

# Ectopic induction of Clb2 in early G<sub>1</sub> phase is sufficient to block prereplicative complex formation in *Saccharomyces cerevisiae*

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**ABSTRACT** Eukaryotic cells ensure the stable propagation of their genome by coupling each round of DNA replication (S phase) to passage through mitosis (M phase). This control is exerted at the initiation of replication, which occurs at multiple origins throughout the genome. Once an origin has initiated, reinitiation is blocked until the completion of mitosis, ensuring that DNA is replicated at most once per cell cycle. Recent studies in several organisms have suggested a model in which S- and M-phase promoting cyclin-dependent kinases prevent reinitiation by blocking the repetition of an early step in the initiation reaction. In budding yeast, this regulation is thought to involve inhibition of prereplicative complex (pre-RC) formation at origins by S and M phase-promoting Clb kinases. To date, however, there has been no direct demonstration that these kinases can perform such an important function. In this report we provide such a confirmation by showing that ectopic induction in G<sub>1</sub> phase of a mitotic Clb, Clb2, is sufficient to inhibit DNA replication and does so by preventing pre-RC formation. This inhibition requires that Clb2 be induced before Cdc6, an initiation protein required for pre-RC formation; once pre-RCs have formed, Clb2 can no longer inhibit initiation. These results support the notion that during the normal cell cycle reassembly of the pre-RC, and hence reinitiation at an origin, is directly inhibited by S and M phase-promoting cyclin-dependent kinases.

To ensure the stable propagation of their genome, eukaryotic cells replicate their DNA precisely once per cell cycle. Rereplication in the absence of an intervening mitosis is strictly prohibited. This tight control is imposed at the multiple replication origins used by the cell to initiate DNA replication; once an origin has fired, reinitiation is prevented until the cell completes mitosis. Such a restriction ensures that only a single round of S phase occurs per cell cycle. It also ensures that, despite the ability of cells to fire origins throughout much of S phase, each individual origin fires once and only once within S phase. Exactly how initiation is controlled with such precision is not understood. Recent advances in our understanding of the mechanism of initiation (see Fig. 1A, Upper) however, have provided clues to understanding this regulation.

Genomic footprinting studies of origins in the yeast *Saccharomyces cerevisiae* suggest that the initiation reaction can be divided into at least two steps (1). Shortly after mitosis, a G<sub>1</sub>-specific genomic footprint appears at origins, suggesting the assembly of a prereplicative complex (pre-RC) in early G<sub>1</sub> in preparation for replication initiation (2). Although the exact identity of the proteins responsible for the prereplicative footprint is unknown, several proteins required for initiation are suspected to be pre-RC components. Most strongly impli-

cated is the initiation protein Cdc6, because it is required for both establishment and maintenance of the prereplicative footprint (3, 4). The periodic expression of Cdc6 shortly after mitosis roughly coincides with the timing of pre-RC assembly. Other putative components of the pre-RC include the six-subunit origin recognition complex (ORC) (5) and the minichromosome maintenance (MCM) family of six sequence-related initiation proteins (6). Although the contribution of ORC and the MCM proteins to the prereplicative footprint remains to be determined, their association with chromatin and origins in G<sub>1</sub> suggests they, too, are part of the pre-RC (7, 8).

Once pre-RCs are assembled at origins, their activation is thought to trigger initiation and entry into S phase. This second step of initiation requires the function of two protein kinases, the Cyclin-dependent kinase (CDK) Cdc28 in association with the B-type cyclins Clb1–6 (9–11), and Cdc7 kinase in association with its regulatory subunit Dbf4 (12), which only become active in late G<sub>1</sub> after passage through START. Sequential waves of Clb/Cdc28 kinases are activated during the cell cycle: Clb5 and Clb6 kinases are activated first in late G<sub>1</sub> to promote S phase by triggering initiation (9, 10); Clbs1–4 kinases are activated later in S and G<sub>2</sub> to promote mitosis (13–15). The distinction between S and M phase-promoting Clb kinases, however, may be due more to their order of appearance rather than their substrate specificity, as members of each class can act redundantly to perform functions of the other (10, 11, 16). Hence, CDKs capable of promoting S phase are continuously present from late G<sub>1</sub> until the end of mitosis (Fig. 1A, Upper), when the Clbs are destroyed (17).

