventing re-replication in *S. pombe*. Further work will be needed to resolve these differences. Nonetheless, in both organisms the regulation of Cdc6p/Cdc18 provides an important link between the cell cycle and the initiation of DNA replication.

C. Detweiler is supported by a NDSEG grant from the Department of Defense and by NIH training grant #5T32 CA09270. J. J. Li is a Lucille P. Markey Scholar, a Searle Scholar, and a Rita Allen Foundation Scholar. We thank P. O'Farrell, E. Blackburn, D. Morgan, A. Murray, and I. Herskowitz for critically reading the manuscript during its preparation.

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(Received 8 November 1996 - Accepted 15 January 1997)