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The Initiation of Eukaryotic DNA Replication:
The Role of Cdc6

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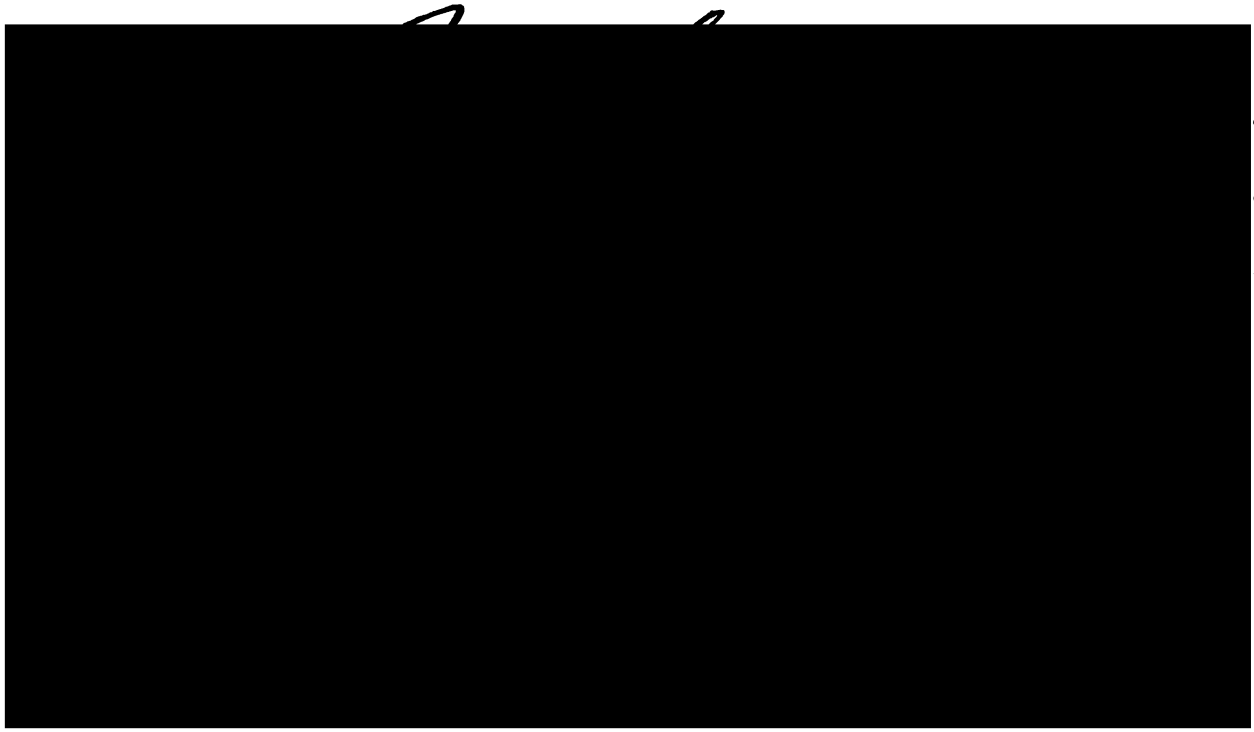

Dedicated to

Sylvia H. Detweiler

John H. Detweiler

Stephen R. Wagner

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The Initiation of Eukaryotic DNA Replication:
The Role of Cdc6

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Cellular proliferation involves the duplication and partitioning of DNA to two daughter cells. This is achieved by passage through the cell cycle, in which a gap period (G1 phase) is followed by DNA replication (S phase), a second gap period (G2 phase), and the distribution of a genomic DNA equivalent to daughter cells in mitosis (M phase) and cytokinesis. Eukaryotic DNA replication occurs exactly once per cell cycle, and is regulated at the level of initiation; the mechanisms by which re-initiation is prevented are unclear. I studied the role of the Cdc6 protein in both promoting and preventing replication initiation in the budding yeast, *Saccharomyces cerevisiae*. I show here that Cdc6 is expressed and can function early in the cell cycle, well before initiation occurs. I demonstrate, by *in vivo* footprinting, that Cdc6 is required to establish and maintain Pre-Replicative Complexes (Pre-RCs), which form at origins of replication in early G1 phase and persist until replication is initiated in S phase. Pre-RCs are shown here to correlate with the ability of cells to replicate, suggesting that they are an intermediate in the initiation reaction. Once initiation has occurred, re-replication is prevented by CDK, the major cell cycle regulatory kinase. I show that CDK (specifically, Cdc28/Clb2) can inhibit replication initiation by preventing Pre-RC formation when ectopically expressed in G1 phase cells before Cdc6. This indicates that CDK can prevent re-replication in S, G2, and M phases by inhibiting either Cdc6 or a Cdc6 dependent event.

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CHAPTER 1

Introduction

During the life cycle of a cell, complex events must be coordinated to precisely duplicate and distribute genomic information to the resulting daughter cells. These events are highly regulated so that each daughter cell receives exactly one copy of the genomic information in the parent cell. To accomplish this, eukaryotic DNA replication occurs once and only once per cell cycle. This thesis concerns the events that lead to DNA replication and how these events are coordinated with the cell cycle.

The Cell Cycle

The cell cycle can be divided into multiple phases. Most eukaryotic cells commit to entering the cell cycle in G1 (Gap 1) phase (Fig. 1-1). This commitment period is called START in *Saccharomyces cerevisiae*. The beginning of DNA synthesis marks the entry into S phase, after which cells enter a second gap period, G2. Duplicated DNA is partitioned and distributed to the daughter cells in mitosis (M phase), which are then separated by cytokinesis to produce two G1 phase cells. Embryonic cell cycles (such as in the model systems of *Xenopus* and *Drosophila*) differ in that they lack both gap phases, but the essential elements of the nuclear cycle, S phase and mitosis, remain intact.

In organisms as diverse as single cell fungi and humans, the major regulators of eukaryotic cell cycle events are members of a family of protein kinases, the cyclin-dependent kinases (CDKs), which are active only when complexed to their cyclin regulatory subuits. In *S. cerevisiae*, there are nine cyclin regulatory subunits (20, 53, 58). The G1 cyclins (Cln1 and Cln2) are important for passage through Start and commitment to another round of cell division. Cyclins Clb5 and Clb6 promote S phase, and Clbs 1-4 are normally required for mitotic events (21, 58).

Cyclins are somewhat functionally redundant. For example, a strain lacking Clb5 and Clb6 is viable because Clb3 and Clb4 can, if necessary, perform the functions of Clb5 and Clb6 (20, 58). Models for how CDKs promote cell cycle progression are therefore based on the sequential expression of the various cyclins (58). In *S. cerevisiae*, Clns 1-3 are expressed in late G1 and promote START, Clbs 5-6 are activated in early S phase and promote DNA replication, and the remaining cyclins, which are expressed from mid-S through mid-M phase, promote mitotic events (47).

DNA Replication Initiation

Studies on bacterial, phage, and viral systems have demonstrated that replication initiation can be conceptually broken down into discrete, molecularly defined steps (13, 35). First, the site on the DNA at which replication will commence, the origin, is recognized by a sequence specific binding protein. Second, additional proteins join the origin binding protein to form an initiation complex. Third, the DNA is unwound, exposing single stranded templates for replication. Fourth, the nucleotide incorporation machinery, including polymerase, primase, and helicase, is loaded onto the unwound DNA. Finally, nucleotide incorporation, called elongation, begins (35).

Hints at how replication initiation is influenced by the cell cycle machinery have been obtained from molecular studies on the viral SV40 system. For example, host cell CDKs promote initiation by phosphorylating T antigen, an SV40 encoded protein that recognizes and unwinds the SV40 origin (44). Furthermore, a T-antigen mutant that lacks a CDK phosphorylation site is unable to promote replication initiation (56). These experiments illustrate that CDKs can directly promote initiation, at least in

the SV40 system, by phosphorylating proteins involved in replication initiation (22).

Eukaryotic Chromosomal Replication Initiation

Compared to bacterial, phage, or viral DNA replication initiation, little is known about the initiation of eukaryotic chromosomal DNA replication. For example, the phrase "origin firing" is used to collectively describe origin unwinding, replication machinery loading, and the start of nucleotide incorporation; we cannot yet separate these events experimentally (13). The regulation of eukaryotic replication differs from that of simpler systems in several important ways. First, the much larger genome of eukaryotes requires that there be multiple origins, compounding the problem of origin identification and firing. Second, eukaryotes generally fire each origin no more than once per S phase; in contrast, viral origins can initiate multiple rounds of replication during a single cell cycle. Third, for unknown reasons, some eukaryotic origins fire early in S phase, and others late, indicating that different origins are differentially regulated (13, 35).

An important clue to how eukaryotic replication is regulated was obtained from early experiments in mammalian tissue culture cells (45). Cells at different stages of the cell cycle were fused and nuclear replication was monitored. The nuclei from G1, but not G2 phase cells, could be driven to replicate DNA when these cells were fused with S phase cells. This indicates that S phase cells contain a factor, called SPF (S-phase Promoting Factor), that can drive G1 phase nuclei into S phase. Furthermore, since G2 phase nuclei were not sensitive to SPF, G1 and G2 phase nuclei differ in their replicative capabilities (52).

Other systems in which replication has been studied include *Xenopus* egg extracts, *Drosophila* embryos and embryonic extracts, the fission yeast *Schizosaccharomyces pombe*, and the budding yeast *Saccharomyces cerevisiae*. *Xenopus* egg extracts have been used to develop an *in vitro* replication system that is biochemically and microscopically tractable (32). The *Drosophila* system supports genetics, cytology, and biochemistry, and boasts a multiplicity of cell cycles (e.g., cycles with multiple rounds of S phase) (18). The two yeasts have the advantage of particularly rapid genetics due to their short life cycles. Furthermore, *S. cerevisiae* has an additional feature that facilitates the study of DNA replication: small, sequence specific origins have been identified (50).

Origins of Replication in Budding Yeast

Replication origins in *S. cerevisiae* were initially identified as sequences that confer high transformation frequencies to plasmids; plasmids that lack origins are not replicated and are effectively lost from a growing population (13). Origins are approximately 120 base pairs long and contain the region of DNA at which bi-directional replication initiates, as demonstrated by two-dimensional (2-D) gel analysis, in which newly replicated DNA is separated from unreplicated DNA based on size and shape (3). Sequence analysis of replication origins revealed that all origins contain an 11-bp consensus sequence that is essential for initiation (Fig. 1-2) (43, 51). In addition, several elements that vary between origins are also important for initiation (Fig. 1-2) (43, 51). Interestingly, not all sequences that can function as an origin on a plasmid actually function as on origin normally in the chromosome, indicating that other factors, besides sequence, help determine where in the genome origin firing occurs (13).

Step 1: Pre-Replicative Complex Formation

The first known step of eukaryotic replication initiation involves the formation of a protein complex, the Pre-Replicative Complex (Pre-RC), at origins of replication (Fig. 1-3). Pre-RCs form only in G1 phase and are characterized in *S. cerevisiae* by *in vivo* footprinting; John Diffley and colleagues showed that in G1 phase cells, origins are more protected from DNaseI, particularly near the consensus sequence, than in G2 phase cells (15). The presence of Pre-RCs in G1 phase was subsequently shown to correlate with the ability of cells to replicate in S phase (11, 49).

Proteins that form the PRE-RC: ORC

Proteins that form the Pre-RC probably include the Origin Recognition Complex (ORC) (Fig. 1-3) (1, 63). ORC is a complex of six proteins that was originally purified from *S. cerevisiae* based on its ability to specifically bind (DNaseI footprint) origin sequences *in vitro* (Fig. 1-2) (2). Members of the complex are required for origin firing in vivo, as demonstrated by 2-D gel analysis (40). Two ORC subunits, Orc1 and Orc5, have ATP binding motifs and apparently bind ATP; ORC ATP binding is required for origin binding. ORC demonstrates weak ATPase activity, but the relationship of this activity to the function of ORC is unknown (2, 13). Homologs of ORC components have been identified in *S. pombe, Xenopus, Drosophila,* and mammals. This is particularly interesting because none of these organisms are known to have small, discrete replication origins; identification of what these ORCs bind may provide clues to the nature of origins in these organisms (13, 46).

Three experiments indicate that ORC binds origins in *S. cerevisiae in vivo*. First, the *in vivo* footprint pattern of purified ORC on origin sequences

closely matches the *in vitro* footprint pattern seen in S, G2, and M phase cells (54). Second, one-hybrid data suggests that the complex interacts with origins *in vivo* (38). Third, immunoprecipitation experiments have shown that ORC associates with origin DNA in S, G2, and M phase cells (1). In these experiments, DNA co-precipitating with ORC proteins after chemical crosslinking *in vivo*, are subjected to PCR to detect the presence of origin sequences. This assay will be referred to as IP-PCR for the remainder of this thesis (1, 61, 63).

CDC6

In addition to ORC, Cdc6 is a likely component of the Pre-RC (Fig. 1-3). Early genetic analysis implicated Cdc6 in DNA replication. The original (recessive) cdc6 mutant was identified in a screen for conditional mutants that are blocked at particular stages of the cell division cycle when shifted to a non-permissive temperature (26). Cdc6 mutant cells arrest at their non-permissive temperature with large buds and unreplicated DNA; mutant cells are unable to incorporate significant amounts of radiolabeled nucleotides indicating an inability to replicate (5, 25). At a semi-permissive temperature, cdc6 mutants fail to maintain plasmids, consistent with a defect in replication. However, plasmids with multiple origins are not lost at a high rate, suggesting that the defect is in the ability to initiate replication (29). The Cdc6 protein contains an NTP binding motif, but NTP hydrolysis has not been convincingly demonstrated (13).

Reciprocal shift experiments also suggest that Cdc6 is involved specifically in replication initiation and not elongation (24). If cdc6 mutant cells are arrested at the non-permissive temperature and then released at the permissive temperature into a drug (hydroxyurea, HU) that blocks

elongation, the cells do not complete S phase. In contrast, if cdc6 mutant cells are arrested with HU at the permissive temperature and released from the HU arrest at the non-permissive temperature, elongation proceeds normally. This indicates that Cdc6 is not involved in elongation and has a role in replication initiation (24). Cdc6 also interacts genetically with at least two subunits of ORC, Orc5 and Orc6, further suggesting a role in replication initiation (38, 40, 41). Finally, CDC6 shows strong homology to ORC1 in a two hundred amino acid region spanning the nucleotide binding motif (13).

MCMs

A third potential component of the Pre-RC are members of the MCM (Mini-Chromosome Maintenance) family of proteins, which share significant sequence identity and contain motifs for nucleotide binding (Fig. 1-3) (13). MCMs were initially isolated in a screen for mutants that could not maintain plasmids (42). These mutants were subsequently shown to be defective in replication, but it was not clear that they played a role in initiation (28, 59, 65). Interest in MCMs was piqued by reports that, in *S. cerevisiae*, MCMs localize to the nucleus in G1 phase and disperse throughout the cell in S, G2, and M phases; G1 phase nuclear localization may suggest an MCM role in preparing the nucleus for the next round of replication (28, 65). It has since been shown in *Drosophila, Xenopus* and mammalian cells that MCMs bind chromatin prior to replication but dissociate from chromatin during replication (8, 36, 62). There is also a recent report that, in *S. cerevisiae*, MCMs are not always bound to chromatin when they are in the nucleus, indicating that MCM chromatin binding may be regulated in this organism as well (67).

Evidence that ORC, Cdc6 and MCMs form the Pre-RC

ORC is a likely component of the Pre-RC because ORC binds chromatin throughout the cell cycle. In *S. cerevisiae*, all six subunits of ORC co-purify with chromatin in G1 phase as well as in S, G2, and M phases, suggesting that ORC is a Pre-RC component (39). Furthermore, in *Xenopus* egg extracts, ORC must be bound to chromatin in order for other initiation proteins to load onto DNA (7). These experiments indicate that ORC is a component of the Pre-RC.

Several experiments indicate that Cdc6 is also part of the Pre-RC. First, Cdc6 is required for the formation and maintenance of the Pre-RC (6, 11). Second, Cdc6 associates with origins in *S. cerevisiae* specifically during G1 phase, as assayed by IP-PCR (1, 63). Finally, since Cdc6 binds chromatin in the *Xenopus in vitro* replication system prior to replication, it is likely that Cdc6 joins ORC at origins in G1 phase (7).

Experiments in two different systems indicate that MCMs are part of the Pre-RC. In the *Xenopus in vitro* system, MCMs bind chromatin in an ORC and Cdc6 dependent manner prior to replication (7, 16). Since in yeast, ORC and Cdc6 appear to form part of the Pre-RC, this indicates that MCMs are also part of the Pre-RC. In yeast, MCMs associate with origins (based on the IP-PCR assay) in G1 phase cells in a Cdc6 dependent manner (1, 63). While not definitive, these experiments suggest that MCMs, along with ORC and Cdc6, bind origins in G1 phase to form the Pre-RC.

Step 2: Origin Firing

The second known step of replication initiation is origin firing, which results in the disassembly of the Pre-RC and subsequent nucleotide incorporation. CDK activity is required for origin firing, and in *S. cerevisiae* CDK/Clb5 becomes active upon passage through START (57).

A second kinase is also required to initiate replication at the G1/S transition (13). Cdc7 and Dbf4 (the regulatory subunit) were both found in screens for cell division cycle mutants, and temperature sensitive mutations in either gene cause cells to arrest late in G1 phase with unreplicated DNA (25, 30, 34, 66). Dbf4 also interacts with origins in a one-hybrid assay (17). Since Dbf4 is expressed only in late G1 and early S phase, it is likely that both proteins are recruited to origins prior to or during the origin firing event (14).

The replication initiation targets of both CDKs and Cdc7 are unknown, although substrates of both kinases abound. Cdc7 can phosphorylate Mcms 2, 3, 4, and 6 *in vitro* (37, 55). Substrates of Clb/CDK include Cdc6, Cdc7, Mcm3, Mcm4, and Orcs 1, 2, and 6 (4, 19, 27, 49, 60, 64, 66, 67). While mutations in putative CDK phosphorylation sites have been made in many of these proteins, no aberration in replication initiation has been observed *in vivo* (10, 60, 64, 66). It is possible that one these proteins is the appropriate substrate of either Cdc7 or Clb/CDK but the critical phosphorylation sites have not yet been identified and mutated. Alternatively, the relevant substrates may have not yet been identified, or phosphorylation of one of several substrates may be sufficient to promote replication.

Origin firing also requires the Cdc45 protein, which has recently been shown to be involved in initiation (23, 31, 48, 68). Pre-RC disassembly does not occur in the absence of Cdc45 activity (48). Furthermore, Cdc45 may associate with origins in late G1 phase, indicating that it may load onto origins after START (1); it is unclear if Cdc45 loads onto origins before or after either Clb/CDK or Cdc7 are activated.

Step 3: Elongation

Origin firing results in Pre-RC disassembly and the recruitment of elongation proteins to origins. Upon Pre-RC disassembly, Cdc6 dissociates from origins, but ORC remains bound (1, 7, 54, 63). The elongation proteins, which include polymerase and primase, assemble into replication forks on either side of the origin and travel bi-directionally away from the origin as nucleotides are incorporated (13). Cdc45, MCMs, and a at least one polymerase are likely part of this elongation complex, as they all migrate away from the origin during S phase based on IP-PCR assays (1). MCMs may provide the helicase activity important for unwinding the template DNA as they can function as helicases *in vitro* (33).

The Prevention of Re-replication

CDKs play a role not only in promoting initiation at the G1/S transition but also in preventing re-replication in S, G2 and M phases (13, 32, 49). The role of Clb/CDK in preventing re-replication was demonstrated by experiments in which artificial inactivation of Clb/CDK in G2 phase cells resulted in an extra round of replication without an intervening mitosis (13, 32, 49). However, it was unclear from these experiments if Clb/CDK prevented re-replication directly. For instance, in *S. cerevisiae*, inhibition of Clb/CDK activity in G2 phase cells resulted in the appearance of several markers of G1 phase events (including the induction of the G1 phase transcriptional program and sensitivity to mating pheromone) before re-replication occurred (9). Thus, it is unclear if Clb/CDK directly prevents re-replication, or if CDK prevents G2 phase cells from entering G1 phase, during which multiple events required for replication occur.

It has been suggested that high Clb/CDK activity levels prevent re-replication directly, based on the correlation between the time at which

mitotic Clb/CDKs are activated and the time at which Cdc6 can no longer promote MCM loading onto origins in *S. cerevisiae* (63). However, it has not directly been shown that high Clb/CDK activity levels prevent an event required for replication initiation.

Experiments in this thesis demonstrate that an early step of replication initiation, Pre-RC formation, is blocked by Clb/CDK. By altering the relative timing of Clb/CDK and Cdc6 expression, we show that a mitotic Clb/CDK can prevent Pre-RC formation and replication in G1 phase cells. This suggests that in the normal cell cycle in S, G2, and M phases, mitotic CDKs may prevent re-replication by blocking Pre-RC formation (12).

It has also been recently demonstrated in the *Xenopus* egg extract system that CDKs inhibit an early step(s) of replication initiation. High levels of CDK activity in interphase extracts prevent MCMs from loading onto chromatin and subsequently prevent DNA replication (32) (Fig. 1-4).

The Thesis

I began the project by confirming the role of Cdc6 in replication initiation, as described in Chapter 2. I performed reciprocal shift experiments and showed that, indeed, Cdc6 functions at an early step in initiation. Characterization of the Cdc6 protein over the cell cycle led to the observation that the protein was expressed early and the hypothesis that it might function early as well. I therefore assessed the role of Cdc6 in an early step of initiation, Pre-RC formation, and found that Cdc6 is required to establish and maintain Pre-RCs.

In Chapter 3, I demonstrated that Clb/CDK can prevent Pre-RC formation and re-replication, suggesting that in the normal cell cycle,

Clb/CDK prevents re-replication by preventing Pre-RC formation. Chapter 4 describes an unsuccessful attempt to determine if Clb/CDK blocks Pre-RC formation by inhibiting Cdc6. The concluding section both briefly reviews the current model of initiation and discusses future directions that the field may take.

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Fig. 1-1. The nuclear division cycle. Cells become committed to enter the cell cycle in G1 by passing through START. At the G1/S transition, DNA replication is initiated and elongation continues throughout S phase. A second gap phase, G2 phase, is followed by Mitosis, in which the DNA is segregated and nuclear division occurs.

G1
start
S
G2
M

Fig. 1-2. Yeast Replication Origins. The 120 base pair ARS1 origin is depicted. The consensensus sequence of box A is found in all origins. The B elements, B1-B3, vary between origins (43). The Origin Recognition Complex, ORC, binds at ARS1 in the region shown (2).