Entry into S phase is tightly correlated with conversion of the prereplicative genomic footprint to a smaller postreplicative footprint that is present at origins for the remainder of the cell cycle (2). This correlation suggests that the pre-RC is disassembled when initiation is triggered, leaving a postreplicative complex bound to origins (Fig. 1A, Upper). Like the pre-RC, the exact structure of the postreplicative complex is unknown. However, the resemblance of the postreplicative genomic footprint to the *in vitro* footprint of purified ORC bound to origins indicates that ORC is a key component of the postreplicative complex (5, 18, 19).

In addition to promoting replication by triggering initiation, cyclin-dependent kinases have also been implicated in the block to rereplication. Inactivation of these kinases in *Drosophila*, *Schizosaccharomyces pombe*, and *S. cerevisiae* enables cells to undergo a second round of S phase in the absence of an intervening mitosis (20–24). We note, however, that at least for the two yeasts, the cells appear to be slipping into the next cell cycle, entering G<sub>1</sub> phase before they repeat S phase (20–22). Hence, these experiments do not address whether

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Abbreviations: ORC, origin recognition complex; MCM, minichromosome maintenance; pre-RC, prereplicative complex; CDK, cyclin-dependent kinase; HA, hemagglutinin epitope.

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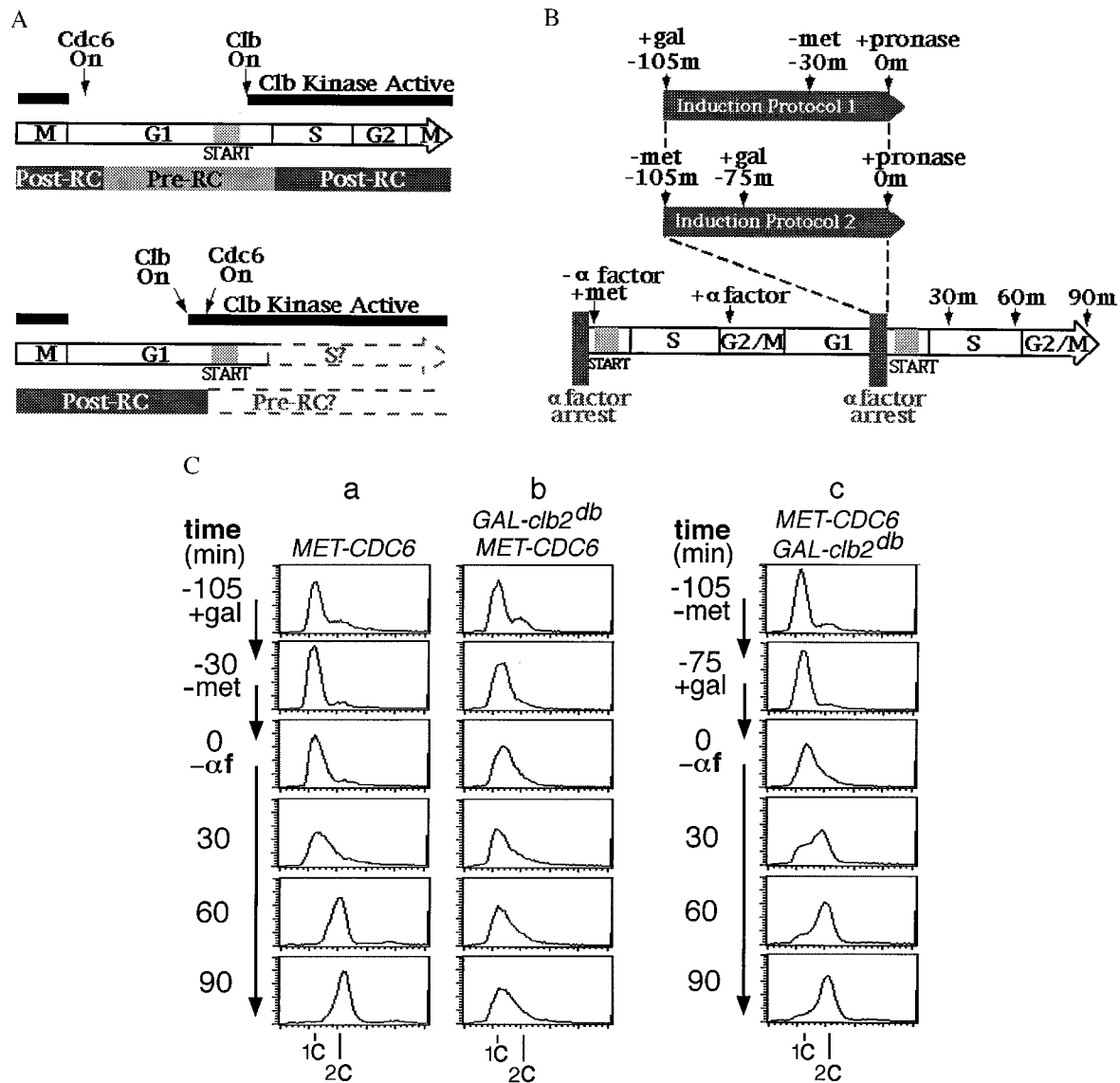


