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The *mcm5-bob1* Bypass of Cdc7p/Dbf4p in DNA Replication Depends on Both Cdk1-Independent and Cdk1-Dependent Steps in *Saccharomyces cerevisiae*

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ABSTRACT

The roles in DNA replication of two distinct protein kinases, Cdc7p/Dbf4p and Cdk1p/Clb (B-type cyclin), were studied. This was accomplished through a genetic and molecular analysis of the mechanism by which the *mcm5-bob1* mutation bypasses the function of the Cdc7p/Dbf4p kinase. Genetic experiments revealed that loss of either Clb5p or Clb2p cyclins suppresses the *mcm5-bob1* mutation and prevents bypass. These two cyclins have distinct roles in bypass and presumably in DNA replication as overexpression of one could not complement the loss of the other. Furthermore, the ectopic expression of *CLB2* in G1 phase cannot substitute for *CLB5* function in bypass of Cdc7p/Dbf4p by *mcm5-bob1*. Molecular experiments revealed that the *mcm5-bob1* mutation allows for constitutive loading of Cdc45p at early origins in arrested G1 phase cells when both kinases are inactive. A model is proposed in which the Mcm5-bob1 protein assumes a unique molecular conformation without prior action by either kinase. This conformation allows for stable binding of Cdc45p to the origin. However, DNA replication still cannot occur without the combined action of Cdk1p/Clb5p and Cdk1p/Clb2p. Thus Cdc7p and Cdk1p kinases catalyze the initiation of DNA replication at several distinct steps, of which only a subset is bypassed by the *mcm5-bob1* mutation.

THE regulation of DNA replication is the result of a two-step mechanism that ensures that S phase is dependent on a prior mitosis and that origins of DNA replication fire once and only once per cycle (for recent reviews, see Kelly and Brown 2000; Sclafani 2000; Lei and Tye 2001). In the first step, a prereplication complex (pre-RC) is assembled onto origins in G1 phase. The origins are bound throughout the cell cycle by a six-member protein complex known as the origin-recognition complex (ORC). The hexameric minichromosome maintenance (Mcm) complex is loaded onto the ORC by Cdc6p, which is produced in G1 phase. The Mcm complex is believed to act as the replicative helicase (Labib and Diffley 2001).

For replication to ensue, two protein kinases, cyclin-dependent kinase (Cdk)1p (also known as Cdc28p) and Cdc7p, must be activated. These kinases both have inactive kinase subunits that are activated by the binding of an unstable regulatory subunit (Nasmyth 1996b; Sclafani 2000). For Cdk1p and Cdc7p, the regulatory subunits are cyclins and Dbf4p, respectively. For Cdk1p, there are three early G1 cyclins (Cln1-3p) and six B-type cyclins (Clb1-6p). Cln1-3p help to regulate the temporal expression of Clb1-6p in a temporal pattern with Clb5p and Clb6p expressed just before S phase, Clb3p and Clb4p at G2 phase, and finally Clb1p and Clb2p in late G2 phase and mitosis. Sic1p is an inhibitor of Cdk1p/Clb5p (Schwob *et al.* 1994). Sic1p degradation in G1 phase is regulated by Cdk1p/Cln1-3p phosphorylation and also by Cdc4p and Cdc34p (Verma *et al.* 1997). For Cdc7p, Dbf4p is absent only in G1 phase due to being targeted by the anaphase promotion complex (APC) for degradation (Cheng *et al.* 1999; Oshiro *et al.* 1999; Weinreich and Stillman 1999; Ferreira *et al.* 2000).