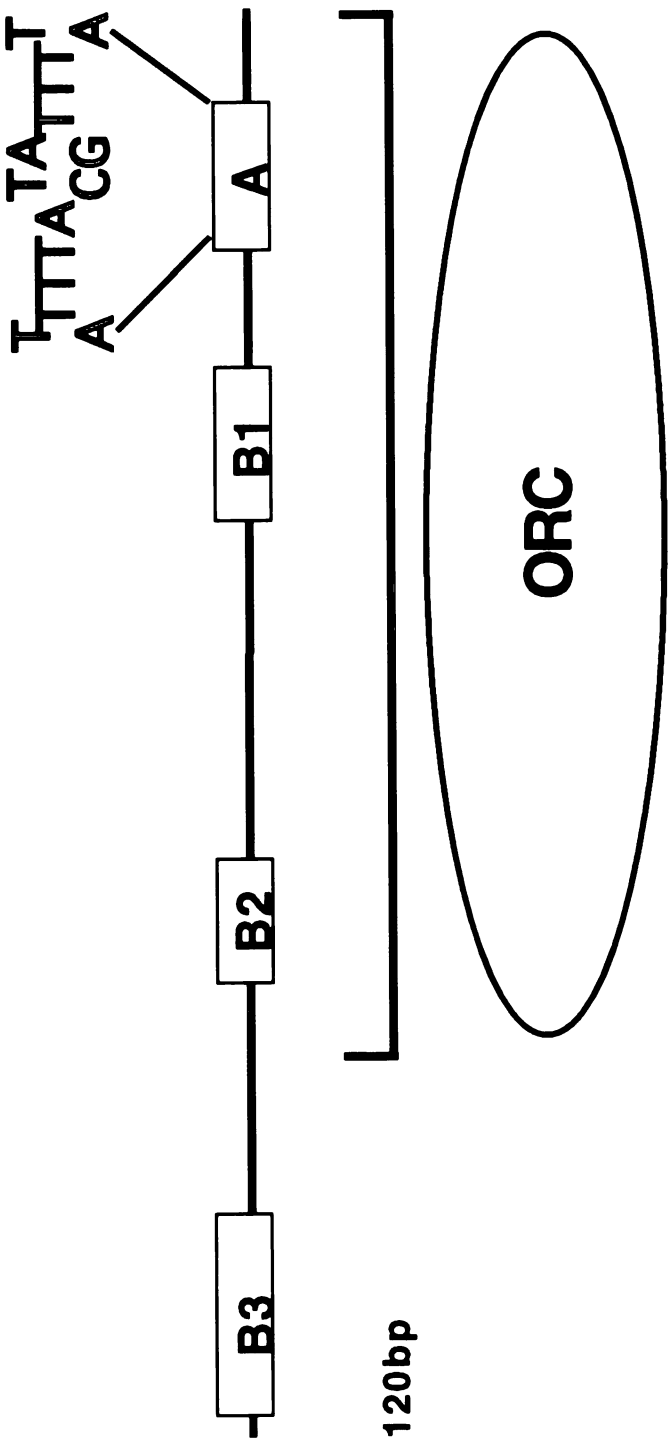

T
TTTA
A
TA
CG
TTT
A
A
B1
B2
B3
ORC
120bp

Fig. 1-3. Step 1 of DNA replication initiation, PRC formation. ORC, a complex of six proteins, is constitutively bound to origins (39). In G1 phase cells, Cdc6 and then MCMs join ORC at origins to form the PRC . The PRC persists throughout G1 phase until origin firing occurs at the G1/S transition and dissembles it (1, 63).

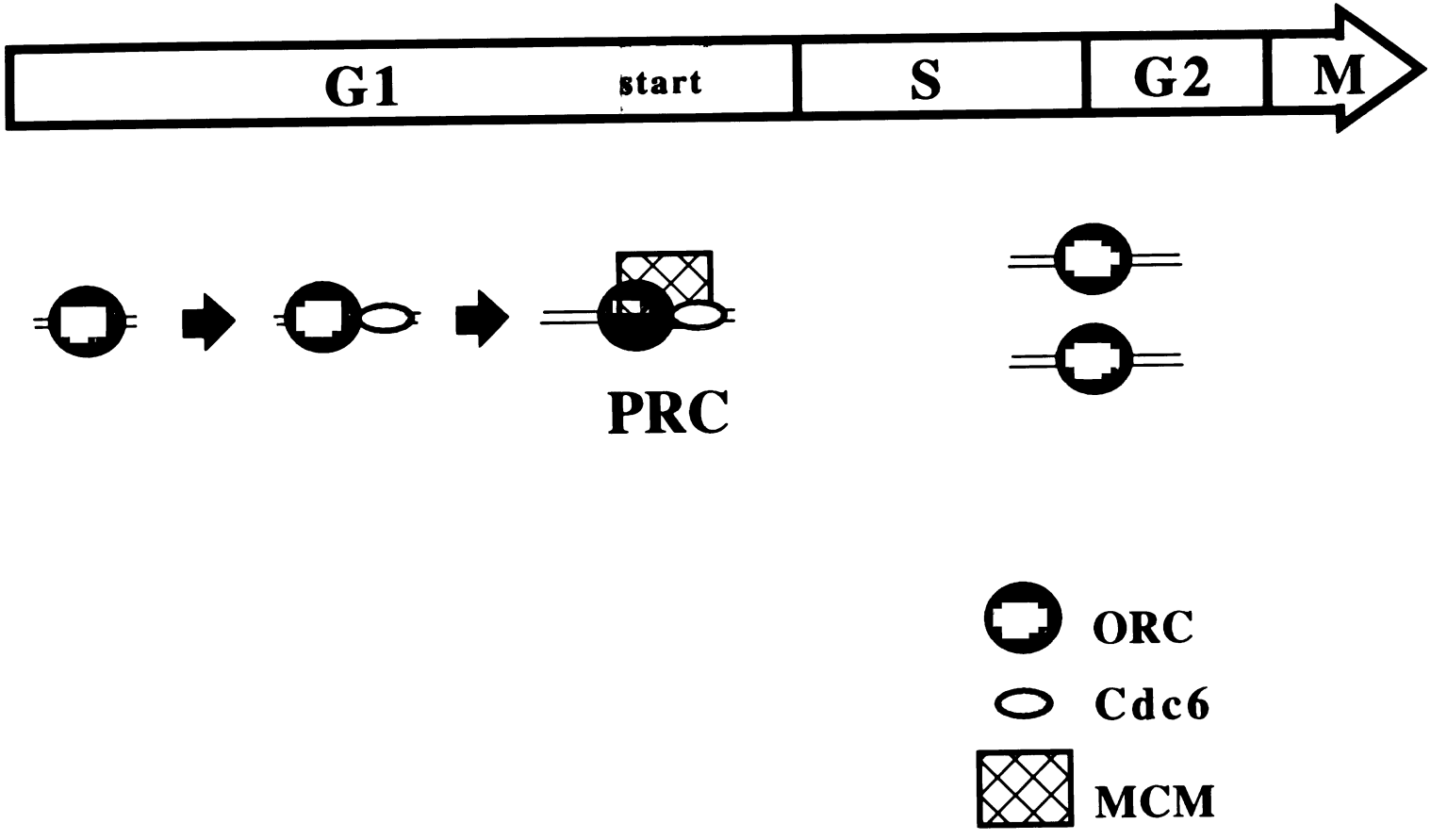
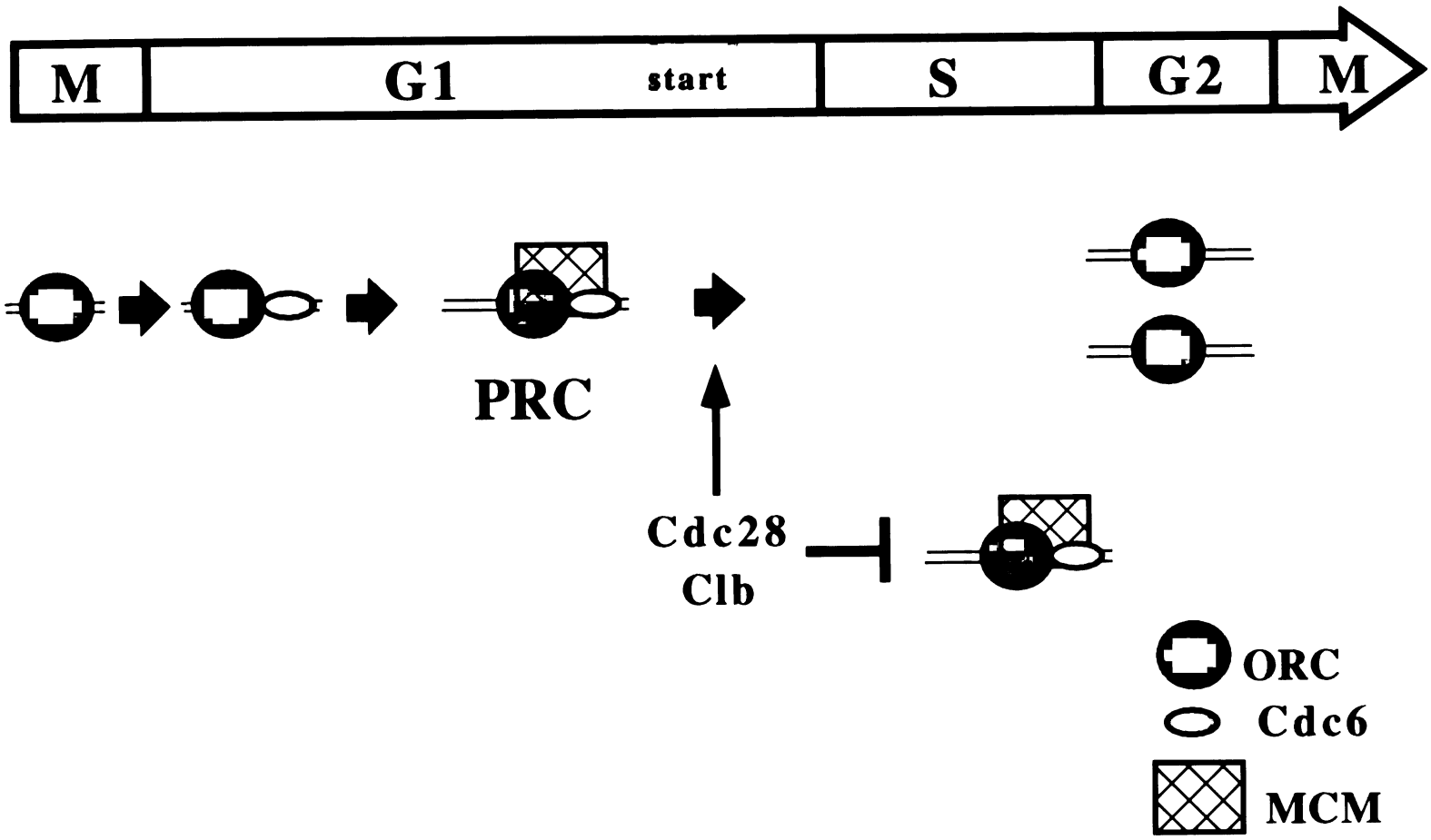

G1
start
S
G2
M
PRC
ORC
Cdc6
MCM

Fig. 1-4. CDK prevents PRC formation in S, G2, and M phase cells. CDK blocks an early step of initiation, such as either Cdc6 or MCM (or both) loading onto origins to form the PRC (12, 63).


M
G1
start
S
G2
M
PRC
Cdc28
Clb
ORC
Cdc6
MCM

CHAPTER 2

Cdc6p Establishes and Maintains a State of Replication Competence during G1 Phase

Detweiler, C. S., and J. J. Li. 1997. Cdc6p establishes and maintains a state of replication competence during G1 phase. J Cell Sci **110**:753-63.

ABSTRACT

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 G1 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 G1. 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 G1 by restricting expression of Cdc6p to this period may be one way of ensuring precisely one round of replication per cell cycle.

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 G1 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 in 39). Yeast origins are bound at a highly conserved consensus sequence (known as the ACS) by a six-subunit origin recognition complex (ORC) (4), whose components are essential for replication initiation (2, 13, 14, 25, 28, 29). This binding cannot be sufficient to induce initiation, however, since ORC binds the origin through much (11), 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 in 39). 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 (19) and ORC (25, 26, 28), and appears to physically associating with ORC in vitro (26). Cdc6p is predicted to be a 58 kD nucleotide binding protein (27). It shares significant sequence identity with Orc1p (3), the largest subunit of ORC, and Cdc18 (23), a homolog of Cdc6p in the fission yeast *Schizosaccharomyces pombe*, which is also required for replication initiation. Transcription of *CDC6* is cell cycle regulated (34); its mRNA normally peaks shortly after mitosis as cells enter G1 (34, 41). An additional peak of mRNA, however, can be observed later in cells that are recovering from a

temporary G1 arrest or delay (6, 34, 40, 41) This later peak of mRNA has been shown to coincide with the transient accumulation of Cdc6p (34), suggesting that the protein is unstable and controlled by the cell cycle regulation of its mRNA. Blocking the induction of Cdc6p in G1 leads to a complete failure to initiate replication (34), 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 G1, and a post-replicative complex in S, G2, and M phase (11). 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 G1 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 G1 and second the activation of this complex at the G1/S boundary (reviewed in 39, 41). 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 (7) 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 G1/S boundary . Because prolonged exposure to the restrictive temperature at a G1 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 media and synthetic media lacking methionine (16) 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) (30), synthetic media was supplemented with 2 mM methionine (+met media). 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 EcoR1-EcoRI *CDC6* genomic fragment cloned into the EcoRI site of pRS306

(16). The sequence ATGCGCGGCCGC containing the NotI 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 (38). To generate pCD22 (*pMET-CDC6*) the region of pCD6 5′ of the *CDC6* ORF from BsgI to the downstream NotI site was replaced with the following sequence: BsgI/SalI blunt end junction--TCTAGAG--nt 386-882 of the *MET3* promoter--GATCCATATGA--NotI 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 NotI site. The *CDC6* constructs on these plasmids were substituted for the wild-type *CDC6* gene by 2-step gene replacement (16) and the desired replacements confirmed by southern analysis (1).

Cell growth, arrest, and release. For examining the expression of Cdc6p in cells synchronously released from mitotic arrest (Fig. 2-1A), 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 media 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 (Fig. 2-1B), 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 (Fig. 2-2A), YJL1473 (*pMET-(HA)3-CDC6 cdc15-1*) cells were arrested at *cdc15* block in SD-met media using the protocol described for YJL1472 in Fig. 2-1A. 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 (Fig. 2-2B), YJL1474 cells (*MATa cdc6-1 bar1*) or YJL310 cells (*MATa 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 G1 cyclin depletion (Fig. 2-3), 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 G1 cyclin depletion (Fig. 2-4), 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 (Fig. 2-6).

To investigate the effect of thermally inactivating Cdc6p on the formation of the pre-replicative complex (Fig. 2-5A), YJL1474 or YJL310 were arrested in mitosis and released as described for Fig. 2-2B 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 G1 arrested cells that had entered the cell cycle in the absence of

Cdc6p (Fig. 2-5B), 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. 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 G1 phase. Once the cells had accumulated at this second arrest point, Cdc6p was induced by changing the media to SRG-met + alpha factor. Arrested cells were harvested for genomic footprint analysis before and after Cdc6p induction.

FACS Analysis. 0.5 ml cells at OD_{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 pH7.5, 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. 1 x 10^8 cells were lysed by vortexing 3 x 90 seconds in 300 µl SDS-Laemli loading buffer (1) containing 300 µl of 0.5 mm glass beads (Biospec Products). The resulting whole cell extracts (60µg) were immunoblotted (1) using the following primary antibodies: 12CA5 anti-HA ascites (Babco) at 1:2000; anti-Sec61p antiserum (a gift from Sylvia Sanders) at 1:1000, or anti-Clb2p antiserum (a gift from Doug Kellogg) at 1:1000. Goat anti-mouse or donkey anti-rabbit antibodies conjugated to HRP (BioRad) were used as secondary antibodies at a dilution of 1:2000. Blots were developed with the Amersham ECL system.

Immunofluorescence. Immunofluorescence was performed essentially as described by Pringle et al. (in 16).. Rat antitubulin 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 (10, 20).

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 (6, 34, 40, 41). Normally, *CDC6* mRNA appears transiently shortly after cells exit mitosis, but if G1 is temporarily delayed before Start, an additional peak of mRNA is observed later in G1 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 G1 daughter cells that were collected by centrifugal elutriation at 4°C and released into the cell cycle at 30°C (34). In these cells, Cdc6p was initially undetectable, appeared in late G1, declined during entry into S phase, and finally reappeared in G1 of the next cell cycle. The first appearance of the protein corresponded to the extra peak of *CDC6* mRNA observed after a G1 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 G1 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. 2-1A 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 G1 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 (34, 41).

To corroborate this analysis, the protein was examined in cells released from a pre-Start G1 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. 2-1B, on release from this arrest, an additional peak of Cdc6p appeared in G1 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 G1 arrest induced by depletion of the G1 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 (6, 34, 40, 41). Taken together, these results indicate that Cdc6p is an unstable protein that normally accumulates between mitosis and Start. Our data also confirms the previous report that a peak of protein is induced in G1 following recovery from a pre-Start G1 delay (34).

Cells become resistant to perturbation of *CDC6* function during the transition from mitosis to Start. The periodic expression of Cdc6p in early G1 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 (34). 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. 2-2A). Although these cells were able to complete mitosis, undergo cell division, and rebud, they accumulated with a 1 n DNA content. (At later timepoints cells with a DNA content < 1 n eventually appeared, echoing previous observations made in both *S. cerevisiae* and *S. pombe* that in the complete absence of*CDC6* (34) or its *S. pombe* homolog *CDC18* (23) 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 (33, 34).

A very different result was obtained in cells that were arrested in G1 before Start. Cells arrested by depletion of the G1 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. 2-3B). Despite this decline, cells underwent S phase with identical kinetics to control cells that were unexposed to methionine (Fig. 2-3A). 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 G1 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. 2-2B). 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. 2-2B). 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 G1 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 G1 cyclins (see materials and methods), then released from the arrest by reinducing *CLN3* at 37°C (Fig. 2-4). 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 G1 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 4 n FACS peak. (This delayed cell separation has been observed in three different strain backgrounds (data not shown) and in cells released from cdc15 arrest (33)). *cdc6-1* cells subjected to the same treatment

progressed through S phase with similar kinetics (Fig. 2-4A); 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 4 n 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. 2-4B). 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. 2-2B). 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.

***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 G1 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. 2-4B). Both nuclear (data not shown) and cell division (Fig. 2-4B, see persistence of 2 n 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. 2-4C and 4D); 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 (17), 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 2 μ origin of replication using the DNAase I genomic footprint assay developed by Diffley and coworkers. 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 G1 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. 2-5A). 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 (33) and an inability to form the pre-RC (7).

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 1 n DNA content in Fig. 2-2B). 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 Material and Methods). Genomic footprinting showed that the post-RC was still present on the 2 μ origin of replication in these cells (Fig. 2-5B), 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. 2-5B). 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 (33). 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 G1 by depletion of G1 cyclins (Fig. 2-6); these footprints correspond to the 0 minutes time point in each of the panels in Fig. 2-4. 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 to the partial loss of replication ability seen in Fig. 2-4B. By two or three hours, the loss of the pre-RC was virtually complete, corresponding to the more severe replication defect seen in Fig. 2-4C and 2-4D. 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 (7). 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 of G1 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 G1 arrest, which does not perturb the ability of cells to execute S phase (Fig. 2-2A), 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 (34). Thus the observation made here and in a previous report (34) 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 (34). 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. 2-2A, -met). Moreover, at a pre-Start G1 arrest, Cdc6p was maintained at high levels but rapidly became undetectable soon after release and well before S phase began (Fig. 2-3B, -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 posttranscriptional 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. 2-1A and 2-1B). We have also confirmed an earlier report (34) that, during the recovery from a pre-Start delay in G1, the protein reappears later in G1 (Fig. 2-1B). 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 (33), which target proteins for degradation by ubiquitination (15 and R. Deshaies, personal communication). We note, however, that this finding is based on studies of *CDC6* under the *GAL1-10* promoter (33), 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 (Fig. 2-2A and 2-3B). One possibility is that Cdc6p becomes more unstable after passage through Start and activation of CDC28 kinase. *SIC1* and *CLN2* (35, 36) 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 (12) and associates

with the kinase in vivo (12, 33) 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, G2, and M). A similar inverse relationship has been observed in *Schizosaccharomyces pombe* between the activity of the CDK cyclinB/cdc2$^+$ and the accumulation of the Cdc6p homolog, Cdc18 (22). 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 G1 cells. Imposing either treatment at mitosis completely blocks subsequent DNA replication (Fig. 2-2), confirming that the activity of newly synthesized Cdc6p is required in each cell cycle for S phase to occur (34). In contrast, imposing these treatments at a pre-Start G1 arrest has either no (Fig. 2-3) or much reduced effect (Fig. 2-4) on S phase progression. 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 G1.

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. 2-4). 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 (7). 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 (Fig. 2-4 and Fig. 2-6). Some of these correlations can also be made by comparing the results obtained by Cocker, et al. (7) with those obtained by Piatti et al (33, 34). 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. 2-7 incorporates the findings from this report and previous studies (7, 11, 26, 33, 34) 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 (7) . Moreover, the rapid decline of Cdc6p around Start helps to confine the establishment of competence to a window in early G1. 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 (26) or their homologs in S. pombe (24) and recent observations of Cdc6 localization in *Xenopus* nuclei (8) 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. 2-7, activation of the pre-replicative complex at the G1/S boundary is thought to trigger initiation, leading to the generation of two daughter origins, each bound by a post-RC (11). This second step of initiation is contingent 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 G1 for replication (21, 36), have been implicated in this step. The proteins encoded by the *MCM* family of genes (37), 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 G1 arrest (via transcriptional repression of pMet-CDC6) has virtually no affect on S phase progression (Fig. 2-3).

Previous studies have demonstrated that B-type cyclin dependent kinases (CDK) in S. cerevisiae (9) and S. pombe (5, 18) 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. have defined a window of time between the M/G1 boundary and the onset of CLB kinase activity in late G1 in which *CDC6* must be transcriptionally active for cells to enter S phase (33). 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 (22).

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 and 6, 33). 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 G1, even though replication is hampered (33). 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 (31, 32) . This finding has led to 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.