FIG. 1. Induction of Clb2<sup>db</sup> in G<sub>1</sub> before Cdc6 expression is sufficient to block DNA replication. (A) Comparison of events in normal cell cycle (Upper) and experimentally manipulated cell cycle (Lower). In the normal cell cycle, disappearance of Clb kinase activity and induction of Cdc6 after mitosis leads to assembly of the pre-RC before START. Clb kinase activity is induced after START, contributing to the triggering of initiation, the entry into S phase, and the apparent disassembly of the pre-RC. To examine what happens if Clb kinase activity appears before pre-RC formation, Clb kinase was prematurely induced before Cdc6 induction in cells arrested before START. (B) Experimental protocol (see *Materials and Methods* for details). Met3p-CDC6 Gal1p-clb2<sup>db</sup> and Met3p-CDC6 cells were released from alpha factor arrest into medium containing methionine. When most of the cells were in G<sub>2</sub>, alpha factor was added back to rearrest the cells in the next cell cycle. At this second arrest either galactose was added before transferring the cells to methionine-free medium (protocol 1) or cells were transferred to methionine-free medium before galactose was added (protocol 2). Following these inductions, cells released from the arrest by addition of pronase (0m). (C) Flow cytometry of cells sampled at times indicated by min (m) in B. Column a: YJL1169 (*Metp-CDC6*) induction protocol 1. Column b: YJL1167 (*Metp-CDC6*, *Galp-clb2<sup>db</sup>*), induction protocol 1. Column c: YJL1167 (*Metp-CDC6*, *Galp-clb2<sup>db</sup>*), induction protocol 2.

these CDKs block rereplication per se or merely prevent cells from reentering G<sub>1</sub> phase.

One way to distinguish between these possibilities would be to determine whether induction of Clb kinases in cells that had already entered G<sub>1</sub> phase can block replication initiation. Such an approach is the basis for the "point of no return" experiments performed by Nasmyth and coworkers. They showed that prolonged delay of Cdc6 induction in cells progressing through G<sub>1</sub> precluded replication initiation. Passage through START and activation of the Clb kinases roughly coincided with the time when Cdc6 lost its ability to load MCM proteins onto origins (7) and to promote initiation (25), i.e., the point of no return. Deletion of *CLB5* and *CLB6*, the first *CLBs* to be expressed, led to a delay in this point of no return. The inference from these temporal correlations is that the S

phase-promoting kinases contribute to a block in pre-RC formation, although pre-RCs were not directly examined by genomic footprinting. A second inference is that this inhibitory function can also be provided by the mitotic (Clb1–4) kinases, which are induced later in the cell cycle. Based on these inferences it has been proposed that the Clb kinases are necessary to prevent pre-RC reassembly and to block reinitiation in S, G<sub>2</sub>, and M phase of the normal cell cycle. Whether these kinases are sufficient to perform this function was not examined.

To directly demonstrate that mitotic Clb kinases can inhibit initiation by preventing pre-RC formation, we have examined the consequences of ectopically inducing Clb kinase in early G<sub>1</sub> before pre-RCs have formed. For this purpose we have placed Cdc6 and Clb2 under distinct regulatable promoters and

thereby developed a system that allows us to independently manipulate the timing of pre-RC formation and Clb kinase activation in cells arrested before START. We show here that induction of Clb2 before (but not after) pre-RC assembly inhibits subsequent replication. We also show that the premature induction of Clb2 blocks pre-RC formation without interfering with Cdc6 induction. These results provide direct evidence that a mitotic CDK is sufficient to block replication initiation in G<sub>1</sub> cells and help confirm the idea that during the normal cell cycle reinitiation is directly blocked in S, G<sub>2</sub>, and M phase by CDK inhibition of pre-RC formation.