Although there is considerable overlap in the function of Clb1-6p (Nasmyth 1996b), the role of Clb5p and Clb6p in DNA replication cannot be substituted by the mitotic Clb2p when all are expressed at physiological levels (Cross *et al.* 1999; Donaldson 2000). These studies employed *clb5::CLB2* constructs in which the *CLB5* promoter is placed upstream of the *CLB2* coding sequence. With *clb5::CLB2*, Clb2p is expressed in G1 phase instead of in late G2 phase and mitosis. In *clb5* mutants, S phase is longer because late origins do not fire (Donaldson *et al.* 1998b). *clb5::CLB2* cannot correct this defect. In *clb5 clb6* mutants, initiation is delayed and now late origins fire presumably due to other *CLBs*. However, neither Clb2p nor Clb4p can completely substitute for Clb5p at late or even at early origins (Donaldson 2000). Clearly, Cdk1p/Clb2p has specificity distinct from that of Clb5p and Clb6p (Donaldson 2000). The fact that the strain in which all six *CLB* genes are deleted is viable if Clb1p is overexpressed indicates that this specificity can be subverted by overexpression of a single Clb protein (Haase and Reed 1999).

Several lines of evidence point to the Mcm complex as the substrate of Cdc7p kinase. Mcm2p is phosphorylated

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both *in vivo* and *in vitro* by Cdc7p/Dbf4p from yeast and mammalian cells (summarized in Sclafani 2000). A mutation in *MCM5* called *mcm5-bob1* can bypass the requirement of Cdc7p/Dbf4p in DNA replication (Jackson *et al.* 1993; Hardy *et al.* 1997). In this regard, *mcm5-bob1* is a bypass suppressor capable of suppressing both *cdc7* and *dbf4* deletions. Cdk1p complexes with any Clb protein are also responsible for blocking rereplication during S phase by phosphorylation of at least Cdc6p, the Mcm complex, and the ORC (reviewed in Kelly and Brown 2000; Nguyen *et al.* 2001). However, the Cdk1p kinase substrate(s) needed to activate DNA replication is unknown. Cdc45p has been shown to load DNA polymerases and other replication proteins onto origins during S phase in yeast *in vivo* (Zou and Stillman 1998, 2000; Aparicio *et al.* 1999) and Xenopus *in vitro* (Mimura and Takisawa 1998; Walter and Newport 2000). In Xenopus, depletion of Cdc7p or inhibition of Cdk2 activity blocks the loading of Cdc45p (Jares and Blow 2000; Walter 2000). Both kinases are also required in yeast for this step (Zou and Stillman 2000), although the order of events is reversed with Cdc7p acting after Cdk1p in yeast (Nougarede *et al.* 2000) and vice versa in Xenopus (Jares and Blow 2000; Walter 2000). One model suggests that phosphorylation of the pre-RC by both kinases results in two events: activation of the Mcm helicase and recruitment of Cdc45p, thus producing binding of the necessary DNA replication proteins and fork movement (Jares *et al.* 2000; Lei and Tye 2001).

Our goal in this report was to investigate the mechanism by which the *mcm5-bob1* suppressor bypasses the requirement for Cdc7/Dbf4 kinase in DNA replication. This analysis has helped to clarify the role of Cdc7p/Dbf4p and Cdk1p/Clbp kinases in DNA replication in eukaryotic cells. It has been shown that changes in chromatin structure of the origin ARS1, which are normally dependent on Cdc7p/Dbf4p, occur constitutively in G1 phase in the *mcm5-bob1* mutant (Geraghty *et al.* 2000). These changes are consistent with unwinding of the origin at the ARS1 B2 element, which is important for stable Mcm2p and perhaps Cdc45p binding (Zou and Stillman 2000). We propose that Mcm5-bob1 protein has a conformation that produces this unwinding without prior phosphorylation by Cdk1/Clb and Cdc7p/Dbfp. This allows for stable binding of Cdc45p to the origin. However, DNA replication cannot occur without the combined action of Cdk1p/Clb5p and Cdk1p/Clb2p.