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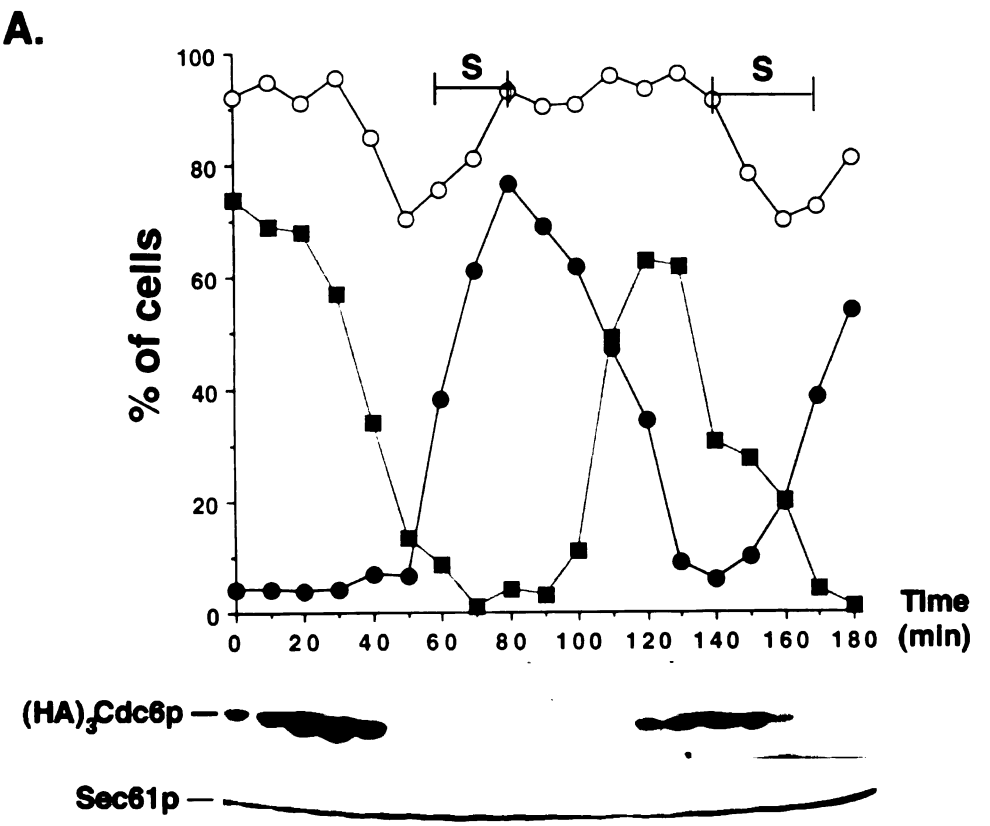
Fig. 2-1. (HA)3Cdc6p is expressed predominately in late mitosis and early G1. (A) *cdc15*-1^{ts} *(HA)3-CDC6* cells were arrested in late anaphase by incubation at 37°C and synchronously released from arrest at 25°C. (B) *(HA)3-CDC6* cells were synchronously released from alpha factor arrest. (HA)3Cdc6p, 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.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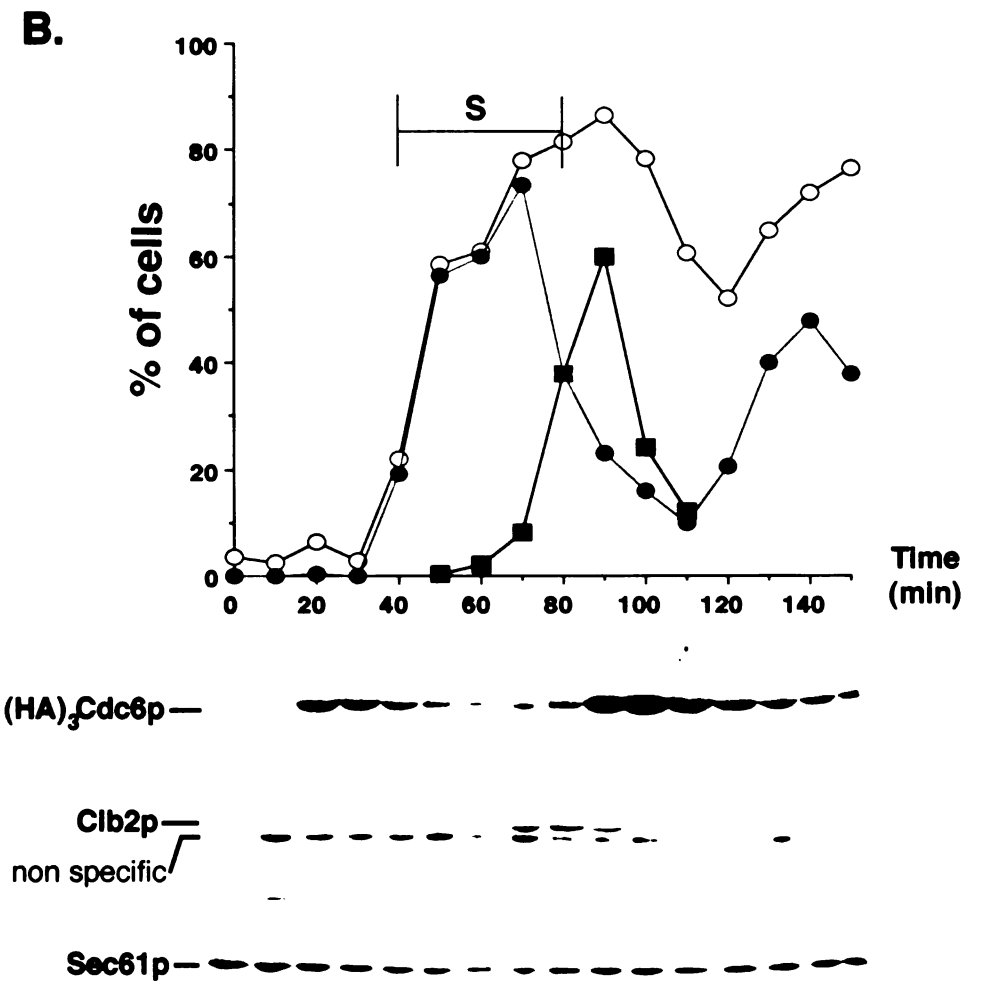
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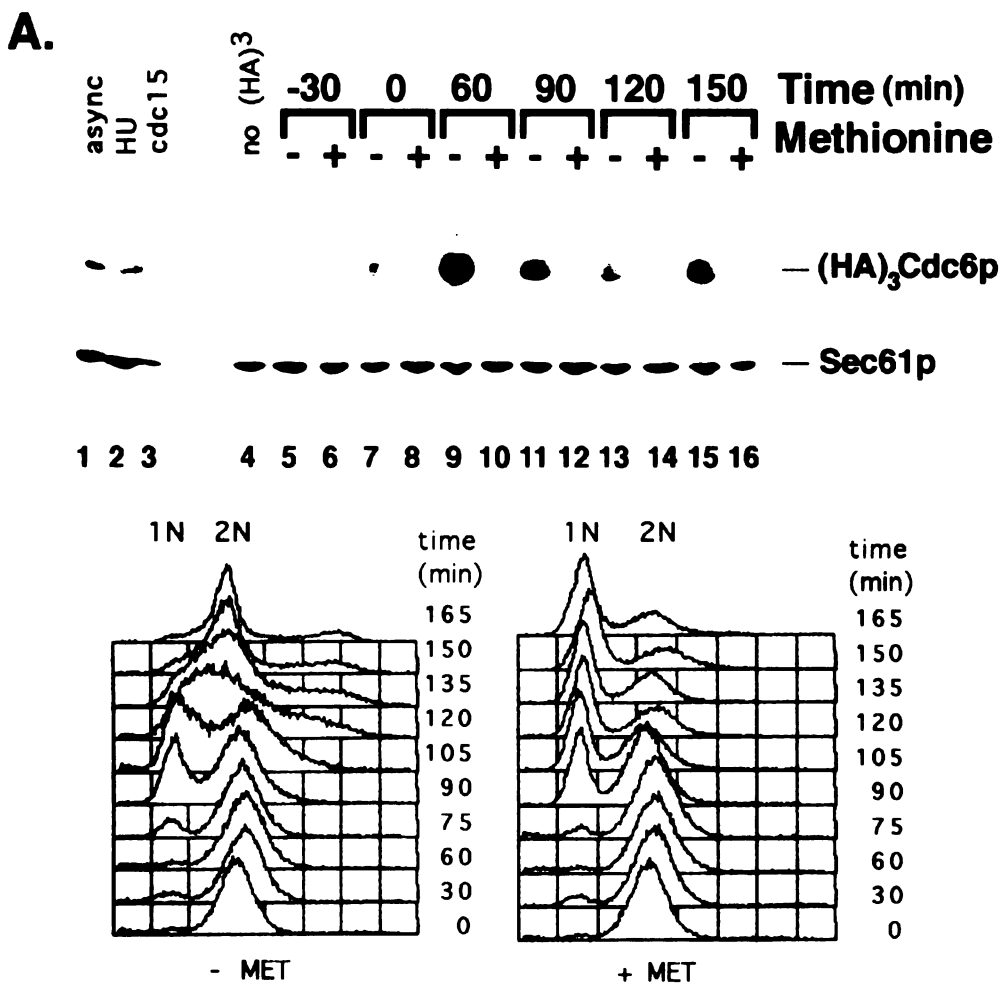
Clb2p
non specific
Sec61p

Fig 2-2. *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.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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
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time (min)
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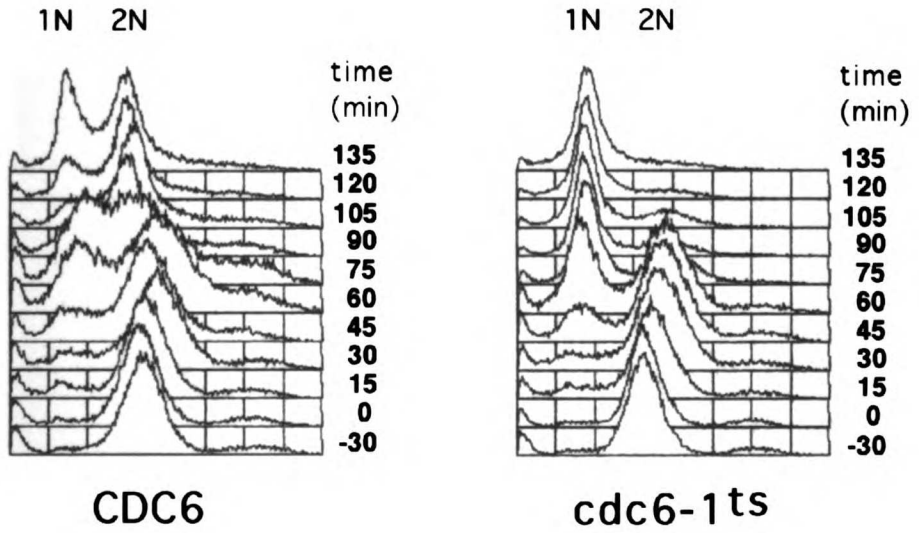
B.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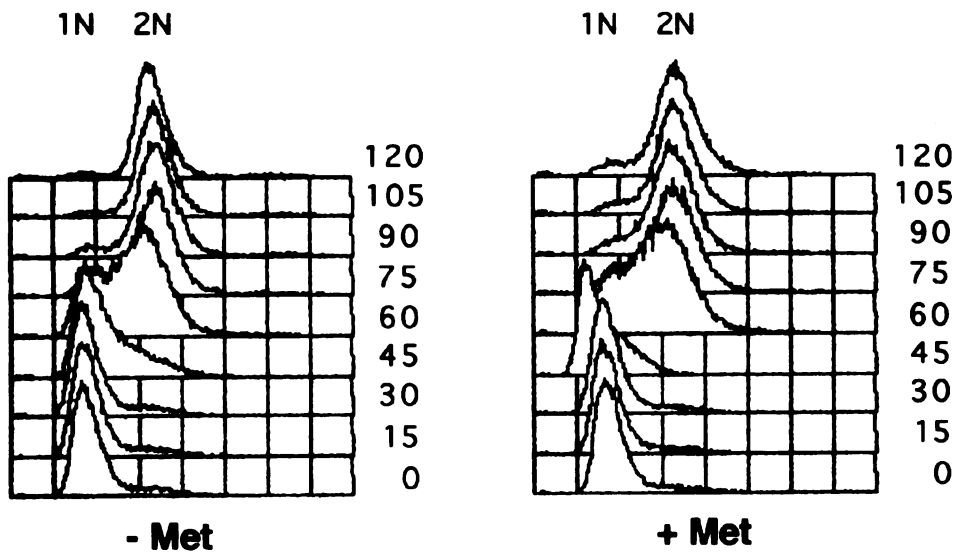
cdc6-1ts

Fig. 2-3. Depletion of Cdc6p at Start does not disrupt replication competence in G1 phase. A *pMET-(HA)3-CDC6* strain was arrested before Start by depletion of G1 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.

A.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+ Met

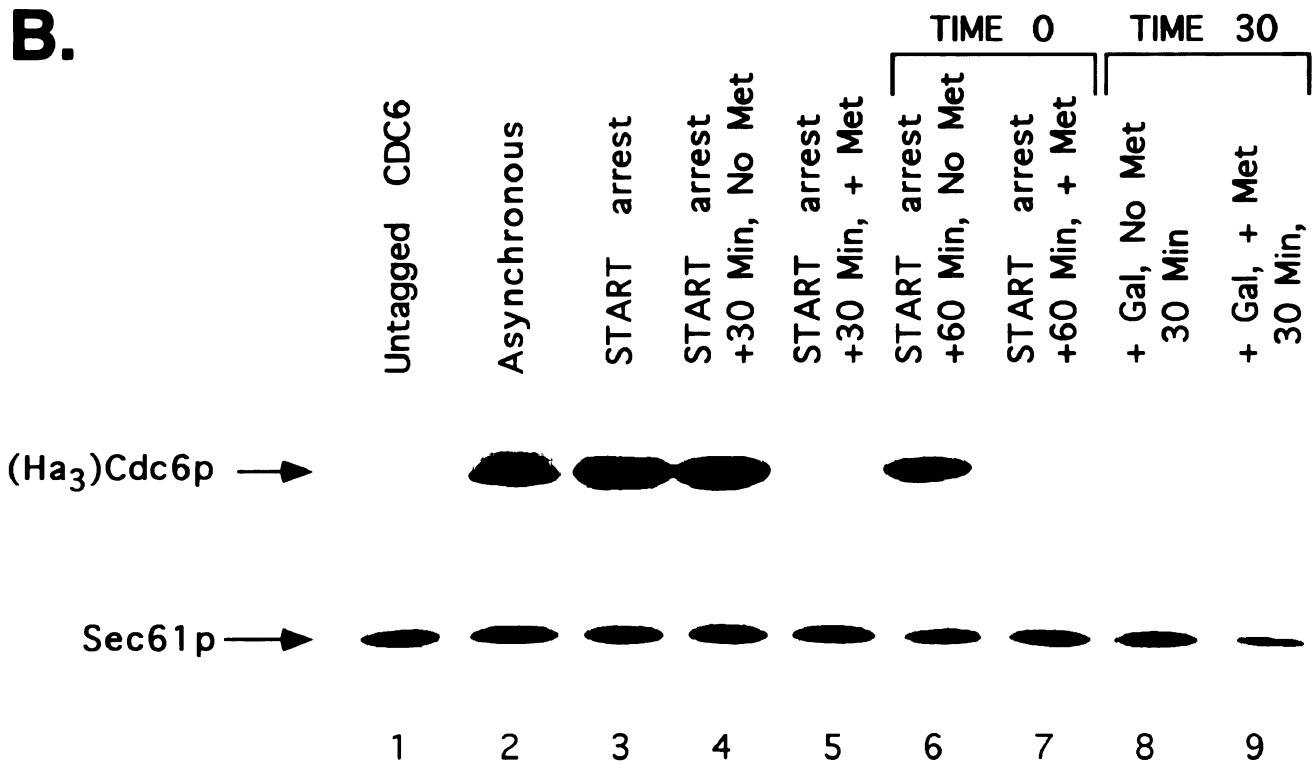

B.
TIME 0
TIME 30
Untagged CDC6
Asynchronous
START arrest
START arrest +30 Min, No Met
START arrest +30 Min, + Met
START arrest +60 Min, No Met
START arrest +60 Min, + Met
+ Gal, No Met 30 Min
+ Gal, + Met 30 Min,
(Ha3)Cdc6p
Sec61p
1
2
3
4
5
6
7
8
9

Fig. 2-4. *CDC6* is required to maintain replication competence in G1 phase. *CDC6*$^+$ and *cdc6-1* cells growing at 23°C were arrested at Start G1 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.

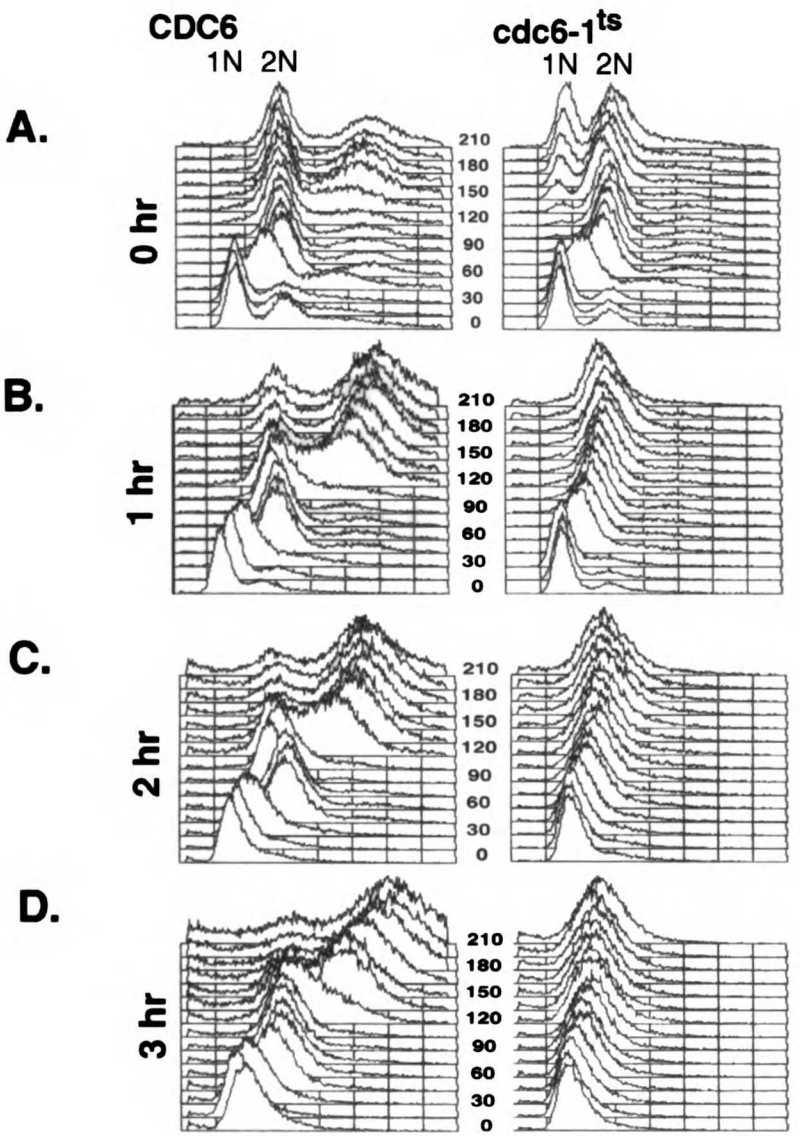
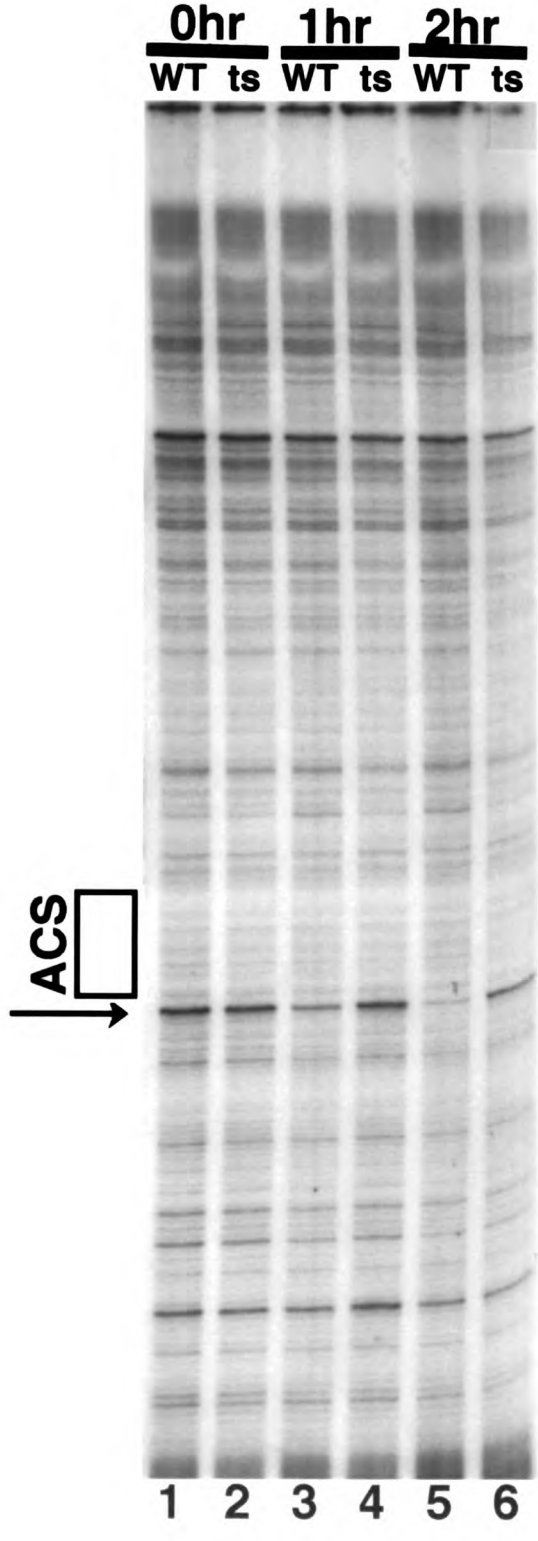

CDC6
1N 2N
cdc6-1ts
1N 2N
A.
0 hr
B.
1 hr
C.
2 hr
D.
3 hr
210
180
150
120
90
60
30
0

Fig. 2-5. *CDC6* is required for formation of the pre-replicative complex in G1 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.

A.



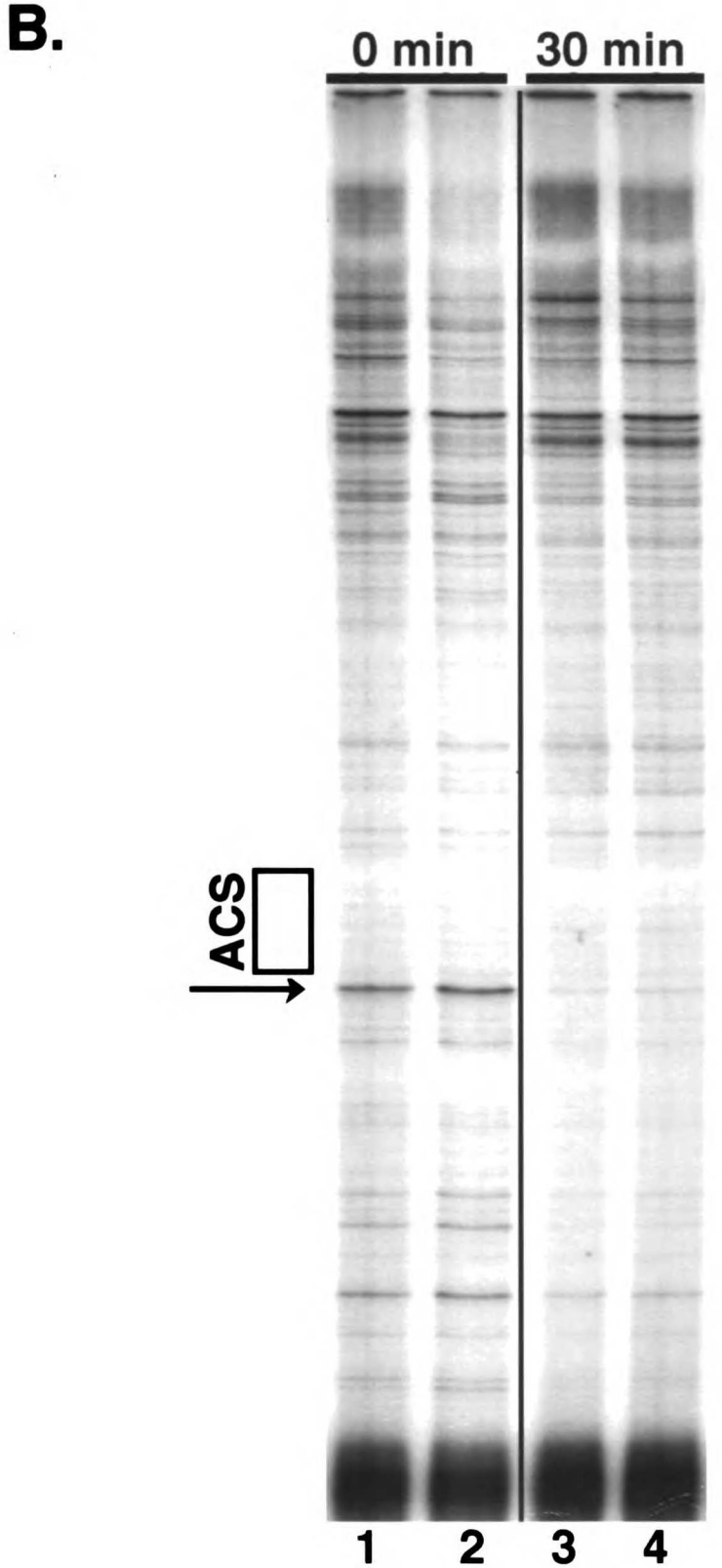

B.
0 min
30 min
ACS
1
2
3
4

Fig. 2-6. *CDC6* is required to maintain the pre-replicative footprint in G1 phase. *CDC6*$^+$ and cdc 6-1 cells growing at 23°C were arrested by depletion of G1 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.