## MATERIALS AND METHODS

**Plasmid and Strain Construction.** Plasmid pCD29 (*Metp-CDC6*) was constructed as follows. The *EcoRI* genomic fragment containing *CDC6* was cloned into a pRS306 (26) derivative whose sole *NotI* site had been destroyed. The sequence CGCGGCCGCATG containing the *NotI* restriction site was then inserted in the *CDC6* ORF just downstream of the translational start codon by oligo-directed mutagenesis. Finally, the fragment 5' of the *CDC6* ORF from *BsgI* to the newly introduced *NotI* site was replaced with a *Sall/NotI* fragment containing the *MET3* promoter and a new translational start codon just upstream of the *NotI* site. pCD59 (*Metp-(HA)<sub>6</sub>-CDC6*) was generated by inserting sequences encoding six tandem copies of the hemagglutinin epitope (HA) in the *NotI* site of pCD29. Plasmid pCD25 (*Galp-clb2<sup>db</sup>*) encodes an 80-aa N-terminal truncation of Clb2 under the control of the *GAL1* promoter (from pAR38; A. Rudner, D. Kellogg, and A. Murray, University of California, San Francisco) cloned into pRS304 (26).

The wild-type *CDC6* gene in yJL310 (*MATa CDC6 bar1::LEU2 ura3 trp1*) was replaced by *Metp-CDC6* (pCD29) by two-step gene replacement (5) to create YJL1184 (*MATa MET3-CDC6 bar1::LEU2 ura3 trp1*). pCD25 (*GalpClb2<sup>db</sup>*) and pRS304 (vector control) were integrated at the TRP1 locus in yJL1184 to create, respectively, yJL1167 (*MATa Metp-CDC6 trp1::Galp-clb2<sup>db</sup>,TRP1 bar1::LEU2 ura3*) and yJL1169 (*MATa Metp-CDC6 trp1::TRP1 bar1::LEU2 ura3*). *Metp-CDC6* in YJL1167 and 1169 was replaced by *Metp-(HA)<sub>6</sub>-CDC6* by two-step gene replacement to generate, respectively, YJL1172 (*MATa Metp-(HA)<sub>6</sub>-CDC6 trp1::Galp-clb2<sup>db</sup>,TRP1 bar1::LEU2 ura3*) and YJL1173 (*MATa Metp-(HA)<sub>6</sub>-CDC6 trp1::TRP1 bar1::LEU2 ura3*).

**Media and Synchronization.** To allow expression from the *MET3* promoter, cells were grown in synthetic complete media lacking methionine and tryptophan (SC-MGT, Trp) (40). This media was supplemented with either 2% raffinose and 2% galactose (SCR-G-Met, Trp) or 2% raffinose (SCR-Met, Trp), depending on whether the *GAL1* promoter was to be induced or not. To repress expression from the *MET3* promoter, 2 mM methionine was added to the synthetic media described above or to rich media (yeast extract/peptone; YEP) (40) supplemented with 2% raffinose and 23 galactose (YEPRG) or just 2% raffinose (YEPR). All cultures were grown at 30°C.

As illustrated in Fig. 1B, cells grown to a density of  $0.5 \times 10^7$  cells ml<sup>-1</sup> in SCR-Met, Trp were arrested with 50 ng ml<sup>-1</sup> alpha factor (see Fig. 1B). These cells were filtered, washed, and resuspended in YEPR and 2 mM methionine (27) to synchronously release them from the arrest while repressing *CDC6* expression from the *MET3* promoter. Approximately 90 min following release, when most of the cells were in G<sub>2</sub>, 50 ng ml<sup>-1</sup> alpha-factor was added back to the cultures to rearrest the cells in the following G<sub>1</sub> phase. Once the cells were rearrested, one of two induction protocols was followed. To induce Clb2<sup>db</sup> before Cdc6 (induction protocol 1), 2% galactose was added for 75 min, then the cultures were filtered, resuspended in SCR-G-Met, Trp containing 50 ng/ml alpha factor, and incubated for an additional 30 min. To induce Cdc6 before Clb2<sup>db</sup>

(induction protocol 2), the cultures were filtered and resuspended in SCR-Met, Trp containing 50 ng/ml alpha factor for 30 min, followed by addition of 2% galactose and incubation for another 75 min. After these inductions, the cultures were released from G<sub>1</sub> arrest by addition of 0.1 μg ml<sup>-1</sup> pronase (to digest the alpha factor) (28) and DNA content monitored by flow cytometry (4).

**Genomic Footprinting.** Genomic footprinting was performed as described (2) with minor modifications. The lysis was performed with  $1-4 \times 10^9$  cells and the primer extensions (5-7 cycles) were performed by using 0.5 μg of total genomic DNA and 1.5 pmol of 5' end-labeled oligonucleotide.