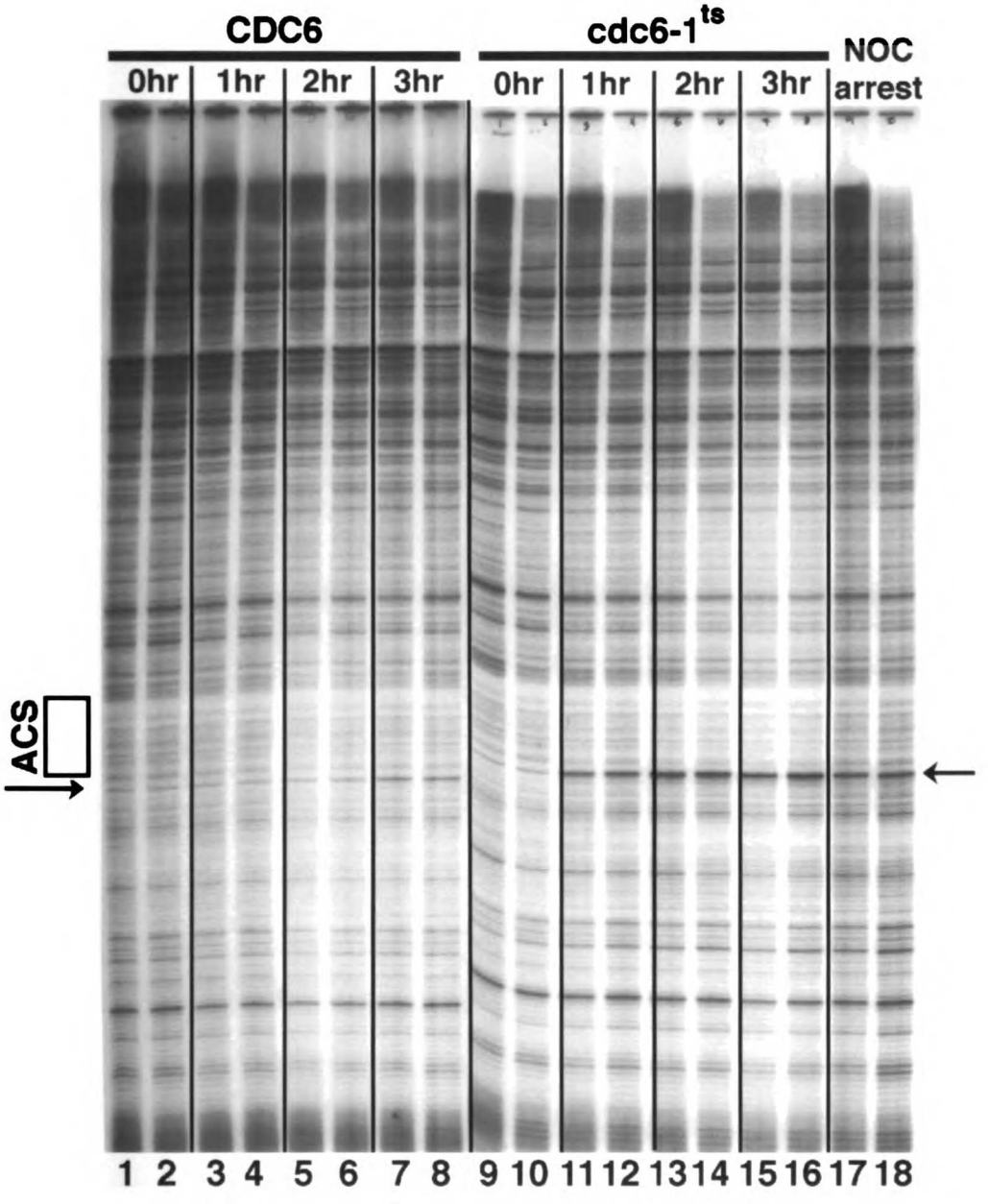

CDC6
cdc6-1ts
NOC
0hr
1hr
2hr
3hr
0hr
1hr
2hr
3hr
arrest
ACS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 2-7. 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).

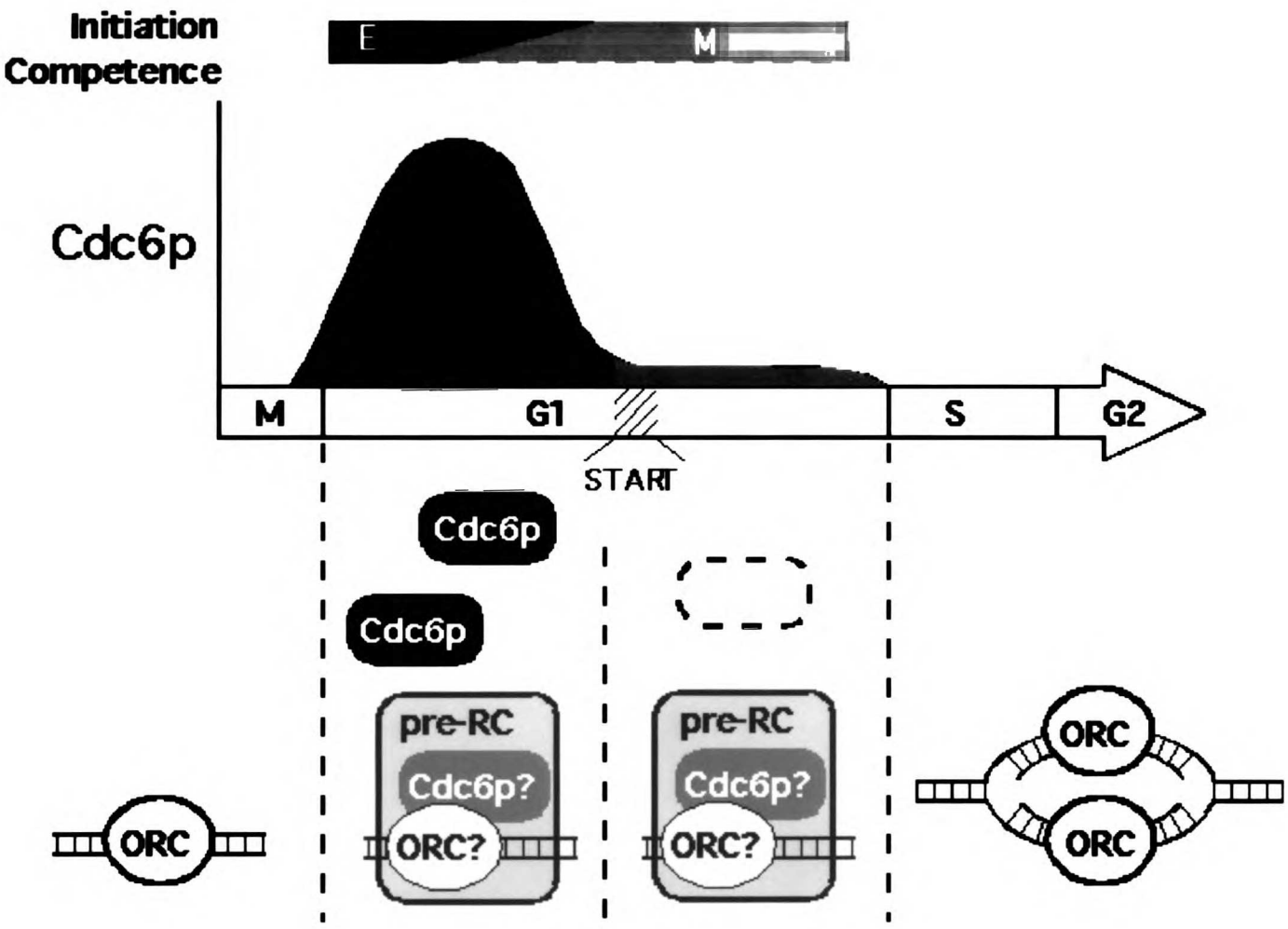

Initiation
Competence
E
M
Cdc6p
M
G1
S
G2
START
Cdc6p
Cdc6p
pre-RC
Cdc6p?
ORC?
pre-RC
Cdc6p?
ORC?
ORC
ORC
ORC

TABLE 2-1. Yeast strains used in this study

Strain	Genotype	Source
YJL310	*MAT**a** leu2-3,112 ura3-52 trp1-289 bar1::LEU2*	R. Deshaies
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::LEU2*	This study
YJL1475	*MAT**a** pMET-(HA)3-CDC6 cln1Δ::URA3 cln2Δ::LEU2 cln3Δ::URA3 ura3-52 trp1-289 l eu2::[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

CHAPTER 3

Ectopic Induction of Clb2 in Early G1 Phase is Sufficient to Block Pre-Replicative Complex Formation in *Saccharomyces cerevisiae*

Detweiler, C. S., and J. J. Li. 1998. Eptopic Induction of Clb2 in Early G1 phase is Sufficient to Block Pre-Replicative Complex Formation in *Saccharomyces cerevisiae*. PNAS, in press.

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, re-initiation 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 (CDKs) prevent re-initiation by blocking the repetition of an early step in the initiation reaction. In budding yeast, this regulation is thought to involve inhibition of pre-replicative 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 G1 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 re-assembly of the pre-RC, and hence re-initiation at an origin, is directly inhibited by S- and M- phase promoting CDKs.

INTRODUCTION

To ensure the stable propagation of their genome, eukaryotic cells replicate their DNA precisely once per cell cycle. Re-replication 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, re-initiation 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. 3-1A, 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 (11). Shortly after mitosis, a G1-specific genomic footprint appears at origins, suggesting the assembly of a pre-replicative complex (pre-RC) in early G1 in preparation for replication initiation (13). Although the exact identity of the proteins responsible for the pre-replicative footprint is unknown, several proteins required for initiation are suspected to be pre-RC components. Most strongly implicated is the initiation protein Cdc6, because it is required for both establishment and maintenance of the pre-replicative footprint (8, 10). 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) (4) and the MCM family of six sequence-related initiation proteins (22). While the

contribution of ORC and the MCM proteins to the pre-replicative footprint remains to be determined, their association with chromatin and origins in G1 suggests they too are part of the pre-RC (2, 39).

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, which only become active in late G1 after passage through START: the cyclin dependent kinase (CDK) Cdc28 in association with the B-type cyclins Clb1-6 (16, 35, 36), and Cdc7 kinase in association with its regulatory subunit Dbf4 (21). Sequential waves of Clb/Cdc28 kinases are activated during the cell cycle: Clb5 and 6 kinases are activated first in late G1 to promote S phase by triggering initiation (16, 36); Clbs1-4 kinases are activated later in S and G2 to promote mitosis (17, 31, 38). The distinction between S- and M- phase promoting Clb kinases, however, may be due more to their order of appearance rather substrate specificity, as members of each class can act redundantly to perform functions of the other (1, 35, 36). Hence, CDKs capable of promoting S-phase are continuously present from late G1 until the end of mitosis (Fig 3-1A, Upper), when the Clbs are destroyed (26).

Entry into S phase is tightly correlated with conversion of the pre-replicative genomic footprint to a smaller post-replicative footprint that is present at origins for the remainder of the cell cycle (13). This correlation suggests that the pre-RC is disassembled when initiation is triggered, leaving a post-replicative complex (post-RC) bound to origins (Fig. 3-1A, Upper). Like the pre-RC, the exact structure of the post-RC is unknown. However, the resemblance of the post-replicative genomic footprint to the *in vitro* footprint of purified ORC bound to origins indicates that ORC is a key component of the post-RC (4, 12, 33).

In addition to promoting replication by triggering initiation, cyclin dependent kinases have also been implicated in the block to re-replication. 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 (5, 9, 18, 19, 34). We note, however, that at least for the two yeasts, the cells appear to be slipping into the next cell cycle, entering G1 phase before they repeat S phase (5, 9, 19). Hence, these experiments do not address whether these CDKs block re-replication per se or merely prevent cells from re-entering G1 phase.

One way to distinguish between these possibilities would be to determine whether induction of Clb kinases in cells that had already entered G1 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 a prolonged delay in Cdc6 induction in cells progressing through G1 would preclude 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 (39) and to promote initiation (29), 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 re-assembly and to block re-initiation in S, G2 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 G1 before pre-RCs have formed. For this purpose we have placed Cdc6 and Clb2 under distinct regulatable promoters and thereby developed a system where we can 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 G1 cells and help confirm the idea that during the normal cell cycle re-initiation is directly blocked in S, G2 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 EcoR I genomic fragment containing *CDC6* was cloned into a pRS306 (37) derivative whose sole Not I 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 Bsg I to the newly introduced Not I site was replaced with a Sal I/Not I fragment containing the *MET3* promoter and a new translational start codon just upstream of the NotI site. pCD59 (*Metp-(HA)$_6$-CDC6*) was generated by inserting sequences encoding six tandem copies of the hemagglutinin epitope

(HA) in the Not I site of pCD29. Plasmid pCD25 (*Galp-clb2^{db}*) encodes an 80 amino acid N-terminal truncation of Clb2 under the control of the *GAL1* promoter (from pAR38; A. Rudner, D. Kellogg and A. Murray) cloned into pRS304 (37).

The wild-type *CDC6* gene in yJL310 (*MAT***a** *CDC6 bar1::LEU2 ura3 trp1*) was replaced by *Metp-CDC6* (pCD29) by 2-step gene replacement (4) to create YJL1184 (*MAT***a** *MET3-CDC6 bar1::LEU2 leu2 ura3 trp1*). pCD25 (*GalpClb2^{db}*)and pRS304 (vector control) were integrated at the TRP1 locus in yJL1184 to create, respectively, yJL1167 (*MAT***a** *Metp-CDC6 trp1::{Galp-clb2^{db},TRP1} bar1::LEU2 leu2 ura3*) and yJL1169 (*MAT***a** *Metp-CDC6 trp1::TRP1 bar1::LEU2 leu2 ura3*). *Metp-CDC6* in YJL1167 and 1169 was replaced by *Metp-(HA)$_6$-CDC6* by 2-step gene replacement to generate, respectively, YJL1172 (*MAT***a** *Metp-(HA)$_6$-CDC6 trp1::{Galp-clb2^{db},TRP} bar1::LEU2 leu2 ura3*) and YJL1173 (*MAT***a** *Metp-(HA)$_6$-CDC6 trp1::TRP1 bar1::LEU2 leu2 ura3*).

Media and synchronization. To allow expression from the *MET3* promoter, cells were grown in synthetic complete media lacking methionine (tryptophan was also left out). This media was supplemented with either 2% raffinose and 2% galactose (SCRG-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 supplemented with the appropriate sugars (YEPR or YEPRG). All cultures were grown at 30° C.

As illustrated in Fig. 3-1B, cells grown to a density of 0.5 x 10^7 cells mL^{-1} in SCR-Met,Trp were arrested with 50 ng mL^{-1} alpha factor (see Fig. 3-1B). These cells were filtered, washed, and resuspended in YEPR + 2 mM methionine (23) 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 G2, 50 ng mL^{-1} α-factor was added back to the cultures to rearrest the cells in the following G1 phase. Once the cells were rearrested, one of two induction protocols was followed. To induce $Clb2^{db}$ before Cdc6 (induction protocol 1), 2% galactose was added for 75 min, then the cultures were filtered, resuspended in SCRG-Met,Trp containing 50 ng/ml alpha factor, and incubated for an additional 30 min. To induce Cdc6 before $Clb2^{db}$ (induction protocol 2), the cultures were filtered and resuspended in SCR-Met,Trp containing 50ng/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 G1 arrest by addition of 0.1 μg mL^{-1} pronase (to digest the alpha factor) (3) and DNA content monitored by flow cytometry (10).

Genomic Footprinting. Genomic footprinting was performed as described (13) with minor modifications. The lysis was performed with 1-4 x 10^9 cells and the primer extensions (5-7 cycles) were performed using 0.5 μg of total genomic DNA and 1.5 pmoles of 5'-end labeled oligonucleotide.

Immunoblot Analysis. Protein extraction and immunoblot analysis was performed essentially as described (28).

RESULTS

$Clb2^{db}$ inhibits replication when expressed in G1 phase before Cdc6. In order to ask whether premature induction of Clb kinase activity in G1 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 G1 phase (Fig. 3-1A, Upper). Hence, we constructed strains where 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 (7) at the *CDC6* locus. By repressing Cdc6 expression we could obtain G1-arrested cells that had not yet formed pre-RCs at their origins (Fig. 3-1A, Lower). This strain also harbors, in addition to the endogenous *Clb2* gene, a truncated version of *CLB2* (*clb2^{db}*) under the control of the galactose-inducible *GAL1* promoter. The protein encoded by the truncated *clb2^{db}* allele lacks the N-terminal cyclin destruction box of Clb2. This truncation allows Clb2^{db} protein to accumulate at a pre-Start arrest, when the full length protein is normally degraded (1). Clb2^{db} apparently accumulates at high enough levels under the *GAL1* promoter to activate Clb kinase activity (1), despite the presence of the CDK inhibitor Sic1 (14, 35) at this point in G1. As a control for the absence of premature Clb kinase activity, YJL1169, which contains *Met3p-CDC6* but lacks *Gal1p-clb2^{db}*, was also constructed.

Fig. 3-1B shows the experimental protocol used to obtain G1 cells that had not assembled pre-RCs at their origins. YJL1167 and YJL1169 cultures grown in SR-Met,Trp (conditions that induce Cdc6 and repress Clb2^{db}) were first arrested in G1 phase of the preceding cell cycle with the mating pheromone α-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). While 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 (8, 30). 90 min into the release almost all the cells had recovered from the initial arrest but had not yet entered mitosis. At this point, α factor was added back to the cultures to collect the Cdc6-depleted cells before START in the following G1 phase.

Once the cells were rearrested, they were subjected to one of two induction protocols (Fig. 3-1B). In the protocol 1, galactose was added first for 75 min to induce (or mock induce) $Clb2^{db}$. The cells were then transferred to methionine-free medium (containing galactose and α-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 (using pronase to digest alpha factor) and monitoring DNA content by flow cytometry every 30 min. YJL1167, which expresses $Clb2^{db}$ upon galactose induction, maintained a nearly 1C DNA content 90 min after arrest release (Fig. 3-1C, column b). In contrast, the control strain YJL1169, which does not express $Clb2^{db}$, replicated and acquired a 2C DNA content within 60 min following the release (Fig. 3-1C, column a). These data indicate that premature expression of $Clb2^{db}$ in G1 phase before Start can inhibit replication.

Induction protocol 2 (Fig. 3-1B) was used to show that the order of $Clb2^{db}$ 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 $Clb2^{db}$ was added 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. 3-1C, column c).

We presume that $Clb2^{db}$ 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.

$Clb2^{db}$ induction prior to Cdc6 inhibits pre-RC formation. Because Cdc6 is required for pre-RC formation (8, 10), 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 2μ origin by DNaseI genomic footprinting at the very beginning and end of induction protocol 1. The most distinctive feature of the post-replicative footprint is the presence of an ORC-induced hypersensitive site near the ORC recognition site (Fig. 3-2, arrow); the absence of this site, on the other hand, is characteristic of the pre-replicative footprint (13). Fig. 3-2 shows that G1-arrested YJL1167 and YJL1169 cells that had been depleted of Cdc6 retained the post-RC footprint as expected (lanes 1,2,5,6) (8, 10) . Induction of Cdc6 expression following mock induction of $Clb2^{db}$ in YJL1169 led to formation of the pre-RC (Fig. 3-2, lanes 3,4). However, when $Clb2^{db}$ was induced prior to Cdc6 expression in YJL1167, the pre-RC failed to form (Fig. 3-2, lanes 7,8). Hence, once $Clb2^{db}$ 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 G1 arrest (data not shown). Thus, we conclude that premature induction of $Clb2^{db}$ can inhibit replication by preventing the Cdc6 dependent formation of pre-RCs.

Inhibition of Pre-RC formation by Clb2^{db} is not mediated by inhibition of Cdc6 induction. One trivial way in which Clb2^{db} 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 post-transcriptional controls (10), possibly through the activity of Clb/Cdc28 kinase. We therefore examined the induction of Cdc6 protein in our experiments by immunoblot analysis.

For this purpose, the *Metp-CDC6* allele in yJL1167 and yJL1169 was replaced with *Metp-(HA)$_6$-CDC6*, to generate YJL1172 and YJL1173 respectively. This new allele expresses Cdc6 tagged with 6 copies of the hemagglutinin epitope (HA). Like the untagged protein, delayed induction of (HA)$_6$-Cdc6 at a pre-Start arrest could make cells competent to replicate their DNA, but not if Clb2^{db} were induced first (data not shown). Despite this difference, (HA)$_6$-Cdc6 accumulated to similar levels in both the presence (YJL1172) and absence (YJL1173) of Clb2^{db} when cells were shifted to methionine-free medium (Fig. 3-3). Furthermore, when methionine was added back to these cultures to repress *Metp-(HA)$_6$-CDC6* transcription, the tagged protein disappeared at roughly equivalent rates in the presence or absence of Clb2^{db} (Fig. 3-3). This result indicates that the stability of (HA)$_6$-Cdc6 was not significantly affected by Clb/Cdc28. Hence, the block to pre-RC formation caused by premature Clb2^{db} expression before Cdc6 induction cannot be attributed to destabilization or insufficient accumulation of Cdc6.

DISCUSSION

Experiments in a number of organisms have led to a model (Figure 3-4) of how replication initiation is triggered and how re-initiation is prevented during each cell cycle. This model rests on two major premises. The first premise is that the initiation reaction can be divided into at least two steps (13): assembly of a pre-replicative complex at the origin and the activation of this complex to trigger initiation. The second premise is that S- and M-phase promoting CDKs affect these steps in opposite ways; they can inhibit the first and promote the second. As cells enter G1, 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 re-initiation. Only at the completion of M phase, when Clbs are destroyed (26), 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 Figure 3-4). 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 re-entry into S phase. However, whether they do so directly by preventing re-initiation 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 CLBs, 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 G1 phase so that the effect of CDKs on G1 entry would not be an issue. Ectopic induction of $Clb2^{db}$, which leads to premature activation of a mitotic Clb kinase (1), 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 G1 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, G2 and M phase to prevent re-initiation. Additional support for this conclusion has recently been obtained in the *Xenopus in vitro* replication system, where both cyclinE/cdk2 and cyclinA/cdc2 have been shown to block loading of MCM proteins onto chromatin and to inhibit initiation, if added early in the reaction (20).

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 (13) (or when a replication fork passes through the origin); this disassembly insures that re-

initiation depends on re-assembly 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 G1 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 $Clb2^{db}$ to prevent initiation when it is induced after Cdc6 is expressed and pre-RCs have assembled (Fig. 3-1C 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 re-replication. 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 (6, 15, 24, 29). Interestingly, overexpression of Cdc18 in *S. pombe* leads to multiple rounds of re-replication within a cell cycle (24, 25, 27). 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 re-replication.

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 re-replication. Overexpression of wild-type Cdc6 or a mutant version lacking the its N-terminal CDK interaction domain (15) does not result in re-replication (32). A similar negative result is also observed with overexpression of a mutant Cdc6 lacking the CDK

consensus phosphorylation sites (C. Detweiler, data not shown). These results suggest that, if Cdc6 is targeted by Clb kinase to prevent re-replication, 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 $Clb2^{db}$ is prematurely expressed or the block to re-initiation 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 (4, 39) and several of them appear to be *in vivo* substrates of the Clb kinases (M. Young and B.-K. Tye, personal communication, and J. Li, 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 re-replication.

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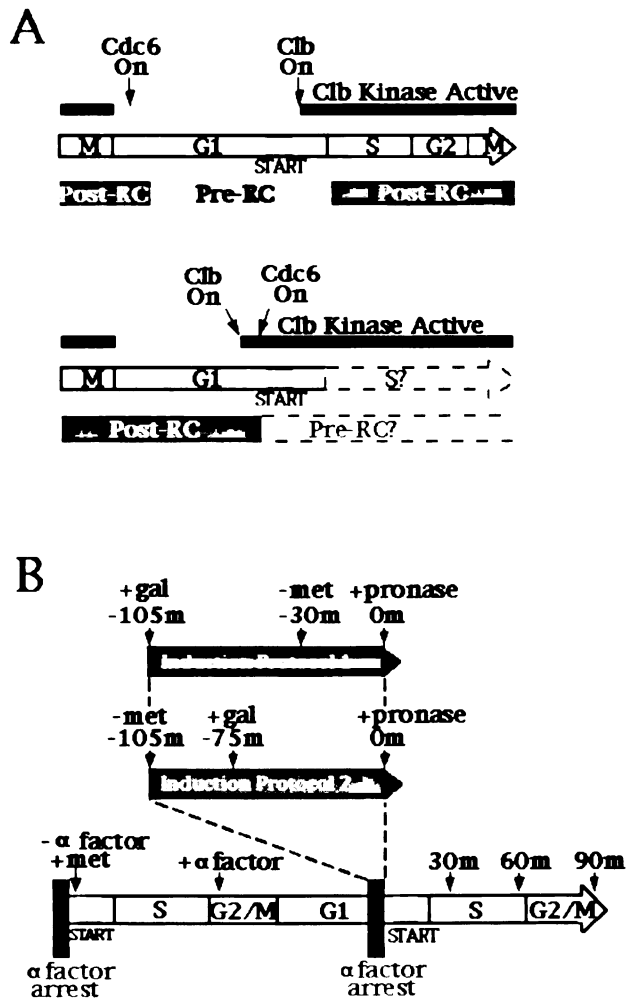
Figure 3-1. Induction of Clb2^{db} in G1 before Cdc6 expression is sufficient to block DNA replication. (A) Comparison of events in normal cell cycle (upper) and experimentally manipulated cell cycle (lower). In the normal cell cycle, disappearance of Clb kinase activity and induction of Cdc6 after mitosis 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-clb2db and Met3p-CDC6 cells were released from alpha factor arrest into medium containing methionine. When most of the cells were in G2, alpha factor was added back to re-arrest 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). Follwing these inductions, cells released from the arrest by addition of pronase (Om). (C) Flow cytometry of cells sampled at times indicated by minutes (m) in panel B. Column a: YJL1169 (*Metp-CDC6*) induction protocol 1. Column b: YJL1167 (*Metp-CDC6, Galp-clb2^{db}*), induction protocol 1. Column c: YJL1167 (*Metp-CDC6, Galp-clb2^{db}*), induction protocol 2.