**Western Blot Analysis.** Protein extraction and immunoblot analysis was performed essentially as described (29).

## RESULTS

**Clb2<sup>db</sup> Inhibits Replication when Expressed in G<sub>1</sub> Phase before Cdc6.** To ask whether premature induction of Clb kinase activity in G<sub>1</sub> phase could prevent initiation from occurring, we wanted to induce Clb kinase before the first step of initiation-pre-RC formation-was executed. Normally, pre-RC formation is completed shortly after mitosis, providing no time for prior induction of Clb kinase in G<sub>1</sub> phase (Fig. 1A, Upper). Hence, we constructed strains in which pre-RC formation could be delayed by manipulating the timing of Cdc6 expression. YJL1167 contains a single copy of *CDC6* under the control of the methionine-repressible *MET3* promoter (30) at the *CDC6* locus. By repressing Cdc6 expression we could obtain G<sub>1</sub>-arrested cells that had not yet formed pre-RCs at their origins (Fig. 1A, Lower). This strain also harbors, in addition to the endogenous *Clb2* gene, a truncated version of *CLB2* (*clb2<sup>db</sup>*) under the control of the galactose-inducible *GAL1* promoter. The protein encoded by the truncated *clb2<sup>db</sup>* allele lacks the N-terminal cyclin destruction box of Clb2, allowing ectopically induced Clb2<sup>db</sup> protein to accumulate at a pre-START arrest, when the full-length protein is normally degraded (16). Clb2<sup>db</sup> apparently accumulates at high enough levels under the *GAL1* promoter to activate Clb kinase activity (16), despite the presence of the CDK inhibitor Sic1 (11, 31) at this point in G<sub>1</sub>. As a control for the absence of premature Clb kinase activity, YJL1169, which contains *Met3p-CDC6* but lacks *Gal1p-clb2<sup>db</sup>*, was also constructed.

Fig. 1B shows the experimental protocol used to obtain G<sub>1</sub> cells that had not assembled pre-RCs at their origins. YJL1167 and YJL1169 cultures grown in SCR-Met, Trp (conditions that induce Cdc6 and repress Clb2<sup>db</sup>) were first arrested in G<sub>1</sub> phase of the preceding cell cycle with the mating pheromone alpha-factor. The cells were then synchronously released from the arrest in the presence of methionine to repress *CDC6* transcription. Because Cdc6 is an unstable protein, transcriptional repression results in a rapid depletion of the protein (data not shown). Although this depletion does not affect completion of the interrupted cell cycle, it does prevent pre-RC formation and replication initiation in the subsequent cell cycle (3, 32). Ninety minutes into the release almost all the cells had recovered from the initial arrest but had not yet entered mitosis. At this point, alpha factor was added back to the cultures to collect the Cdc6-depleted cells before START in the following G<sub>1</sub> phase.

Once the cells were rearrested, they were subjected to one of two induction protocols (Fig. 1B). In the protocol 1, galactose was added first for 75 min to induce (or mock induce) Clb2<sup>db</sup>. The cells were then transferred to methionine-free medium (containing galactose and alpha-factor) for 30 min to induce Cdc6. These cells remained unbudded, indicating that they had not passed through START. After the induction, the ability of these cells to replicate was assessed by releasing them from the arrest (by using pronase to digest alpha factor) and monitoring DNA content by flow cytometry every 30 min. YJL1167, which expresses Clb2<sup>db</sup> upon galactose induction,



maintained a nearly 1C DNA content 90 min after arrest release (Fig. 1C, column b). In contrast, the control strain YJL1169, which does not express Clb2<sup>db</sup>, replicated and acquired a 2C DNA content within 60 min following the release (Fig. 1C, column a). These data indicate that premature expression of Clb2<sup>db</sup> in G<sub>1</sub> phase before START can inhibit replication.

Induction protocol 2 (Fig. 1B) was used to show that the order of Clb2<sup>db</sup> and Cdc6 induction is critical for replication to be inhibited. In this protocol, cells were first transferred to methionine-free medium for 30 min to induce Cdc6 before Clb2<sup>db</sup> was induced with galactose. This protocol preserved the order in which Cdc6 expression and Clb2 kinase activation normally occur during the cell cycle. Under these circumstances YJL1167 cells replicated with relatively normal kinetics, acquiring a 2C DNA content within 60 min after release from the alpha factor arrest (Fig. 1C, column c). We presume that Clb2<sup>db</sup> could not inhibit replication in these cells because the prior induction of Cdc6 had allowed them to complete a Clb/Cdc28-sensitive replication function. Collectively, these data argue that Clb2/Cdc28 activity is sufficient to inhibit a Cdc6-dependent replication step but cannot disrupt that step once it has been executed.