A
Cdc6
On
Clb
On
Clb Kinase Active
M
G1
S
G2
M
START
Post-RC
Pre-RC
Post-RC
Clb
On
Cdc6
On
Clb Kinase Active
M
G1
S?
START
Post-RC
Pre-RC?
B
+gal
-105m
-met
-30m
+pronase
0m
-met
-105m
+gal
-75m
+pronase
0m
Induction Protocol 2
- α factor
+met
+α factor
30m
60m
90m
S
G2/M
G1
S
G2/M
START
START
α factor
arrest
α factor
arrest

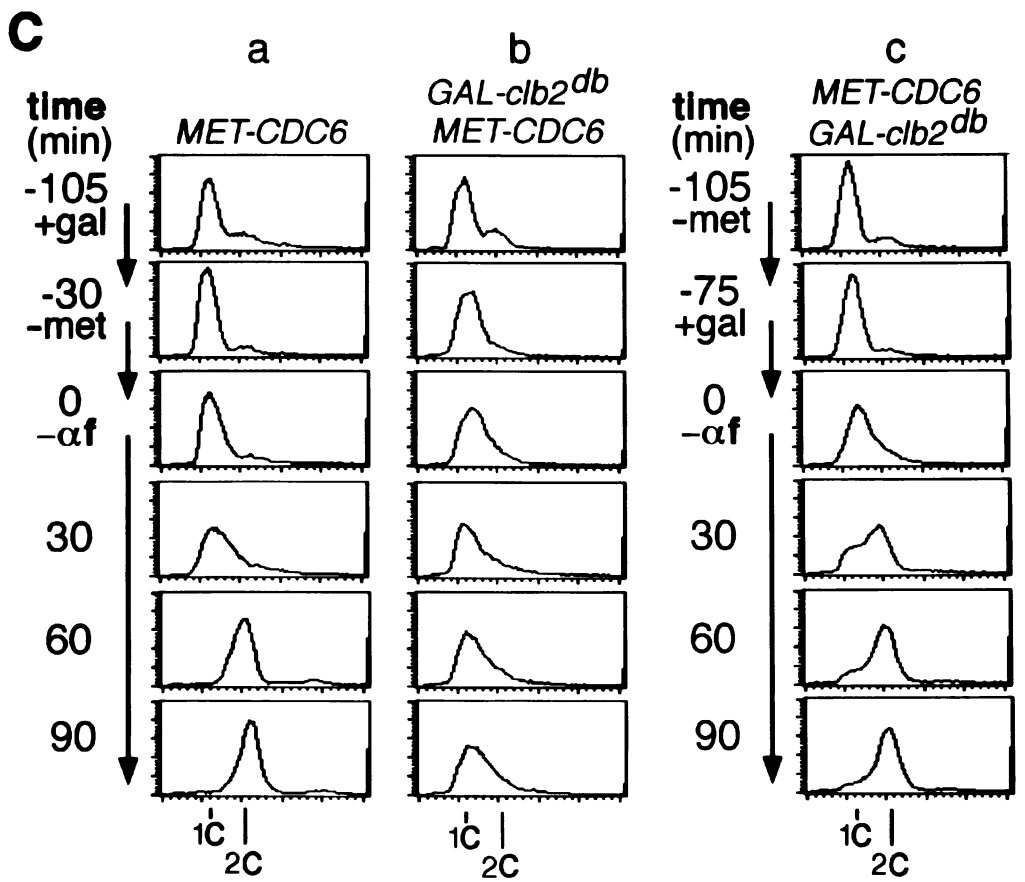

C
a
b
c
time
(min)
MET-CDC6
GAL-clb2db
MET-CDC6
MET-CDC6
GAL-clb2db
-105
+gal
-30
-met
0
-αf
30
60
90
-105
-met
-75
+gal
0
-αf
30
60
90
1C
2C
1C
2C
1C
2C

Figure 3-2. Induction of Clb2^{db} in G1 before Cdc6 expression is sufficient to block pre-RC formation. *Metp-CDC6* (yJL1169, lanes 1-4) and *Metp-CDC6 Galp-clb2^{db}* (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^{db} induction (lanes 1,2,5,6) and after Cdc6 induction (lanes 3,4,7,8). Each sample was treated with two different concentrations of DNaseI and loaded in adjacent lanes. The ORC-induced hypersensitive site characteristic of the the post-replicative footprint is indicated by the arrow.

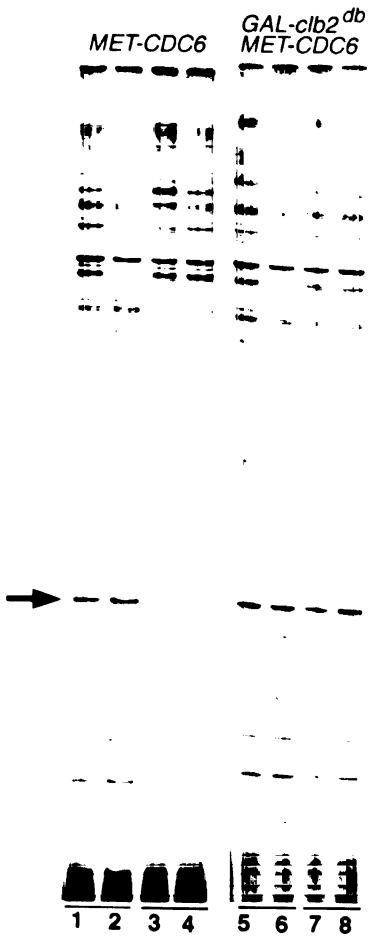
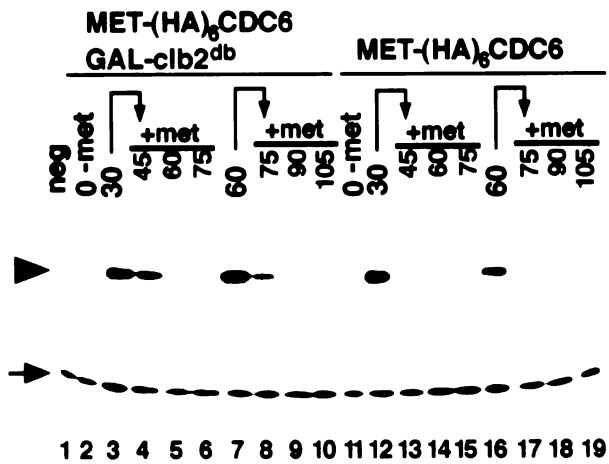

MET-CDC6
GAL-clb2 db
MET-CDC6
1 2 3 4 5 6 7 8

Figure 3-3. $Clb2^{db}$ does not inhibit Cdc6 by preventing Cdc6 accumulation. *Metp-(HA)$_6$-CDC6 GAL-clb2^{db}* (yJL1172, lanes 2-10) and *Metp-(HA)$_6$-CDC6* (yJL1173, lanes 11-19) cells were subjected to induction protocol I as depicted in Fig. 1B but were not released from the arrest so that $(HA)_6$-Cdc6 accumulation (arrowhead) could be monitored by immunoblotting (lanes 2,3,7 and lanes 11,12,16). 30 and 60 min after induction of the $(HA)_6$-Cdc6, 2 mM methionine was added back to a portion of the cultures to repress *(HA)$_6$-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 SR-Leu. Sec61 was immunoblotted as an internal loading control (arrow).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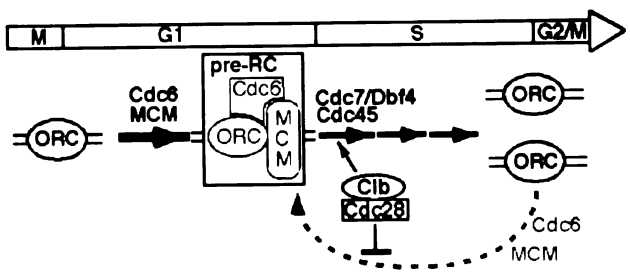
GAL-clb2db
MET-(HA)6CDC6
neg
0
-met
30
+met
45
60
75
60
+met
75
90
105
0
-met
30
+met
45
60
75
60
+met
75
90
105
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 3-4. A model for Clb/Cdc28 kinase inhibition of re-replication. In G1 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 G1 triggers replication initiation and disassembly of the pre-RCs. The continual presence of Clb/Cdc28 during S, G2, and M phases also blocks re-assembly of the pre-RC, preventing re-initiation until the Clbs are destroyed at the end of mitosis.


M
G1
S
G2/M
pre-RC
Cdc6
Cdc6
MCM
ORC
ORC
M
C
M
Cdc7/Dbf4
Cdc45
ORC
ORC
Clb
Cdc28
Cdc6
MCM

CHAPTER 4

Attempt to Determine if CLB/CDK can Directly Inhibit Cdc6

ABSTRACT

Clb/CDK can inhibit Pre-RC formation in G1 phase cells (Chapter 3). We attempted to determine if the target of Clb/CDK is Cdc6, a protein that is a likely component of the Pre-RC. Our approach involved constructing mutants of Cdc6 that we predicted would be insensitive to Clb/CDK inhibition. We thought that such mutants may promote re-replication upon over -expression and/or allow Pre-RC formation in the presence of Clb/CDK. We found no evidence of the former and were unable to determine the latter for technical reasons.

INTRODUCTION

In the previous chapter we showed that Clb/Cdc28 kinase can block pre-RC formation when ectopically induced before Cdc6. This suggests that Clb/Cdc28 kinase may directly inhibit Cdc6, and that Clb/Cdc28 kinase might contribute to a block to re-replication in S, G2 and M phase by inhibiting Cdc6 in a normal cell cycle. We tried to test this model by engineering mutant forms of Cdc6 that we predicted would be insensitive to the kinase. The first Cdc6 mutant had the five Cdc28 consensus phosphorylation sites (S/TXPK/R) altered, whereas the second mutant lacked the domain of Cdc6 that is required for the Cdc6 interaction with Cdc28 *in vitro* (8). We tested these mutants for sensitivity to Clb/Cdc28 kinase in two distinct ways.

First, we asked if expression of the mutant proteins in a wildtype background promotes over replication. The rationale was that if Clb/Cdc28 inhibition of Cdc6 is important for preventing over-replication, expression of a Cdc6 mutant that is insensitive to Clb/Cdc28 would promote over-replication. We thought it important to express the mutant protein from a strong, constitutive promoter in order to maintain high levels of the protein

throughout the cell cycle; Cdc6 is normally expressed at high levels in early G1 phase, and degraded at the G1/S transition (7). However, wildtype Cdc6 is not limiting for replication, since over-expression of Cdc6 does not promote over replication in S. cerevisiae (4, 11) indicating that there is at least one other mechanism of preventing re-replication. If the second mechanism for preventing re-replication involves Clb/Cdc28 kinase inhibition of Cdc6, then over expression of the Cdc6 mutants would be expected to promote over-replication.

The second approach we used to ask if Cdc6 could be inhibited by Clb/Cdc28 kinase involved reversing the relative timing of Clb kinase and Cdc6 expression in G1 phase (Chapter 3). Normally, Cdc6 is induced in early G1 phase and CLB is induced in late G1 phase. In the experiments we ectopically induced $clb2^{db}$ (a stable form of Clb2) before Cdc6, and then asked if, upon release from G1 phase, DNA replication could occur. By using this assay we could isolate the effect of Clb/Cdc28 kinase on Cdc6 dependent events, since in the experiment, G1 phase events that were sensitive to Clb/Cdc28 kinase but that were Cdc6 independent could occur before $clb2^{db}$ was induced.

MATERIALS AND METHODS

Plasmid Construction

Cdc6-5a and cdc6-5d were generated by site directed oligo mutagenesis using a single-stranded phage CDC6 template. Fragments containing the mutated sequences were subcloned into either pJL496 or pCD3 and assayed by restriction digest and sequencing to generate pWG6 and pWG7.
The oligos used to generate cdc6-5a are as follows (lower case nucleotides indicate the introduced mutations):

oJL406(MunI)(CGCTTAGTTGGAGcaATTGGTATAGCTGAC),

oJL412(EagI)(AGGTCGTGGAGGatccGCTGGAGCATCGTCAAATAG),

oJL408(NarI)(CTGCAGTTTTTCTGGcGccGATTCTGGTGTAAC),

oJL414(NheI)(GATCCTCTTGTTGGatctAGaAGAAACCGCTTTTC),

oJL415(NarI)(CGATTTCTTTACCGGAtccGTAGTTGGCGTCAAAG)

The oligos used to generate cdc6-5d are as follows:

oJL411 (MunI) CGCTTAGTTGGAtcaATTGGTATAGCTGAC

oJL412 (BamHI) AGGTCGTGGAGGatccGCTGGAGCATCGTCAAATAG

oJL413 (BspEI) CTGCAGTTTTTCcGGatcTGATTCTGGTGTAAC

oJL414 (XbaI) GATCCTCTTGTTGGatctAGaAGAAACCGCTTTTC

oJL415 (BamHI) CGATTTCTTTACCGGAtccGTAGTTGGCGTCAAAG

Plasmids constructed by Wendy Gilbert include pWG30 (Galp-HA3cdc6-5a) pWG32 (Galp-HA3) and pWG31(Galp-cdc6-5d). Plasmids constructed by Nataliya Libina include pNL1101(Galp-HA3CDC6).

The pCD31(Metp-cdc6-5a, URA3) integrating plasmid was generated by cutting pCD29 (Metp-CDC6, (5)) with Not1 and Bcl1 and isolating the 7.4 kb fragment, into which the 1.3 kb Not1-Bcl1 fragment of pWG6 was inserted.

The pCD74(Metp-ubi-cdc6-5a, URA3) integrating plasmid was generated by PCR (5' primer, GTATGCGGCCGCCAGATTTTCGTCAAGACTTTGACCG; 3' primer, GATAGCGGCCGCTATGCATGGTAGCGGTCATCCTACCACCTCTT AGCCTTAG) the ~204 bp ubiquitin fragment of pPW66R (6), cutting it with NotI, and cloning it into pCD31 (Metp-cdc6-5a) at the NotI site upstream of the cdc6-5a open reading frame.

The integrating plasmids pCD78/79(Metp-Δntcdc6, URA3) and pCD80/81 (Metp-Δntcdc6-2a, URA3) were constructed by replacing the 146 bp NotI-PstI fragment of pCD4 (CDC6, C, A, LEU2 (5)) with two hybridized oligos (CGTTGCGGCCGCCTGCAGTTGC and GCAACTGCAGGCGGCCGCAACG)

that had been cleaved with NotI and PstI to generate pCD76 (Δntcdc6, C, A, LEU2). PCD76 was cut with NotI and SphI and the 556 bp fragment was cloned into the 8142 bp fragment of either pCD29 (Metp-CDC6) or pCD31 (Metp-cdc6-5a) cut with NotI and SphI to generate pCD78/79 and pCD80, respectively.

The pCD84 (Metp-ubi-Δntcdc6, URA3) and pCD82/83 (Metp-ubi-Δntcdc6-2a, URA3) integrating plasmids were generated by inserting the NotI-cleaved ubiquitin PCR product (described above) into the NotI sites of, respectively, pCD78(Metp-Δntcdc6) and pCD80(Metp-Δntcdc6-2a).

The pCD98/99/100/101 2 micron plasmids were generated by cutting pJL1101, with SpeI and inserting the 2.6 kb fragment into pRS426 (14) at the SpeI site. In pCD98/99, cdc6 is transcribed in the opposite direction as URA3. In pCD100/101, cdc6 is transcribed in the same direction as URA3.

Strain Construction

yCD252/254 (*MAT***a** *Metp-cdc6-5a trp1::TRP1 bar1::LEU2 leu2 ura3*) were generated by a 2-step gene replacement of the *Metp-CDC6* gene by *Metp-cdc6-5a* (pCD31) in yCD236 (Chapter 3).

yCD246/257/258 (*MAT***a** *Metp-cdc6-5a trp1::{Galp-clb2^{db},TRP1} bar1::LEU2 leu2 ura3*) were generated by a 2-step gene replacement of the *Metp-CDC6* gene by *Metp-cdc6-5a* (pCD31) in yCD233 (Chapter 3).

yCD300/301 (*MAT***a** *Metp-ubi-cdc6-5a trp1::TRP1 bar1::LEU2 leu2 ura3*) were generated by a 2-step gene replacement of *Metp-cdc6-5a* in yCD254 with *Metp-ubi-cdc6-5a* (pCD74).

yCD304/305 (*MAT***a** *Metp-ubi-cdc6-5a trp1::{Galp-clb2^{db},TRP1} bar1::LEU2 leu2 ura3*) were generated by a 2-step gene replacement of *Metp-cdc6-5a* in yCD257 with *Metp-ubi-cdc6-5a* (pCD74).

yCD306 (*MAT***a** *Metp-Δntcdc6 trp1::TRP1 bar1::LEU2 leu2 ura3*) was generated by a 2-step gene replacement of *Metp-CDC6* in yCD235 (Chapter 3) with *Metp-Δntcdc6* (pCD79).

yCD309 (*MAT***a** *Metp-Δntcdc6 trp1::{Galp-clb2db, TRP1} bar1::LEU2 leu2 ura3*) was generated by a 2-step gene replacement of *Metp-CDC6* in yCD233 (Chapter 3) with *Metp-Δntcdc6* (pCD79).

yCD312 (*MAT***a** *Metp-Δntcdc6 2a trp1::TRP1 bar1::LEU2 leu2 ura3*) was generated by a 2-step gene replacement of *Metp-cdc6-5a* in yCD254 with *Metp-Δntcdc6 2a* (pCD80).

yCD318 (*MAT***a** *Metp-Δntcdc6-2a trp1::{Galp-clb2db, TRP1} bar1::LEU2 leu2 ura3*) was generated by a 2-step gene replacement of *Metp-cdc6-5a* in yCD257 with *Metp-Δntcdc6 2a* (pCD80).

Media and Manipulations

YEP and synthetic media (S) with amino acids added (C) (9) were supplemented with dextrose (D), galactose (G), and/or raffinose (R) as indicated to a final concentration of 2%.

Experiments in which Galp-CDC6 was induced were performed as follows: Cells were grown to log phase in SR-U and arrested in G1 by the addition of alpha factor (50 ng/mL) for 2.5 hours. CDC6 constructs were induced by the addition of galactose (2% final) for 30 minutes. Cells were then released from G1 arrest by filtration and resuspension in YEPRG with nocodazole (20ug/mL). DNA content was monitored at 30 min intervals for 5 h and the footprint pattern at the 2 micron origin of replication was assessed 3 hours after release.

To arrest Metp-CDC6 dependent cells in G1 phase, alpha factor was added (50 ng/mL) to log phase cells in SRC-MLT. When greater than 95% of

cells were unbudded (approximately 2 hrs), samples were processed for footprinting. DNA content analysis confirmed that G1 phase arrest was achieved with this procedure.

Experiments in which clb2^{db} was ectopically induced in G1 phase before Cdc6 were performed as previously described (Chapter 3 and Fig. 4- 4). Briefly, log phase cells were alpha factor (50 ng/mL) arrested in the absence of methionine, and released from the arrest in the presence of methionine. After most of the cells had recovered from the arrest (approximately 70% budded), alpha-factor was again added to arrest cells in the next G1 (-105 min); the cells had progressed through a complete cell cycle in the presence of methionine, ideally depleting Cdc6. At -105 min, galactose was added, followed 75 min later by the removal of methionine (-30 min). Cells were then released from alpha-factor arrest at time 0 and monitored for DNA content at 30 min intervals.

Footprinting, FACS analysis and Immunoblotting

Samples were processed for footprinting as previously described (3). FACS analysis was performed as previously described(5). Immunoblotting was carried out as previously described (5).

RESULTS

Over-expression of cdc6-5a does not promote re-replication or detectable pre-RC formation

In the hopes of preventing potential Cdc6 phosphorylation by Clb/Cdc28 kinase, we (Wendy Gilbert and I) constructed a mutant form of CDC6, cdc6-5a, in which the five CDC28 consensus phosphorylation sites (S/TPXK/R) have been mutated to alanine. The equivalent aspartic acid mutant was also made (cdc6-5d) as a control for protein charge. We cloned an

epitope version of each mutant ((HA)^3-cdc6-5a or (HA)^{3}cdc6-5d) downstream of the highly inducible Gal promoter and transformed the constructs into a wildtype yeast strain (yJL310) on low copy plasmids to create the strains yCD238-241. As controls, we used both wildtype CDC6 (GAL-(HA)^{3}CDC6) and the vector (GAL-(HA)^3) to create the strains yCD242-245. All eight of these strains formed colonies of similar sizes when grown on dextrose, but on galactose, strains carrying (HA)^{3}CDC6 formed smaller colonies than strains with (HA)^{3}cdc6-5a, (HA)^{3}cdc6-5d, or (HA)^3 for reasons we do not understand (Fig. 4-1).

To determine if over expression of cdc6-5a or cdc6-5d could promote over replication in a wildtype background, the mutant constructs were induced in synchronized cells and DNA replication was monitored (Fig. 4- 2). Strains (yCD238, yCD240, yCD242, yCD244) containing a galactose inducible wildtype or mutant CDC6 were grown to log phase in the absence of galactose and were then arrested in G1 phase with alpha factor. The CDC6 constructs were induced for 30 minutes by the addition of galactose, after which cells were released from the G1 phase arrest and allowed to progress to G2/M phases. DNA content was monitored at hour intervals for 5 hrs, (Fig. 4-2A) and the footprint pattern at the 2 micron origin of replication was assessed 3 hours after release (Fig. 4-2B).

Induction of the (HA)^{3}CDC6 wildtype and mutant constructs had no discernible effect on DNA content or the kinetics of S phase; no obvious over-replication was observed relative to the vector control. Three hours after release from alpha factor, footprint analysis revealed a hypersensitive site, characteristic of a post-RC, at the 2 micron origin in all four strains (Fig. 4-2B, lanes 1-8), indicating that inappropriate G1 footprint formation did not occur. However, the *in vivo* footprinting assay is not sensitive enough to detect a

small number of Pre-RCs (13). An independent lab obtained similar results upon Gal promoter induction of wildtype CDC6 (12). We did not determine in our experiments, whether the CDC6 mutants were, in fact, expressed upon induction, but the plasmids in question have been shown (by anti-HA immunoblotting) to produce products with the predicted SDS-page gel mobility upon induction (10). We conclude that over expression of CDC6, cdc6-5a, or cdc6-5d has no obvious effect on either DNA replication or complex formation at origins.

Attempt to determine the effect of ectopic clb2^{db}expression on cdc6-5a

In order to determine whether cdc6-5a is insensitive to ectopic Clb induction in G1, we constructed strains (yCD252, 254, 246, 257, 258) with a single copy of cdc6-5a under a promoter that is repressed in the presence of methionine; these strains lack a wildtype copy of CDC6. We found that Metp-cdc6-5a dependent strains formed larger colonies but had similar DNA content profiles as Metp-CDC6 strains (Fig. 4-3A). Metp-cdc6-5a dependent strains also formed pre-RCs at 2 micron origins of replication in G1 phase (Fig. 4-3B) and supported an approximately normal S phase: upon induction of cdc6-5a, replication occurred within 30 minutes (between the 30m and 60m time points, Fig. 4-4Ca), which is comparable to the time course of Metp-CDC6 induced replication (Chapter 3, Fig. 4-1).