**Clb2<sup>db</sup> Induction Prior to Cdc6 Inhibits Pre-RC Formation.** Because Cdc6 is required for pre-RC formation (3, 4), this event is the most obvious step that could be inhibited by Clb2/Cdc28 kinase. To test this we monitored pre-RC formation at the 2 micron origin by DNase I genomic footprinting at the very beginning and end of induction protocol 1. The most distinctive feature of the postreplicative footprint is the pres-

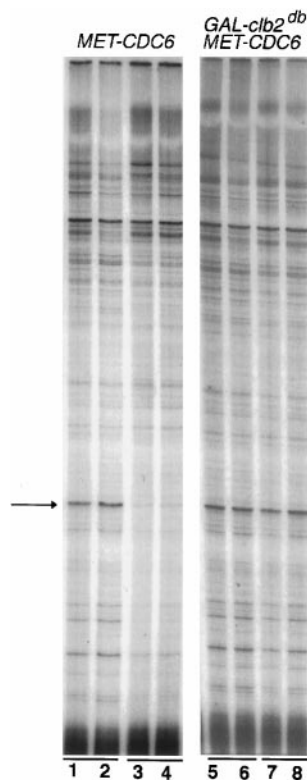


FIG. 2. Induction of Clb2<sup>db</sup> in G<sub>1</sub> before Cdc6 expression is sufficient to block pre-RC formation. *Metp-CDC6* (yJL1169, lanes 1–4) and *Metp-CDC6 Galp-clb2<sup>db</sup>* (yJL1167, lanes 5–7) cells were subjected to induction protocol 1 as described in Fig. 1B. Samples for footprint analysis of the 2 micron replication origin were taken at the second alpha factor arrest before Clb2<sup>db</sup> induction (lanes 1, 2, 5, and 6) and after Cdc6 induction (lanes 3, 4, 7, and 8). Each sample was treated with two different concentrations of DNase I and loaded in adjacent lanes. The ORC-induced hypersensitive site characteristic of the postreplicative footprint is indicated by the arrow.

ence of an ORC-induced hypersensitive site near the ORC recognition site (Fig. 2, arrow); the absence of this site, on the other hand, is characteristic of the prereplicative footprint (2). Fig. 2 shows that G<sub>1</sub>-arrested YJL1167 and YJL1169 cells that had been depleted of Cdc6 retained the postreplicative footprint as expected (lanes 1, 2, 5, and 6) (3, 4). Induction of Cdc6 expression following mock induction of Clb2<sup>db</sup> in YJL1169 led to formation of the pre-RC (Fig. 2, lanes 3 and 4). However, when Clb2<sup>db</sup> was induced prior to Cdc6 expression in YJL1167, the pre-RC failed to form (Fig. 2, lanes 7 and 8). Hence, once Clb2<sup>db</sup> was induced, pre-RC formation was blocked. We also confirmed in these experiments that YJL1167, but not YJL1169, was severely impaired in its DNA replication on release from the G<sub>1</sub> arrest (data not shown). Thus, we conclude that premature induction of Clb2<sup>db</sup> can inhibit replication by preventing the Cdc6-dependent formation of pre-RCs.

**Inhibition of Pre-RC Formation by Clb2<sup>db</sup> Is Not Mediated by Inhibition of Cdc6 Induction.** One trivial way in which Clb2<sup>db</sup> could have prevented pre-RC formation in our experiments was by preventing Cdc6 induction or accumulation. During the normal cell cycle, Cdc6 abundance is regulated in part by posttranscriptional controls (4), possibly through the activity of Clb/Cdc28 kinase. We therefore examined the induction of Cdc6 protein in our experiments by Western blot analysis.

For this purpose, the *Metp-CDC6* allele in yJL1167 and yJL1169 was replaced with *Metp-(HA)<sub>6</sub>-CDC6*, to generate YJL1172 and YJL1173, respectively. This new allele expresses Cdc6 tagged with six copies of the HA epitope. Like the untagged protein, delayed induction of (HA)<sub>6</sub>-Cdc6 at a pre-START arrest could make cells competent to replicate their DNA, but not if Clb2<sup>db</sup> were induced first (data not shown). Despite this difference, (HA)<sub>6</sub>-Cdc6 accumulated to similar levels in both the presence (YJL1172) and absence (YJL1173) of Clb2<sup>db</sup> when cells were shifted to methionine-free medium (Fig. 3). Furthermore, when methionine was added back to these cultures to repress *Metp-(HA)<sub>6</sub>-CDC6* transcription, the tagged protein disappeared at roughly equivalent rates in the presence or absence of Clb2<sup>db</sup> (Fig. 3). This result indicates that the stability of (HA)<sub>6</sub>-Cdc6 was not significantly affected by Clb/Cdc28. Hence, the block to pre-RC formation caused by premature Clb2<sup>db</sup> expression before Cdc6 induction cannot be attributed to destabilization or insufficient accumulation of Cdc6.