In addition to Metp-cdc6-5a, the strains yCD246, yCD257, and yCD258 also have an inducible clb2^{db} construct, with which we attempted to determine whether cdc6-5a is insensitive to Clb/Cdc28 kinase inhibition. Using the system developed in Chapter 3, the experiment aimed to alter the relative timing of Cdc6 and Clb induction. Normally, Cdc6 is expressed in early G1, before Clb is induced in late G1 (Fig. 4-4A, top). We attempted to

express Clb before cdc6-5a (Fig. 4-4A, bottom) by inducing Galp-clb2^{db} before cdc6-5a (Fig. 4-4B, -105 min. and -30 min., respectively). To this end, cells were arrested in G1 phase under conditions that repress Metp-cdc6-5a; depleting cdc6-5a in this step of the experiment is critical. While the cells are still arrested in G1 phase, clb2^{db} was ectopically induced, followed 75 minutes later by cdc6-5a induction. Upon release from G1 arrest (0 min.), DNA content was monitored at 30 min. intervals.

In a control strain lacking clb2^{db}, replication upon release from G1 phase required induction of cdc6-5a(-met), indicating that the protocol successfully depleted much of the cdc6-5a protein in G1 phase by -105m (Fig. 4-4C, a vs. b). Footprinting experiments also revealed a hypersensitive site, indicating that Pre-RCs had not formed, when Metp-cdc6-5a cells entered G1 in the presence of methionine (corresponds to Fig 4C, -105 min., data not shown). However, if a small percentage of origins had Pre-RCs, the footprinting assay would not be sensitive enough to detect them.

Apparently, not all of the cdc6-5a was actually depleted since upon clb2^{db} induction a significant amount of replication occurred whether or not cdc6-5a was induced (Fig. 4-4C, c vs. d). Some of this replication occurred immediately after clb2^{db} induction (-30 min.), in the presence of alpha factor, consistent with published results that ectopic induction of clb2^{db} in G1 phase will over-ride an alpha-factor induced arrest and promote DNA replication (1). Thus, ectopic induction of clb2^{db} suggested that the mutant protein was not fully depleted. Residual cdc6-5a may have formed PRCs at a small number of origins (undetectable by the footprinting assay), enough to promote significant replication upon induction of clb2^{db}. We therefore cannot determine from this experiment whether Clb/Cdc28 kinase can inhibit cdc6-5a.

We next attempted to make a less stable form of cdc6-5a, as our ability to deplete wildtype Cdc6 using the same protocol (Chapter 3) argues that the mutant cdc6 was more stable than wildtype Cdc6. We fused ubiquitin to cdc6-5a, such that the cleavage of ubiquitin from cdc6-5a would reveal an N-terminal amino acid (arginine) that subjects proteins to rapid turnover by the N-end degradation pathway (6). Yeast strains in which the CDC6 locus has been replaced with Metp-ubi-cdc6-5a (to yield yCD300, yCD301, yCD304, yCD305) failed to grow, as expected, in the presence of methionine. In the absence of methionine, Metp-ubi-cdc6-5a strains grow as well as their cdc6-5a counterparts (data not shown).

When Metp-ubi-cdc6-5a cells were arrested in G1 phase in the presence of methionine, induction of ubi-cdc6-5a (achieved by the removal of methionine) supported complete replication within 60 minutes of release from the arrest (Fig. 4-5a). However, when Metp-ubi-cdc6-5a cells were released without ubi-cdc6-5a induction, significant replication still occurred, indicating the presence of residual ubi-cdc6-5a (Fig. 4-5b). Furthermore, ectopic induction of $clb2^{db}$ in G1 phase induced nearly complete replication by 90 minutes after release from alpha factor with or without ubi-cdc6-5a induction again suggesting that not all of the ubi-cdc6-5a had been depleted (Fig. 4-5c vs. d). A comparison of the -30 min. time points of Fig. 4-4c and d with Fig. 4-5c suggest that ubi-cdc6-5a was somewhat better depleted than cdc6-5a, since immediately after $clb2^{db}$ induction little replication occurred in Fig. 4-5c relative to Fig. 4-4c and d. In spite of the apparently decreased stability of ubi-cdc6-5a, residual activity interfered with our ability to determine whether ectopic $clb2^{db}$ can inhibit ubi-cdc6-5a.

Attempt to determine the effect of ectopic clb2^{db} expression on Δntcdc6 and Δntcdc6-2a

A second mutant form of Cdc6 that we predicted would be insensitive to inhibition by Clb/Cdc28 kinase was constructed. This mutant lacked the N-terminal 46 amino acids of Cdc6, which are both necessary and sufficient for Cdc6 and Clb/Cdc28 kinase to interact *in vitro* (8). We predicted that if the N-terminus of Cdc6 were critical for interaction with Clb/Cdc28 kinase, then Δntcdc6 would uncouple S phase from mitosis. However, overexpression of Δntcdc6 in a wildtype background did not promote over replication (data not shown and (7)).

We attempted to test the ability of clb2^{db} to inhibit the activity of a Δntcdc6 and Δntcdc6-2a (cdc6-5a without the N terminal 46 amino acids; only two of the five CDC28 consensus phosphorylation sites remain). Δntcdc6 and Δntcdc6-2a were placed under the control of the MET promoter in strains both with and without Galp-clb2^{db} (yCD309, 318 and yCD306, 312, respectively). Both Δntcdc6 and Δntcdc6-2a supported viability as well as their full length counterparts (Fig 6) and strains dependent upon either construct cannot grow in the presence of methionine (data not shown).

Unfortunately, neither Δntcdc6 nor Δntcdc6-2a appeared to be fully depletable in G1 phase cells. Figure 4-7 depicts data from experiments in which we attempted to deplete Δntcdc6 nor Δntcdc6-2a in G1 phase arrested cells. Columns a (-met) and b demonstrate that Δntcdc6 does not need to be re-induced in G1 phase in order for replication to occur upon release from alpha factor. Although neither the rate nor the extent of replication is optimal, nearly equivalent amounts of replication occur by 90 minutes whether or not the mutant cdc6 is induced. Ectopic induction of Galp-clb2^{db} exacerbates the situation, as, upon induction of clb2^{db}, replication began

immediately in the Δntcdc6-2a strain (Fig. 4-7e, time -30) and was nearly completed in both strains by time 0. This suggests that neither Δntcdc6 nor Δntcdc6-2a were depleted in the experimental protocol. Consistent with published reports, these experiments suggest that Δntcdc6 and Δntcdc6-2a proteins are more stable than wild-type Cdc6 and cannot therefore be used in our experimental system (7, 8).

We next constructed ubi-Δntcdc6 and ubi-Δntcdc6-2a, in the hopes that these proteins would be less stable than their non-ubiquitinated counterparts. Unfortunately, neither clone (under control of the Metp), could support growth, either in single copy or on a high copy plasmid (Table 1). We conclude that neither ubi-Δntcdc6 nor ubi-Δntcdc6-2a can supply adequate Cdc6 activity.

Finally, since Metp-ubi-Δntcdc6 and Metp-ubi-Δntcdc6-2a may have failed to support growth because the mutant proteins do not accumulate in the nucleus (a portion of the CDC6 NLS is within the sequence that was deleted), we (Joachim Li) cloned an NLS into ubi-Δntcdc6-2a. Since Metp-ubi-NLS-Δntcdc6-2a on a high copy plasmid failed to support cell growth, we conclude that ubi-NLS-Δntcdc6-2a is not a functional protein (Table 1).

DISCUSSION

We were unable to determine if mutant forms of Cdc6 that lack Cdc28 consensus phosphorylation sites, the N-terminus of Cdc6, or both, are sensitive to inhibition by Cdc28/Clb kinase. The closest our experiments come to addressing this is in Fig. 4-5c, in which induction of $clb2^{db}$ before ubi-cdc6-5a results in a slight kinetic delay in replication relative to 4-5a, which depicts a strain lacking $clb2^{db}$. In Fig. 4-5c, replication is not completed until 90 minutes after release from alpha factor arrest, whereas in 4-5a, replication

is completed 60 minutes after release, hinting that Cdc28/Clb kinase can inhibit ubi-cdc6-5a. However, since a significant amount of ubi-cdc6-5a dependent replication occurred in 4-5c (compare to Fig. 4-5d) the experiment is uninterpretable. We therefore do not know if any of the mutants can be inhibited by Clb/Cdc28 kinase and have no evidence indicating that wildtype Cdc6 can be directly inhibited by Clb/Cdc28 kinase.

It has been suggested that having used rho° (lacking significant mitochondrial DNA) strains would have reduced the background DNA replication. However, since the majority of background replication occurs only upon induction of $clb2^{db}$, and since mitochondrial DNA replication is likely to be Clb/Cdc28 independent, the use of rho° strains would probably not have reduced the background replication enough to determine if Cdc28/Clb kinase inhibits the Cdc6 mutants.

A limitation of our experiments in which Cdc6 was depleted in G1 phase was that we used FACS analysis, a measure of DNA content, to determine whether or not initiation occurred. Changes in DNA content reflect the extent of elongation, an event that is downstream, both temporally and physically of initiation; only a small number of origins may need to fire to produce a relatively large amount of elongation. A more direct assay of initiation may have enabled us to more cleanly show that wildtype Cdc6 is sensitive to $clb2^{db}$ induction, since $clb2^{db}$ induction may have prevented an origin from firing, for example, in eighty five percent of the cells (Chapter 3). A more direct assay may also have made it clear earlier in the course of the experiments that we were unable to deplete mutant cdc6 in G1 phase cells. For these two reasons, we briefly explain two examples of assays that more directly reflect initiation.

Two dimensional (2-D) gel analysis uses the differential shape and molecular weight of origin DNA at which varying degrees of replication have occurred to distinguish between fired and unfired origins, and thereby more directly measures origin firing. This assay could be used in the experiments in which Cdc6 is depleted in G1 phase cells by collecting frequent samples (5 min.) upon clb2^{db} induction. The samples could be pooled and processed for 2-D gel analysis, in which several origins could be assessed. If, after clb2^{db} induction, the data showed that wildtype Cdc6 failed to promote origin firing, it would be consistent with the results in Chapter 3.

A second assay that may have been useful is the IP-PCR assay, in which MCM association with origins could be used as a marker of the ability of an origin to fire (2, 15). MCMs associate with origins in G1 of the cell cycle in a Cdc6 dependent manner (2, 15). Thus, if induction of clb2^{db} before Cdc6 prevents MCM association with origins in G1, it would extend the results in Chapter 3 by suggesting that MCM loading is a (perhaps the) critical step that is blocked by Clb/Cdc28 kinase. This would be consistent with published results showing that the inability of Mcm7 to associate with origins correlates with the timing of Clb induction (15).

1. **Amon, A., S. Irniger, and K. Nasmyth.** 1994. Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell **77**:1037-50.

2. **Aparicio, O. M., D. M. Weinstein, and S. P. Bell.** 1997. Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell **91**:59-69.

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5. **Detweiler, C. S., and J. J. Li.** 1997. Cdc6p establishes and maintains a state of replication competence during G1 phase. J Cell Sci **110:**753-63.

6. **Dohmen, R. J., P. Wu, and A. Varshavsky.** 1994. Heat-inducible degron: a method for constructing temperature-sensitive mutants. Science **263:**1273-6.

7. **Drury, L. S., G. Perkins, and J. F. Diffley.** 1997. The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. Embo J **16:**5966-76.

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9. **Guthrie, C., and G. R. Fink (ed.).** 1991. Guide to yeast genetics and molecular biology., vol. 194. Academic Press, New York.

10. **Libinia, N., and W. Gilbert.** personal communication. .

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13. **Santocanale, C., and J. F. Diffley.** 1996. ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in Saccharomyces cerevisiae. Embo J **15:**6671-9.

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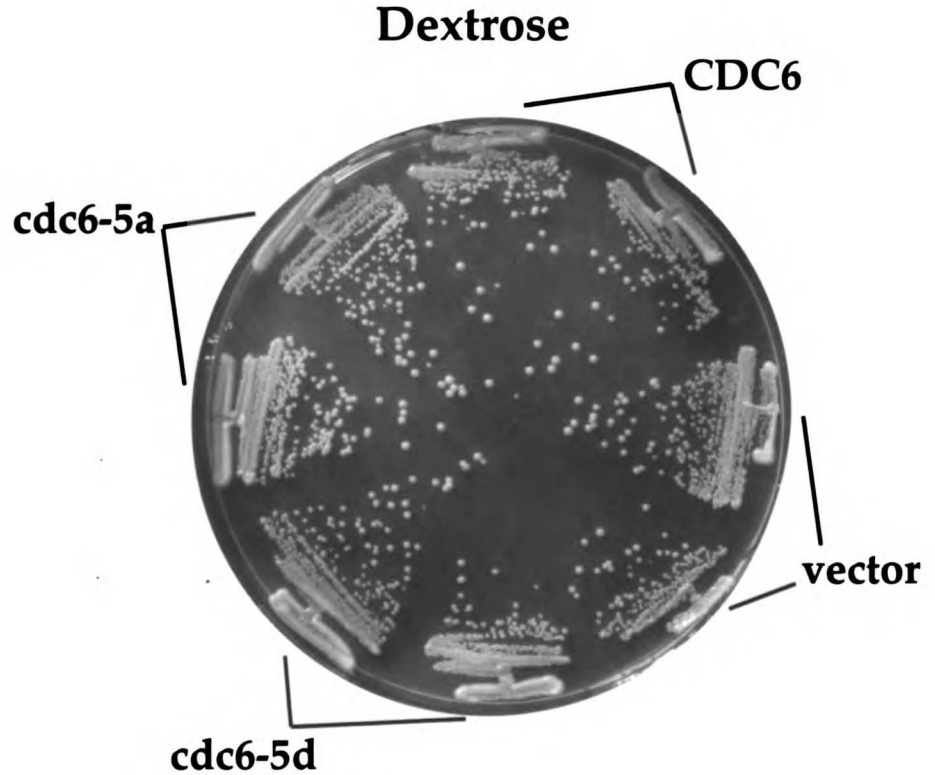
Figure 4-1. The effect of over-expression of wildtype and mutant Cdc6 on growth. Clockwise from top: relative 30°C growth rates of strains with plasmids carrying Galp driven $(HA)^3$CDC6 (yCD245, yCD244), vector (yCD243, yCD242), $(HA)^3$cdc6-5d (yCD241, yCD240), or $(HA)^3$cdc6-5a (yCD239,238). Two independent transformants per plasmid are shown. Top, two days, SDC-URA. Bottom, four days, SGC-URA.


Dextrose
CDC6
cdc6-5a
vector
cdc6-5d

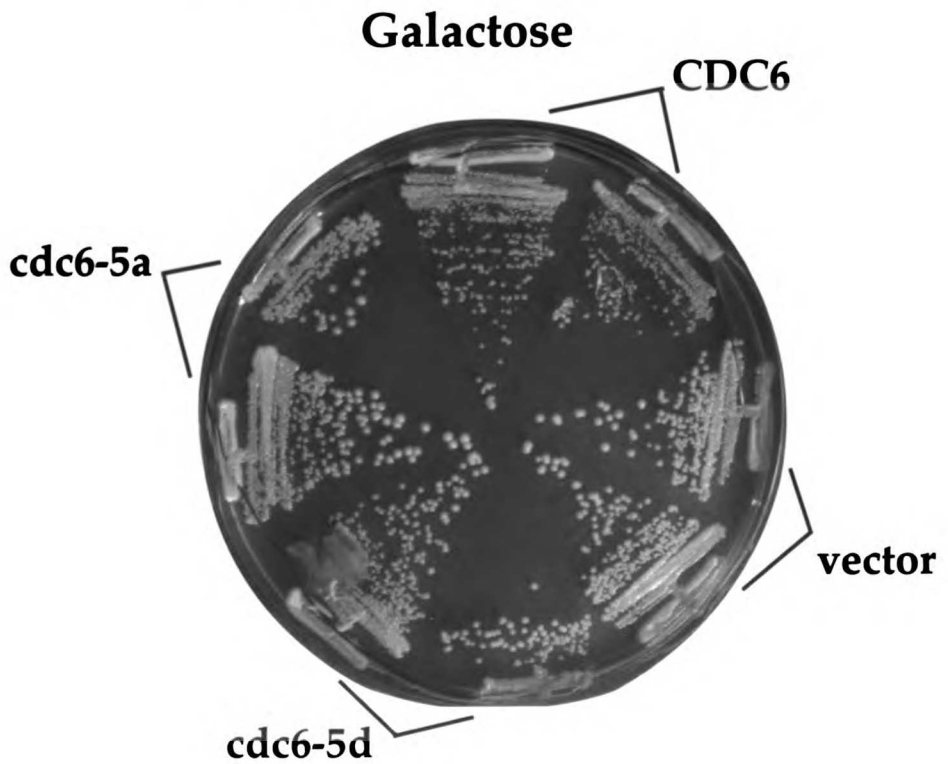
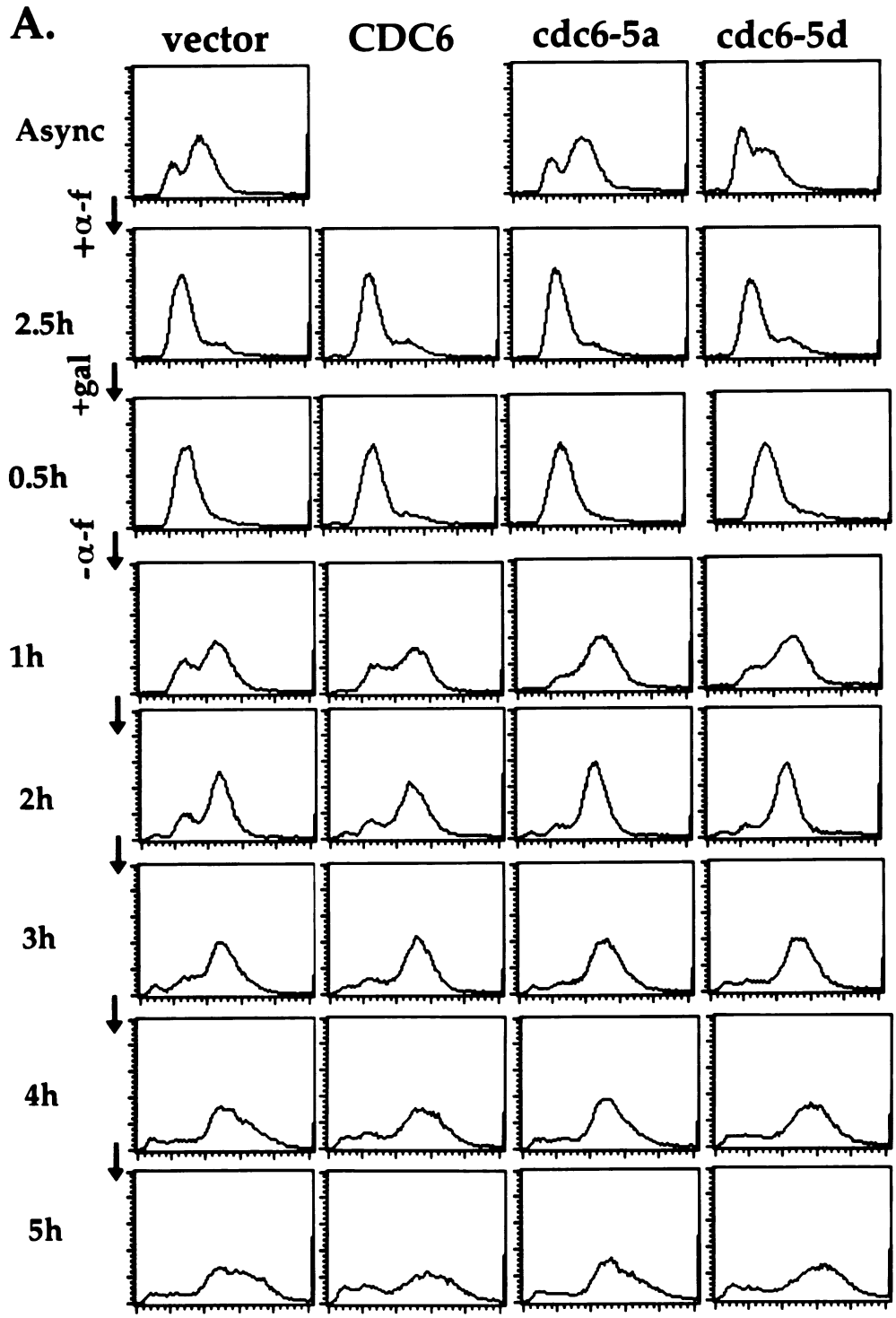

Galactose
CDC6
cdc6-5a
vector
cdc6-5d

Figure 4-2. Over-expression of cdc6-5a or cdc6-5d does not interfere with DNA replication and does not promote pre-mature pre-RC formation in G2 arrested cells. Strains with plasmids carrying Galp driven $(HA)^3$, $(HA)^3$CDC6, $(HA)^3$cdc6-5a, or $(HA)^3$cdc6-5d were alpha-factor arrested for a total of 3 hours. During the last 30 minutes of arrest, galactose was added. Upon release from alpha factor in the presence of both galactose and nocodazole, (A) replication was monitored at 1 hour intervals and (B) the 2 micron origin footprint was assessed 3 hours after release. Two footprint samples (different DNaseI concentrations) were processeed per strain; arrow indicates hypersensitive site. αf, alpha factor; G1, G1 phase arrested MET-CDC6 (yCD233). Strains: vector, yCD242; CDC6, yCD244; (HA)3cdc6-5a, yCD238; (HA)3cdc6-5d, yCD240.


A.
vector
CDC6
cdc6-5a
cdc6-5d
Async
+α-f
2.5h
+gal
0.5h
-α-f
1h
2h
3h
4h
5h

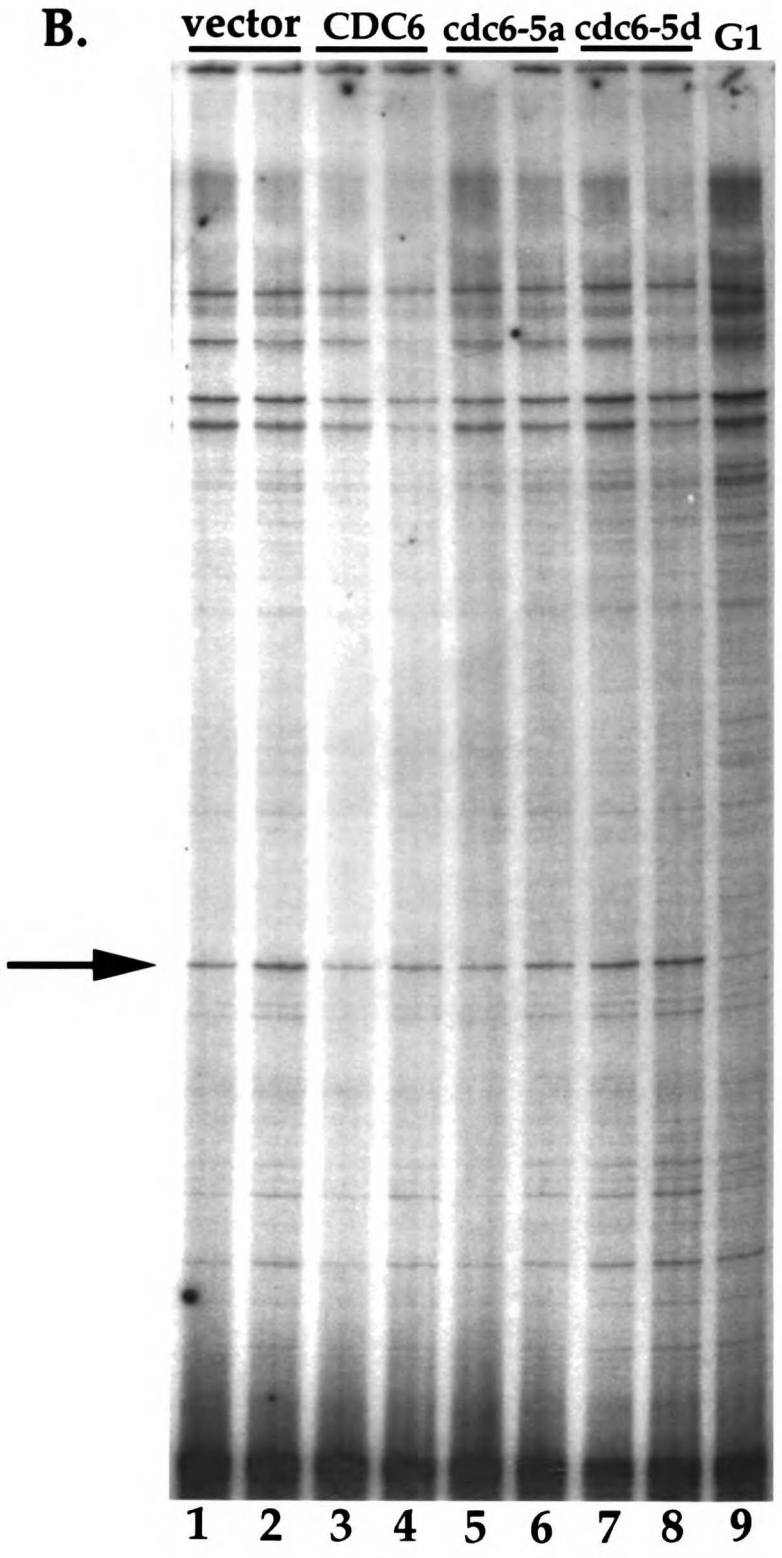
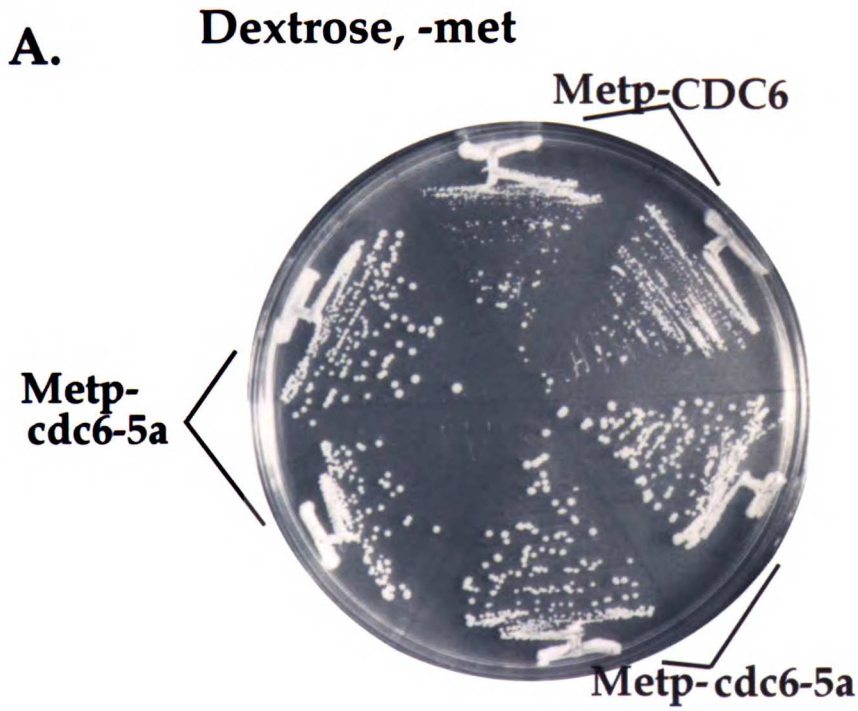
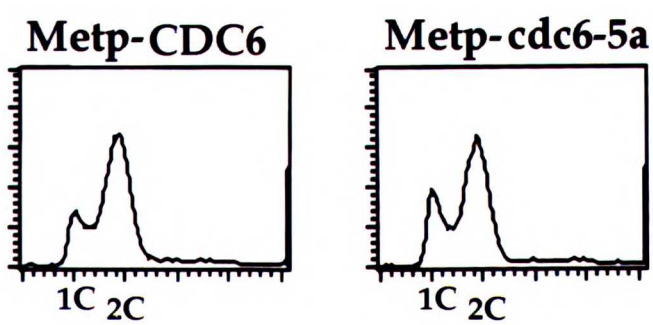

B.
vector
CDC6
cdc6-5a
cdc6-5d
G1
1 2 3 4 5 6 7 8 9

Figure 4-3.A. Relative growth rates and DNA content of Metp-cdc6-5a and Metp-CDC6 dependent strains. A. Top, two days growth at 30°C on SDC-MET. Bottom, DNA content of asynchronous, log phase cells, grown in SR-MLT. Strains, clockwise from top: Metp-CDC6 (yCD235, yCD233), Metp-cdc6-5a (yCD257, yCD254, yCD252, yCD246). DNA content strains: Metp-CDC6 (yCD235), Metp-cdc6-5a (yCD254). B. Footprint pattern at the two micron origin of replication Metp-CDC6, Metp-cdc6-5a, or Metp-cdc6-5d dependent cells arrested in G1 phase with alpha factor. Duplicate samples were processed for footprinting (Arrow denotes hypersensitive site). G2, Metp-CDC6 cells arrested in G1 in the absence of Cdc6. G1, Metp-CDC6 cells arrested in G1 in the presence of Cdc6. ddG, dideoxy-GTP ladder.


A.
Dextrose, -met
Metp-CDC6
Metp-
cdc6-5a
Metp-cdc6-5a


Metp-CDC6
Metp-cdc6-5a
1C 2C
1C 2C

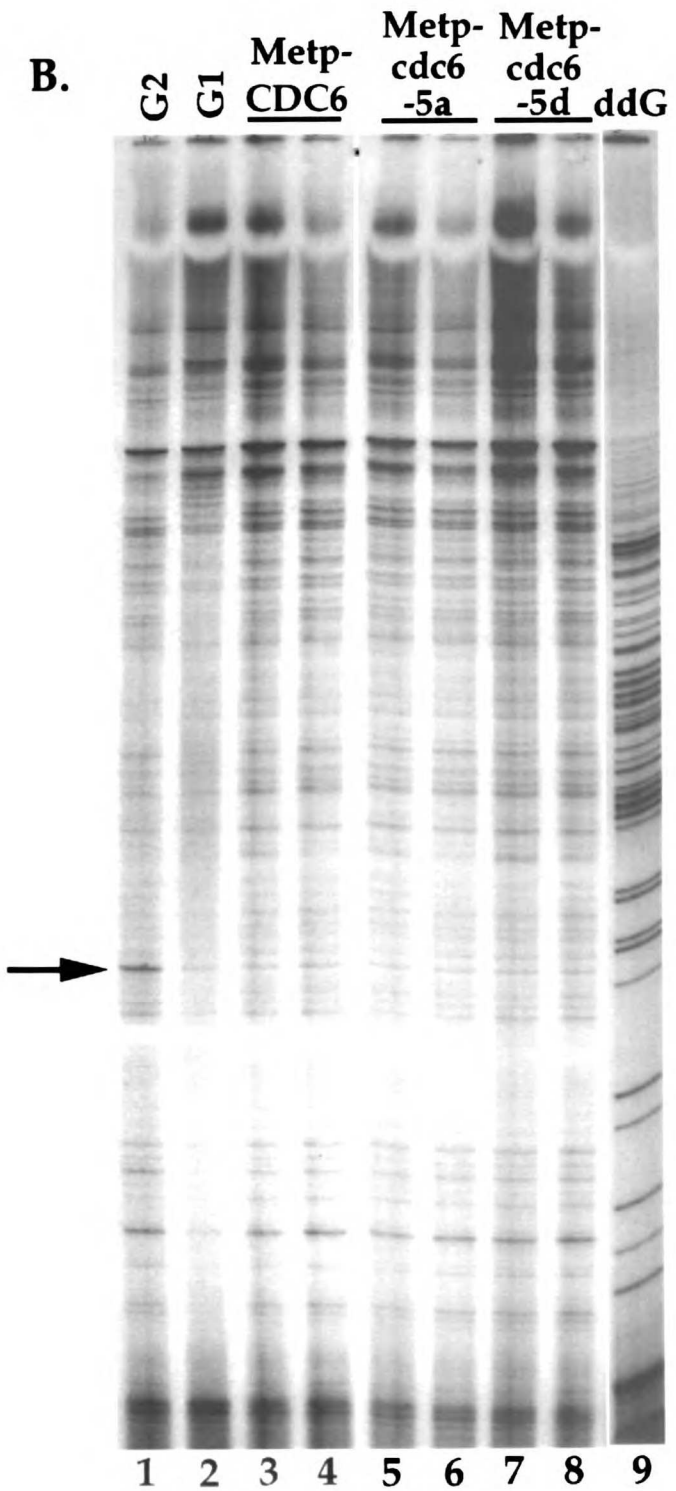
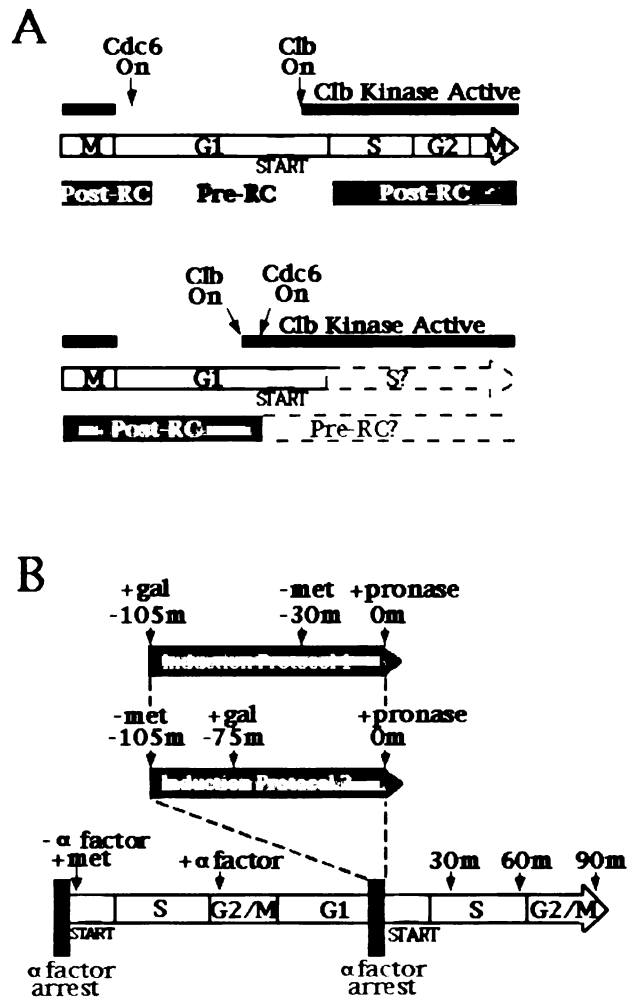

B.
G2
G1
Metp-CDC6
Metp-cdc6-5a
Metp-cdc6-5d
ddG
1
2
3
4
5
6
7
8
9

Figure 4-4. Metp-cdc6-5a is not depleted from G1 phase arrested cells that have been treated with methionine. A. Rationale behind the experiments which attempt to deplete Cdc6 in G1 phase arrested cells. Top depicts normal cell cycle, in which Cdc6 is induced in G1 phase before Cdc28/Clb kinase. Bottom depicts experimental cell cycle, in which Clb kinase is induced before Cdc6 in G1 phase. B., C. Induction protocol and results. Metp-cdc6-5a, Gal-clb2^{db} and Metp-cdc6-5a cells were arrested in G1 phase with alpha factor and released under conditions that repress the Met promoter (-α factor, +met). After the majority of cells had recovered, alpha factor was added back to the media to arrest cells in the following G1 (+αf). Upon achieving G1 arrest, galactose was added (+gal, -105 m). In a and c, methionine was removed 75 minutes later (-met, -30 m). In b and d methionine was not removed (-30 m). All samples were released from alpha factor arrest thirty minutes later (+ pronase, 0 m) and replication was monitored at 30 min intervals thereafter. m, minutes. 1C= 1 genomic DNA content; the line was detemined by the position of the 1C peak at -105m.


A
Cdc6
On
Clb
On
Clb Kinase Active
M
G1
S
G2
M
START
Post-RC
Pre-RC
Post-RC
Clb
On
Cdc6
On
Clb Kinase Active
M
G1
S?
START
Post-RC
Pre-RC?
B
+gal
-105m
-met
-30m
+pronase
0m
-met
-105m
+gal
-75m
+pronase
0m
- α factor
+met
+α factor
30m
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G1
S
G2/M
START
START
α factor
arrest
α factor
arrest

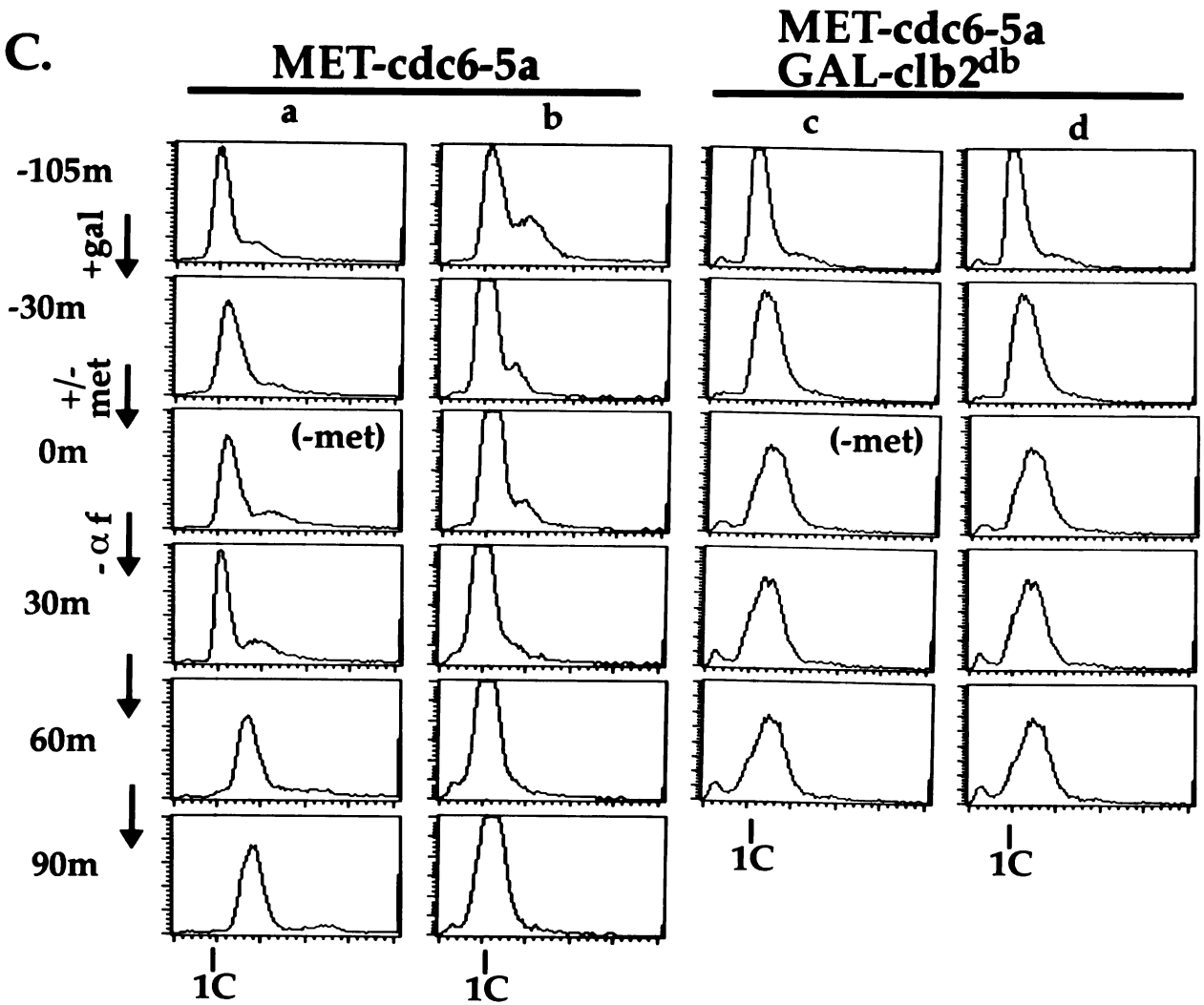

C.
MET-cdc6-5a
MET-cdc6-5a
GAL-clb2db
a
b
c
d
-105m
+gal
-30m
+/-
met
0m
(-met)
(-met)
-αf
30m
60m
90m
1C
1C
1C
1C

Figure 4-5. Met-ubi-cdc6-a is not depleted from G1 arrested cells that have been treated with methionine. Metp-ubi-cdc6-5a cells were arrested in G1 phase in the presence of methionine (see methods). At -105 min, galactose was added, followed by the removal of methionine at -30 min from a and c; methionine was not removed from samples b and d. All samples were released from alpha factor arrest thirty minutes later and replication was monitored at thirty minute intervals. 1C= 1 genomic DNA content; the line was detemined by the position of the 1C peak at -105m.

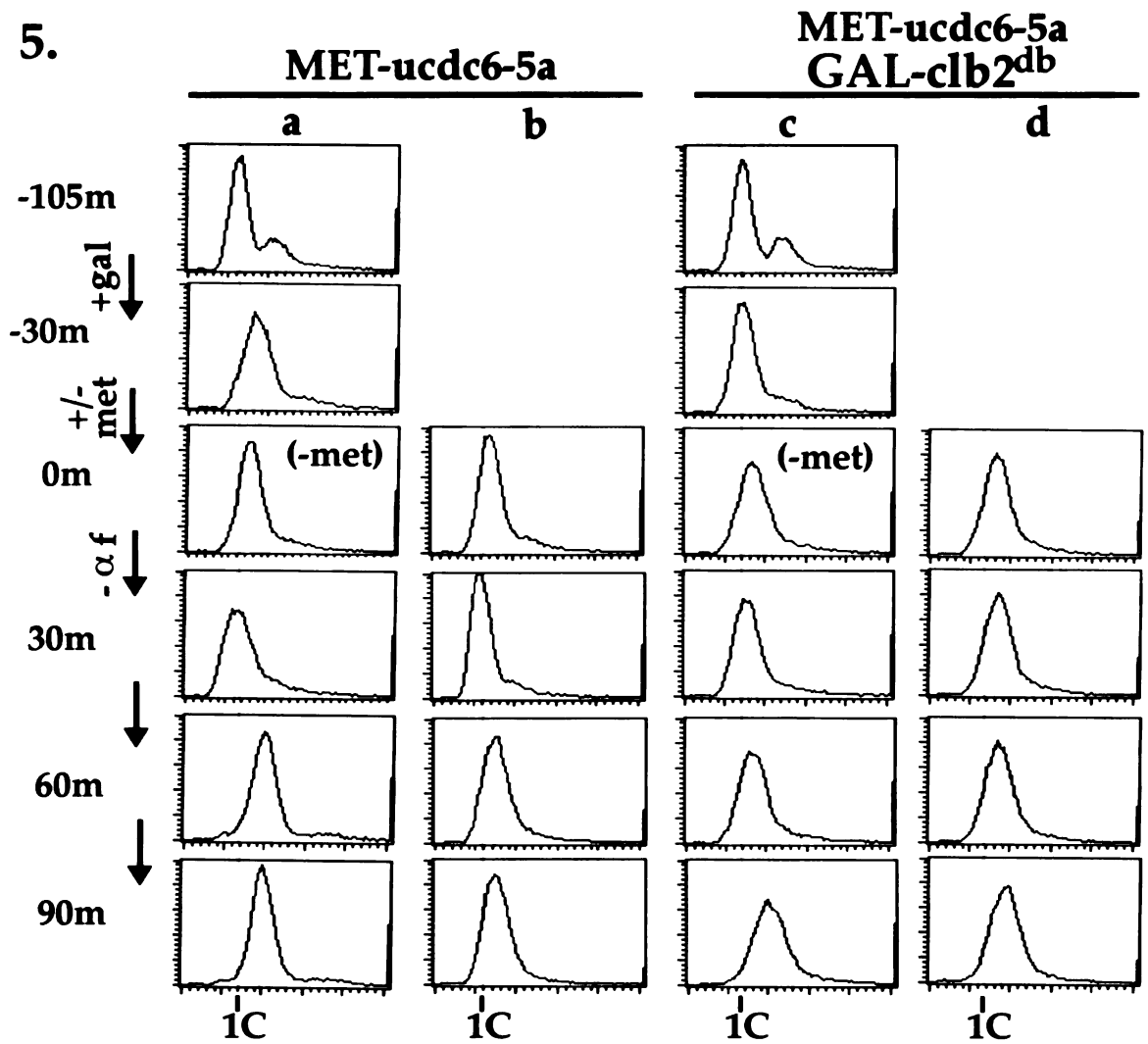
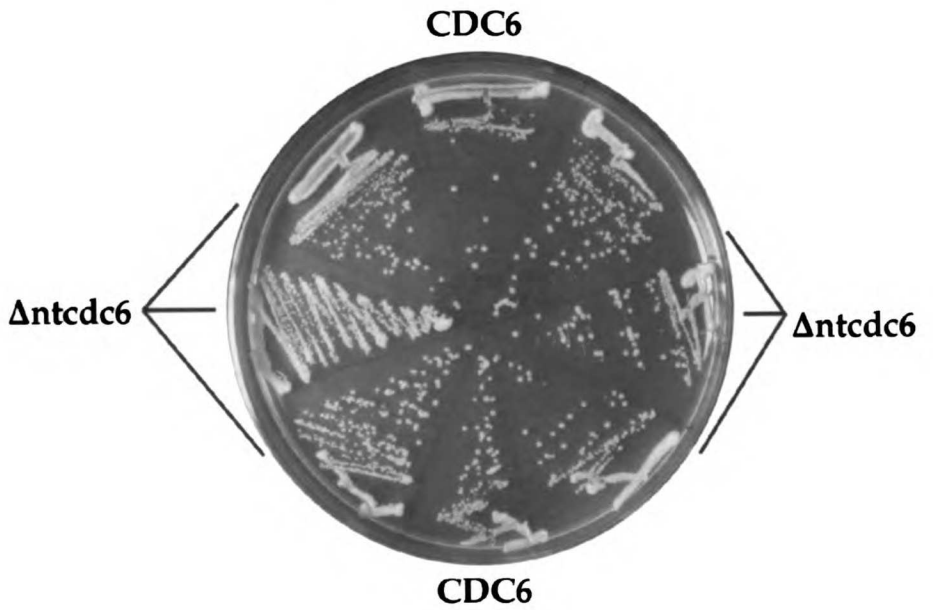

5.
MET-ucdc6-5a
MET-ucdc6-5a
GAL-clb2db
a
b
c
d
-105m
+gal
-30m
+/-
met
0m
(-met)
(-met)
- α f
30m
60m
90m
1C
1C
1C
1C

Figure 4-6.A. Relative growth rates of Metp-CDC6 and Metp-Δntcdc6 dependent strains. Metp-CDC6, top and bottom (yCD235, yCD233); Metp-Δntcdc6, clockwise from right of top (yCD309, yCD310, yCD311) and clockwise from left of bottom (yCD306, yCD307, yCD308). Two days growth at 30°C on SDC-MET. B. Relative growth rates of Metp-cdc6-5a and Metp-Δntcdc6-2a dependent strains. Metp-cdc6-5a, top and bottom (yCD254, yCD257). Clockwise from right of top, Metp-$(HA)^3$cdc6-5a (yCD319), Metp-Δntcdc6-2a (yCD318, yCD317, yCD316 and yCD315, yCD314, yCD313, yCD312). Two days growth at 30°C on SDC-MET.