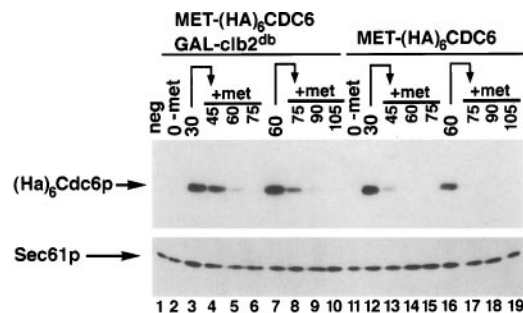


FIG. 3. Clb2<sup>db</sup> does not inhibit Cdc6 by preventing Cdc6 accumulation. *Metp-(HA)<sub>6</sub>-CDC6 GAL-clb2<sup>db</sup>* (yJL1172, lanes 2–10) and *Metp-(HA)<sub>6</sub>-CDC6* (yJL1173, lanes 11–19) cells were subjected to induction protocol 1 as depicted in Fig. 1B but were not released from the arrest so that (HA)<sub>6</sub>-Cdc6 accumulation (arrowhead) could be monitored by Western blot analysis (lanes 2, 3, 7, and lanes 11, 12, 16). Thirty and sixty minutes after induction of the (HA)<sub>6</sub>-Cdc6, 2 mM methionine was added back to a portion of the cultures to repress (HA)<sub>6</sub>-CDC6 expression; proteins levels were monitored at 15 min intervals by immunoblotting (arrowhead, lanes 4–6, 8–10, 13–15, 17–19). Lane 1, *CDC6* (YJL312) cells grown in SCR-Leu. Sec61 was immunoblotted as an internal loading control (arrow).

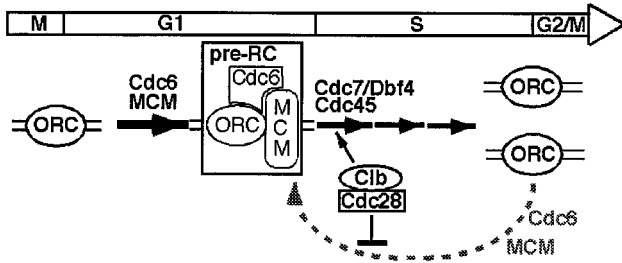


FIG. 4. A model for Clb/Cdc28 kinase inhibition of rereplication. In  $G_1$  phase when Clb/Cdc28 is absent, Cdc6 and Mcm proteins join ORC to form the pre-RC at origins. Activation of Clb/Cdc28 kinase activity late in  $G_1$  triggers replication initiation and disassembly of the pre-RCs. The continual presence of Clb/Cdc28 during S,  $G_2$ , and M phases also blocks reassembly of the pre-RC, preventing reinitiation until the Clbs are destroyed at the end of mitosis.

## DISCUSSION

Experiments in a number of organisms have led to a model (Fig. 4) of how replication initiation is triggered and how reinitiation is prevented during each cell cycle. This model rests on two major premises. (i) The initiation reaction can be divided into at least two steps: assembly of a prereplicative complex at the origin and the activation of this complex to trigger initiation (2). (ii) S and M phase-promoting CDKs affect these steps in opposite ways; they can inhibit the first and promote the second. As cells enter  $G_1$ , the absence of Clb kinase activity (as well as the induction of Cdc6) allows the formation of pre-RCs before START. Passage through START then turns on the Clb kinases, which activate the pre-RCs and simultaneously prevent any further assembly of these complexes, thereby precluding reinitiation. Only at the completion of M phase, when Clbs are destroyed (17), can pre-RCs reform to begin a new round of initiation in the next cell cycle.

Clearly, an important component of this model is the idea that S and M phase-promoting CDKs can inhibit pre-RC formation (see Fig. 4). Support for this idea comes primarily from two types of experiments, neither of which directly demonstrates this inhibitory activity of CDKs. First, loss of S and M phase CDK activity has been shown to lead to a second round of S phase in the absence of an intervening mitosis, suggesting that CDK activity normally prevents reentry into S phase. However, whether they do so directly by preventing reinitiation of replication or indirectly by preventing reentry into the next cell cycle is not addressed in these experiments. A second source of support comes from experiments showing that delayed Cdc6 induction eventually leads to a block in initiation. The loss of the ability of Cdc6 to promote initiation (termed "the point of no return") roughly coincides with the passage of cells through START and the induction of Clb kinase activity. Deletion of the S phase-promoting CLB5 and CLB6—leads to a delay in the point of no return that correlates with the later induction of the mitotic CDKs (Clb1–4 kinase). Because Cdc6 has been shown to be required for pre-RC formation, it has been inferred from this temporal correlation that both S and M phase Clb kinase activity can inhibit pre-RC formation.

In this report we provide direct evidence that a mitotic CDK can inhibit pre-RC formation. To do this we started with cells that were already in  $G_1$  phase so that the effect of CDKs on  $G_1$  entry would not be an issue. Ectopic induction of Clb2<sup>db</sup>, which leads to premature activation of a mitotic Clb kinase (16), was shown to inhibit pre-RC formation and replication initiation. Because we are seeing the effects of inappropriate induction of CDK activity rather than loss of CDK activity, these experiments also suggest that Clb kinase activity is sufficient to prevent pre-RC formation. For example, the passage through

START, which involves activation of the  $G_1$  CDKs Cln1/Cdc28 and Cln2/Cdc28, apparently is not required to prevent pre-RC assembly; we observed this block in cells that were prevented from passing through START with alpha factor. Our results directly confirm that S and M phase promoting CDKs can inhibit pre-RC formation and strongly support the notion that they normally do so in S,  $G_2$  and M phase to prevent reinitiation. Additional support for this conclusion has recently been obtained in a *Xenopus in vitro* replication system, in which either cyclinE/cdk2 or cyclinA/cdc2 can block loading of MCM proteins onto chromatin and inhibit initiation, if added early in the reaction (33).

Two properties of the pre-RC are critical for this model to work. One is that the pre-RC must disassemble when initiation is triggered (2) (or when a replication fork passes through the origin); this disassembly insures that reinitiation depends on reassembly of the pre-RC. It is not known whether pre-RC disassembly at an origin is in fact inextricably linked to the triggering of initiation at that origin. To date, however, these two events have not been uncoupled during the  $G_1$  to S phase transition. The second key property of the pre-RC is its resistance to Clb kinase inhibition once formed. This property is demonstrated here by the failure of Clb2<sup>db</sup> to prevent initiation when it is induced after Cdc6 is expressed and pre-RCs have assembled (Fig. 1C, column c).

How do the S and M phase-promoting CDKs prevent pre-RC formation? An obvious possibility is that the kinases target proteins required for pre-RC formation and inactivate them by phosphorylation or physical association. Studies in *S. cerevisiae* and *S. pombe* point toward Cdc6 and its *S. pombe* homolog Cdc18 as promising targets of CDKs in the block to rereplication. Both homologs associate with S and M phase-promoting CDKs *in vivo*, have multiple consensus CDK phosphorylation sites [(S/T)PX(K/R)], and are phosphorylated by these kinases *in vitro* (25, 34–36). Interestingly, overexpression of Cdc18 in *S. pombe* leads to multiple rounds of rereplication within a cell cycle (34, 37, 38). This observation has led to the suggestion that Cdc18 is the rate-limiting component for replication initiation in *S. pombe* and that it is the primary target for CDK inhibition in the block to rereplication.

In striking contrast to *S. pombe*, however, experiments performed in *S. cerevisiae* have failed to implicate Clb kinase phosphorylation of Cdc6 or association with Cdc6 in the block to rereplication. Overexpression of wild-type Cdc6 or a mutant version lacking the its N-terminal CDK interaction domain (35) does not result in rereplication (39). A similar negative result is also observed with overexpression of a mutant Cdc6 lacking the CDK consensus phosphorylation sites (C.S.D., data not shown). These results suggest that, if Cdc6 is targeted by Clb kinase to prevent rereplication, it is probably only one of several redundant targets used in *S. cerevisiae*. Such redundancy would explain why attempts to prevent Clb kinases from targeting Cdc6 are not sufficient to relieve either the block to initiation when Clb2<sup>db</sup> is prematurely expressed or the block to reinitiation during the normal cell cycle. Other potential targets of the Clb kinases that could provide this redundancy include the ORC subunits and the MCM proteins. These proteins are strong candidates for components of the pre-RC (5, 7) and several of them appear to be *in vivo* substrates of the Clb kinases (ref. 41, and J.J.L., unpublished data). Further study, however, will be needed to determine whether Cdc6, ORC, and MCM proteins are important targets of Clb kinases in the block to rereplication.

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