A. **Dextrose, -met**



B. **Dextrose, -met**

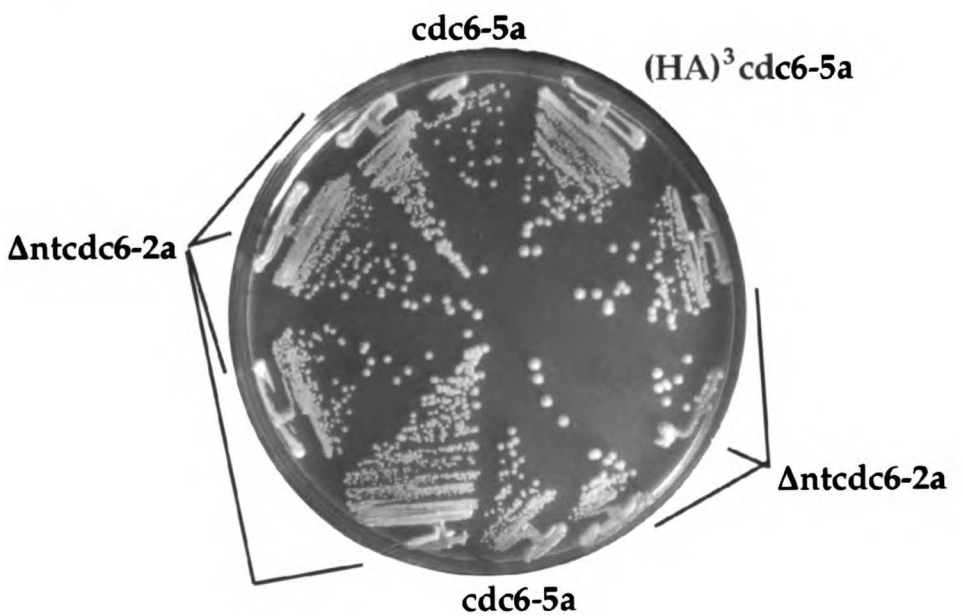


Figure 4-7. Δntcdc6 and Δntcdc6-2a are not depleted in G1 phase arrested cells. Cells were arrested in G1 phase in the presence of methionine according to the protocol in Figure 4. Cultures were split at -30 minutes and methionine was removed from a, c, and e; methionine was not removed from b, d, and f. All cultures were released from alpha factor arrest at time 0 and replication was monitored at thirty minute intervals after release. 1C, 1 genomic DNA content; the line was detemined by the position of the 1C peak at -105m.

7.

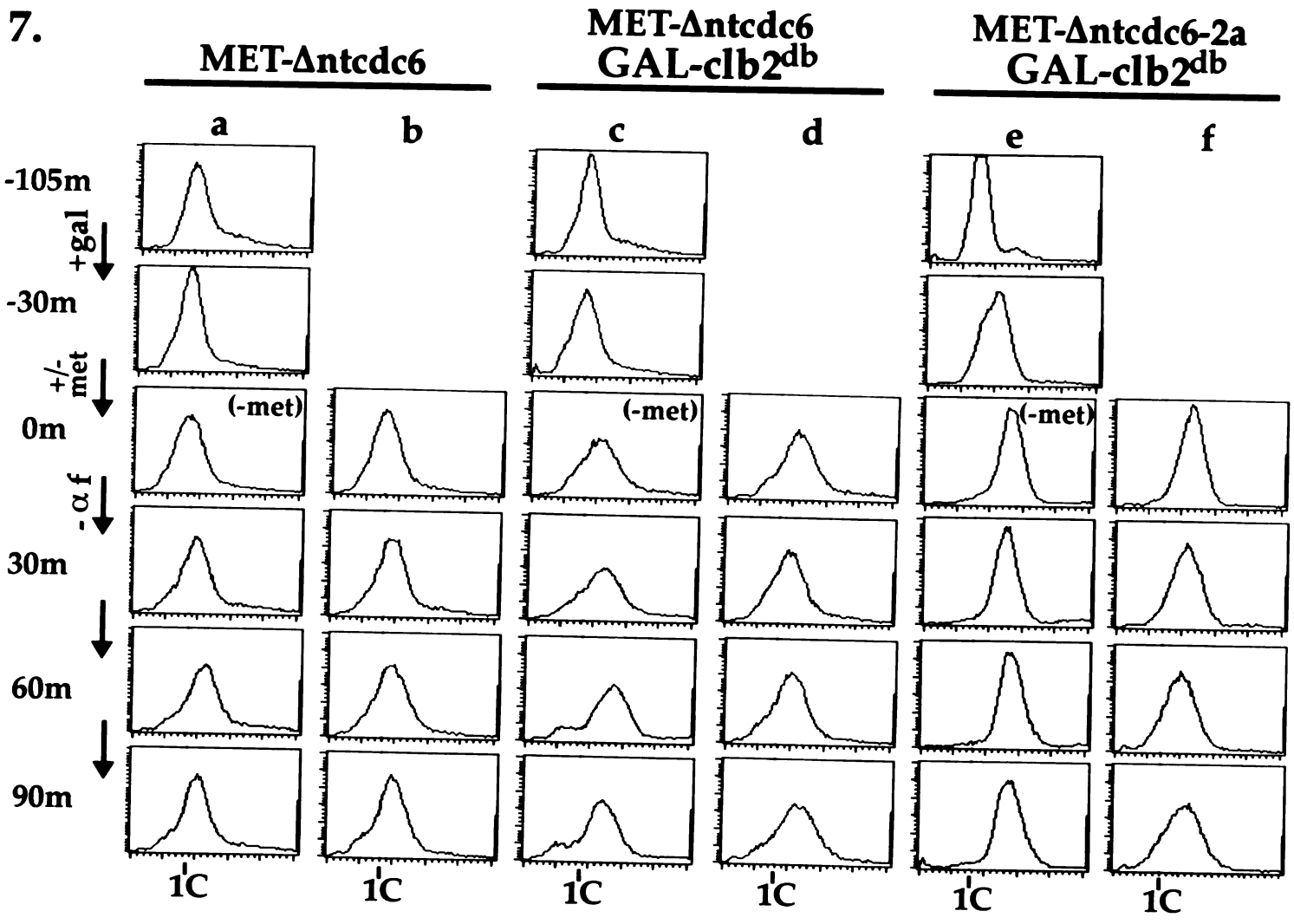

MET-Δntcdc6
MET-Δntcdc6 GAL-clb2db
MET-Δntcdc6-2a GAL-clb2db
a
b
c
d
e
f
-105m
+gal
-30m
+/- met
0m
- α f
30m
60m
90m
(-met)
(-met)
(-met)
1C
1C
1C
1C
1C
1C

Table 4-1

SUMMATION OF RESULTS

construct	plasmid	functional? (low copy)	functional? (high copy)	depletable from G1 cells?	sensitive to ectopic clb2^{db}?
METp-CDC6	pCD29	Y	ND	Y	Y
METp-cdc6-5a	pCD31	Y	ND	N	
METp-ubi-cdc6-5a	pCD74	Y	ND	N	
METp-Δntcdc6	pCD78/79	Y	ND	N	
METp-Δntcdc6-2a	pCD80/81	Y	ND	N	
METp-ubi-Δntcdc6	pCD84	N	N		
METp-ubi-Δntcdc6-2a	pCD82/83	N	N		Y, Yes
METp-ubi-NLS-Δntcdc6-2a	pJL1101	ND	N		N, No ND, no data

Chapter 4 Strain Table

yJL	yCD	genotype	source
1211/1212	242/243	Mata trp1-289 leu2-3,112 ura3-52 bar1::LEU2 [pGAL-$(HA)^3$]	this study
1213/1214	244/245	Mata trp1-289 leu2-3,112 ura3-52 bar1::LEU2 [pGAL-$(HA)^3$-CDC6]	this study
1215/1216	238/239	Mata trp1-289 leu2-3,112 ura3-52 bar1::LEU2 [pGAL-$(HA)^3$-cdc6-5a]	this study
1217/1218	240/241	Mata trp1-289 leu2-3,112 ura3-52 bar1::LEU2 [pGAL-$(HA)^3$-cdc6-5d]	this study
1167	233	Mata pMET-CDC6 trp1::[pGAL-clb2db TRP1] leu2-3,112 ura3-52 bar1::LEU2	chapter 3
1169	235	Mata pMET-CDC6 trp1-289 leu2-3,112 ura3-52 bar1::LEU2	chapter 3
1179/1178	252/254	Mata pMET-cdc6-5a trp1-289 leu2-3,112 ura3-52 bar1::LEU2	this study
1219/1176/1177	246/257/258	Mata pMET-cdc6-5a trp1::[pGAL-clb2db TRP1] leu2-3,112 ura3-52 bar1::LEU2	this study
1220/1221	300/301	Mata pMET-ubi-cdc6-5a trp1-289 leu2-3,112 ura3-52 bar1::LEU2	this study
1222/1223	304/305	Mata pMET-ubi-cdc6-5a trp1::[pGAL-clb2db TRP1] leu2-3,112 ura3-52 bar1::LEU2	this study
1224	306	Mata pMET-Δntcdc6 trp1-289 leu2-3,112 ura3-52 bar1::LEU2	this study
1225	309	Mata pMET-Δntcdc6 trp1::[pGAL-clb2db TRP1] leu2-3,112 ura3-52 bar1::LEU2	this study
1226	312	Mata pMET-Δntcdc6-2a trp1-289 leu2-3,112 ura3-52 bar1::LEU2	this study
1227	318	Mata pMET-Δntcdc6-2a trp1::[pGAL-clb2db TRP1] leu2-3,112 ura3-52 bar1::LEU2	this study

CHAPTER 5

Conclusion

Key aspects of DNA replication initiation are not yet understood. The molecular events of initiation, including Pre-Replicative Complex (Pre-RC) formation, kinase activation in late G1 phase, and Pre-RC disassembly upon origin firing remain unclear. Also, while it is clear that CDKs prevent rereplication, an apparent target of CDK activity, i.e., a protein that must be phosphorylated by CDK in order for initiation to occur only once per S phase, has been identified in only one organism. While this CDK substrate (Cdc18/Cdc6) is conserved between organisms, it is unclear if its probable role in preventing rereplication is also conserved. A description of these issues and possible approaches follow.

What are the molecular events of DNA replication initiation?

Pre-RC Formation

In the current model, replication initiation can be broken down into at least three major steps. First, upon entry into G1 phase, replication proteins join the Origin Recognition Complex (ORC) at origins to form the Pre-RC (8). Pre-RCs remain intact until passage through START, the late G1 phase cell cycle commitment period in budding yeast. Pre-RCs were initially defined by a characteristic *in vivo* footprinting pattern observed at origins of replication only in G1 phase, and later shown to be an important early step in replication initiation (6, 9, 21).

The proteins that make up the Pre-RC probably include ORC, Cdc6, and Mini Chromosome Maintenance (MCM) family members. ORC is a likely component of the Pre-RC because it co-purifies with chromatin from yeast cells arreated in G1 phase (18). Furthermore, in the *Xenopus* system, ORC must be bound to chromatin in order for Cdc6 and then Mcm2 to load onto the chromatin (5). Cdc6 is likely a Pre-RC component because it is required

for the establishment and maintenance of the characteristic G1 phase *in vivo* footprint that represents the Pre-RC (4). Additionally, Cdc6, Mcm4, and Mcm7 were shown by an immunoprecipitation-PCR (IP-PCR) assay to associate with origins specifically in G1 phase; IP-PCR involves crosslinking proteins and DNA, immunoprecipitating a particular protein, reversing the crosslinks, and determining if origin DNA is in the precipitate by PCR (1, 30).

In spite of the apparent completeness of this model, not all of the putative Pre-RC components have been definitively shown to bind origins in G1 phase. Only two of the six MCM family members have been shown to associate with origins in G1 phase (1, 30). In spite of their sequence homology, the remaining MCMs may not behave similarly (12).

It is also unclear if other proteins contribute to the Pre-RC. For instance, there are conflicting results as to whether or not Cdc45, which is required for origin firing and the disassembly of the Pre-RC, associates with origins prior to passage through START (20, 28, 32). Finally, other replication proteins such as polymerases and primases may also contribute to Pre-RC formation. Systematic application of the IP-PCR assay would help determine whether other elongation proteins may also be part of the Pre-RC.

Unfortunately, the IP-PCR assay, cannot definitively show that proteins associated with origins in G1 phase are part of the Pre-RC. An alternative approach is to determine if each putative Pre-RC component is required for Pre-RC formation and maintenance by *in vivo* footprinting. Although this has been attempted both with MCM family members and ORC components, definitive results, have not been published (25).

Kinase Activation

The second step of replication requires two kinases, Clb/CDK and Cdc7, both of which are activated upon passage through START (13, 27). Clb/CDK and Cdc7 probably promote initiation by phosphorylating replication proteins, but we do not know which proteins are the critical substrates. Both kinases can phosphorylate putative components of the Pre-RC. For example, Cdc7 can phosphorylate MCMs *in vitro* (17, 26). Substrates of Clb/CDK include Cdc6, Orc2, and Orc6, and likely substrates include the MCMs (2, 11, 31). There is no evidence, however, that phosphorylation of any of these proteins is important for initiation. Thus, Clb/CDK and/or Cdc7 may promote initiation by phosphorylating as yet unknown substrates or by phosphorylating multiple substrates.

Approaches to identifying additional Clb/CDK or Cdc7 replication substrates could include systematic testing of other known replication proteins for *in vitro* phosphorylation by either kinase. The phosphorylation sites on each putative substrate could then be identified by phospho-peptide mapping and eliminated by site directed mutagenesis. The substrate mutants could be tested *in vivo* for an inability to support replication initiation. It may be critical to combine substrate mutants in different genes: perhaps the phosphorylation sites on both Cdc6 and MCMs need to be removed in order to prevent kinase-activated initiation.

Functional approaches to substrate identification could include genetic screens for genes whose products may interact with either kinase. For example, mutations that confer synthetic lethality with either Clb/CDK or Cdc7, but not both, may be in genes encoding substrates of the respective kinase. Putative substrates would need to be cloned; if the gene products demonstrated Clb/CDK or Cdc7 kinase dependent phosphorylation, the phosphorylated amino acids could be identified and mutated. The mutant

derivative could be assayed *in vivo* for failure to promote replication initiation, which would indicate that phosphorylation of this particular substrate is necessary for origin firing.

Pre-RC Disassembly

Concomitant with kinase activation and entry into S phase is Pre-RC disassembly, initially assayed by the loss of the characteristic G1 phase footprint pattern at origins of replication. Pre-RC disassembly is likely required for the commencement of nucleotide incorporation (6, 9, 21). This situation is analogous to that of lambda phage initiation, in which components of the complex that forms at the origin before initiation must be removed from the origins before nucleotide incorporation can begin (29).

The molecular events of Pre-RC disassembly are unclear but do not appear to involve the displacement of ORC. *In vivo* footprinting, IP-PCR, and immunofluoresence data all indicate that ORC remains bound to origins during elongation (1, 3, 24, 30). This suggests that a second ORC must load onto a duplicated origin during or immediately after initiation.

Unlike ORC, Cdc6 dissociates from the DNA very early in S phase, probably when Pre-RCs disassemble (5, 30). Mcm4 and Cdc45, in contrast, neither dissociate from the DNA nor remain bound to origins. Instead, they appear to move along the DNA with the replication fork (1). Since MCMs gradually dissociate from the DNA as replication proceeds, they may come off the DNA when replication in a particular region of the genome is completed through the convergence of replication forks from adjacent origins (1, 15, 22, 23).

Towards an in vitro replication system

While existing assays, including *in vivo* footprinting, immunofluoresence, IP-PCR, and *in vitro* replication in embryonic systems, have been very informative, the field ultimately needs an *in vitro*, origin-specific replication system to dissect the biochemistry of initiation. Several factors argue for the development of such a system in *S. cerevisiae*. First, *S. cerevisiae* features origin specific firing, whereas embryonic replication systems replicate DNA indiscriminately. The sequence specificity of origins has allowed for the observation of the second critical feature of *S. cerevisiae* replication, the formation of an initiation intermediate, the Pre-RC. The development of an *in vitro* Pre-RC formation assay would be an important step towards *in vitro* replication. Third, the many well-studied replication mutants in *S. cerevisiae* will enable the authenticity of an *in vitro* replication system to be relatively easily determined.

Once the components of the Pre-RC are known, the first step towards an *in vitro* replication system, *in vitro* Pre-RC formation, may be possible. Attempts to re-create a Pre-RC *in vitro* could utilize purified or partially purified components. Initial experiments could theoretically monitor Pre-RC formation by a gel shift assay. For example, addition of Pre-RC components may decrease the mobility of the ORC-origin complex (crosslinking of proteins to DNA may be necessary prior to the gel shift separation step). An *in vitro* Pre-RC footprinting assay may help confirm that a synthetic Pre-RC protects origin DNA similarly to an *in vivo* Pre-RC, indicating that the Pre-RC has been faithfully re-created *in vitro*.

An *in vitro* Pre-RC assay may also be useful for determining the effect of the protein kinases Clb/CDK and Cdc7, on the Pre-RC. Substrates of the kinases could be revealed by the addition of radiolabeled ATP along with either kinase. Substrates could be identified based on immunoreactivity or

mass spectrometry and the relevance of their phosphorylation could be assessed by identifying and mutating the phosphorylated amino acids. Furthermore, if the addition of S phase extract or kinases to *in vitro* assembled Pre-RCs resulted in Pre-RC disassembly, analogous to the *in vivo* situation, this system would also permit analysis of the physical changes that occur upon Pre-RC disassembly.

Once established, an *in vitro* Pre-RC assay would also enable dissection of the architecture of the Pre-RC. For example protein-protein and protein-DNA crosslinking experiments may help determine which components bind each other and which contact the DNA.

What protein(s) does CDK target to prevent re-replication?

Once the carefully regulated steps of origin firing have been executed, a cell must ensure that re-initiation will not occur until the next cell division cycle. The mechanism to prevent re-replication requires CDK and likely involves the negative regulation of one or more proteins specifically required for initiation. In only one model system, however, is a likely target of CDK known.

In *S. pombe*, Cdc18, the homolog of Cdc6, is a target of CDK mediated replication inhibition. Supporting evidence includes the observation that over-expression of Cdc18 induces multiple rounds of DNA replication in the absence of nuclear division, suggesting that negative regulation of Cdc18 is important for limiting replication to a single round (19). Since Cdc18 is likely phosphorylated by CDK *in vivo* and mutation of the CDK consensus sites within Cdc18 renders the protein refractory to inhibition by CDK (albeit, upon over-expression of the mutant cdc18), it appears that CDK prevents replication by inhibiting Cdc18 (14). The effect of CDK phosphorylation on

Cdc18 is to render Cdc18 unstable; in the absence of Cdc18, Pre-RCs cannot (presumably) re-form at origins (16).

In *S. cerevisiae*, the situation appears to be somewhat different. First, over-expression of Cdc6 does not appear to result in over-replication, indicating that Cdc6 is not the limiting factor for initiation (7, 10, 21). Second, while Cdc6 can be a target for CDK (specifically, Clb/CDKs) *in vitro*, mutation of the putative CDK phosphorylation sites within Cdc6 does not appear to affect the regulation of replication. *In vivo*, CDK can inhibit Pre-RC formation and Mcm7 loading onto origins, both of which are Cdc6 dependent events, but it is not known if Cdc6 or MCMs are directly inhibited by CDK; it is possible that CDK inhibits only Cdc6 (albeit, independently of the Cdc28 consensus phosphorylation sites within Cdc6), only Mcm7, both, or neither (1, 4, 6, 30).

Conclusion

In conclusion, the future of the eukaryotic replication field lies in the development of biochemical assays that will enable workers to make better use of the many genetic tools available. Since an *in vitro* replication assay is neither trivial to develop nor necessarily possible (outside of the architecture of the nucleus), it makes sense to continue to break down the problems of initiation and re-replication prevention into as many discrete steps as possible. These discrete steps may not only be more amenable to re-creation in the test tube, but have and will continue to give us clues to how eukaryotic systems regulate replication.

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APPENDIX I

Does Cdc6 associate with origins?

INTRODUCTION

The Cdc6 requirement for the establishment and maintenance of the pre-RC suggests that Cdc6 binds origins, either directly or indirectly (4). However, an alternative explanation is that Cdc6 loads other proteins onto origins to form a labile complex that is continually in need of re-loading by Cdc6. To distinguish between these two models, we attempted to develop an *in vivo* assay for Cdc6 association with origins based on the one-hybrid system(6, 8)

The one-hybrid system seeks to determine if a protein and a particular DNA sequence associate with each other in vivo. The basic components of this system are the protein in question, the DNA target, and the reporter gene (6). In our experiments, the protein (bait) consists of Cdc6 fused to a transcriptional activation domain and under the control of a constitutive promoter (ADH3p-GAD-CDC6). The target DNA sequence was eight tandem copies of the ACS (ARS consensus sequence) from ARS1, a strong *in vivo* origin (6). The multimermized ACS was inserted in the promoter region of a chromosomal reporter gene, such that if Gad-Cdc6 binds to the ACS, either directly or indirectly, the activation domain fused to Cdc6 will promote transcription of the downstream reporter gene, LACZ. LacZ expression is assayed colorometrically (2).

MATERIALS AND METHODS

Plasmids and Strains

pCD47/48 (ADH3p-GAD-CDC6) were generated by blunt ending the 1.5 kb NotI-SpeI fragment of pCD3 (5) and ligating it to pGAD2F (3) that had been BamHI cut and blunt ended.

yJL371 and yJL372 were generated as previously described (6).

B-galactosidase filter assays were performed essentially as described (2). Briefly, patches of each strain were grown on selective media and replica-plated to filters which were flash-frozen in liquid nitrogen, thawed and developed in Z buffer with .009% X-gal and 10mM B-mercaptoethanol at 30°C overnight (2).

RESULTS

A strain (yJL371/372) with eight direct repeats of the ARS 1 consensus sequence upstream of a lacZ reporter gene was transformed with a GAD-CDC6 fusion protein on a low copy plasmid (Fig. 5-1). The GAD-CDC6 construct could provide Cdc6 activity since it was capable of supporting viability in a strain lacking the chromosomal copy of CDC6. A B-galactosidase assay was used to determine if GAD-CDC6 could promote lacZ production from the reporter strain (2). Two additional plasmids, GAD (pGAD2F) and GAD-Δ134orc6 served as a negative and positive control, respectively, for transcriptional induction (3, 6).

Independent transformants of the reporter strain with each plasmid were patched onto media selective for the plasmid and replica-plated onto filters. The filter patches were tested for B-galactosidase activity, an assay in which dark blue indicates high levels of LacZ production and therefore a strong interaction between the protein and DNA, and white or light blue reflects low levels of LACZ transcription and thus little interaction between the bait and target. Fig. 1 illustrates that GAD-Cdc6 promoted little reproducible lacZ expression above the GAD negative control, and considerably less lacZ expression than Δ134orc6, which is a relatively weak positive control (6). This indicates that this assay does not reproducibly reveal GAD-Cdc6 association with origins.

CONCLUSIONS

Since we were unable to convincingly detect GAD-Cdc6 origin association, our experiments do not indicate whether Cdc6 can associate with origins. Were they to be repeated, it may be worth constructing a reporter strain with multiple copies of a complete origin instead of simply the ARS consensus sequence. In addition, it would be useful, perhaps, to construct a reporter strain that lacks wildtype Cdc6, which may compete with GAD-Cdc6 for binding at the ACS upstream of the reporter gene.

However, since these experiments were conducted, another assay has been developed to determine if proteins can associate with origin sequences *in vivo*. This technique, IP-PCR, requires formaldehyde crosslinking in whole cell lysates, followed by immunoprecipitation of Cdc6. The precipitate crosslinks are then chemically reversed and PCR with origin specific primers is performed (1, 7). Tanaka et. al. have shown that via this assay that Cdc6 associates with origins in G1 phase cells (7). Interestingly, in a similar experiment, another lab was unable to detect Cdc6-origin association, although they could show that other proteins (MCM family members) associate with origins in G1 phase cells (1). In conclusion, Cdc6 most likely associates with origins in G1 phase, but further experiments (such as IP-PCR without prior crosslinking) are necessary to confirm these results and determine their biological relevance.

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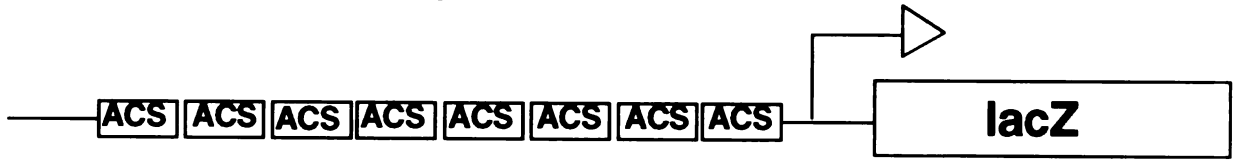
Figure AI-1. A. Summary of one-hybrid system results. Diagram of the eight tandem copies of ARS1 used as a target sequence in the one-hybrid assay upstream of the reporter gene, lacZ. B. Summary of results. The Gal Activation Domain (GAD) fused either to CDC6, nothing (negative control) or the C-terminus of ORC6 (positive control). Patches of each strain were assayed for B-galactosidase activity in sextuplicate (GAD-CDC6) or triplicate (GAD and GAD-ORC6).

Yeast Strains (target)



Construct/plasmid

B-galatosidase activity