MATERIALS AND METHODS

Yeast strains, media, and plasmids: Yeast strains were grown in yeast extract/peptone/dextrose (YPD) with 2% glucose or in synthetic defined (SD) minimal media supplemented with appropriate amino acids and 2% glucose (Sclafani *et al.* 1988). For galactose induction, YP with 2% raffinose as carbon source was used and galactose was added to 2% for induction (Oshiro *et al.* 1999). We have found that raffinose can be substituted with 2% sucrose and the same level of induction occurs. All yeast strains used in this study are listed in Table 1. All strains are congenic with A364a (Hartwell 1967). Standard genetic methods were used for strain construction and tetrad analysis (Sherman *et al.* 1979) and transformation of yeast strains was performed by the lithium acetate method (Ito *et al.* 1983). The presence of the *mcm5-bob1* mutation was assayed by complementation and/or papillation assays (Jackson *et al.* 1993; Hardy *et al.* 1997). Briefly, *mcm5-bob1* is recessive so *cdc7ts/cdc7ts mcm5-bob1/+* diploids are Tsm−. These diploids will papillate to Tsm+ due to the high rate of mitotic recombination (5–10%) at the proximal rDNA on chromosome XII, which produces *mcm5-bob1* homozygotes (Jackson *et al.* 1993; Hardy *et al.* 1997).

All plasmids used in this study are listed in Table 1. All disruptions were produced by one-step gene disruption (Rothstein 1983) in the A364a genetic background. All disruptions were verified by Southern genomic hybridization (Sclafani and Fangman 1984). All disruptions were also crossed to wild-type strains and the disruption segregated predominantly 2:2 in the resultant asci. Additional strains were produced from the disruptions using standard genetic techniques. The *trp1::hisG* mutation was added to strain 820 to produce strain 876 as described (Alani *et al.* 1987). For *clb1*, a 3.2-kb *Eco*RI-*Xho*I fragment from plasmid scb1::URA3 that contains an insertionally inactivated *clb1::URA3* allele (Ghiara *et al.* 1991) was transformed into strain P253 to generate strain 947 by selecting for Ura^+. For *clb2::LEU2*, genomic DNA was purified (Holm *et al.* 1986) from strain G144 (Shellman *et al.* 1999) and used for amplification of the 3-kb *clb2::LEU2* fragment (Surana *et al.* 1991) by PCR using forward and reverse primers, 5′ CGTTGGATCAAGCACTGAGG 3′ and 5′ CCCTCTTCTCATTCATGCAAGG 3′, respectively. The DNA was purified (QIAGEN, Valencia, CA) and transformed into *leu2*− yeast selecting for Leu^+. For *clb5*, a 4.8-kb *Xho*I-*Spe*I *clb5::ARG4* fragment from plasmid clb5::ARG4ΔBspE1 (Epstein and Cross 1992) was transformed into *arg4*− yeast selecting for Arg+. For *clb3*, the *Pvu*II fragment from pCD3 (Fitch *et al.* 1992) was transformed into *trp1*− yeast selecting for Trp+. For *clb4*, the *Pvu*II fragment from pCD4 (Fitch *et al.* 1992) was transformed into *his3*− yeast selecting for His+. For *clb6*, the 4.5-kb *Bgl*II fragment from *clb6::ADE1* plasmid (Basco *et al.* 1995) was transformed into *ade1*− yeast selecting for Ade+. For *sic1*, the 3-kb *Eco*RI-*Hin*dIII *sic1::URA3* fragment from plasmid pMDM203 (Schwob *et al.* 1994) was transformed into *ura3*− yeast selecting for Ura+.

During the course of this work, we discovered that many ARS-containing vectors are unable to be maintained in *mcm5-bob1 cdc7ts* strains at the restrictive temperature. This is probably because *mcm5-bob1* is inefficient at origin firing when Cdc7p is absent and the plasmids have only one origin as opposed to normal chromosomes, which have many origins (R. A. Sclafani, S. Hunt, B. Brewer and W. Fangman, unpublished results). We found that vectors with 2μ origins are stable and integrating vectors can be used as they are replicated passively using chromosomal origins. Therefore, we had to produce or use 2μ or integrating constructs. Plasmid pGal-CLB2 (Fitch *et al.* 1992) was integrated into strain 876 to produce strain 914 by targeting it to integrate at the *trp1::hisG* locus by digestion with *Bsm*F1 and selection for Trp+ after transformation. Plasmids pRAS531, pRAS532, and pRAS533 were produced by ligation of the *Cla*I-*Xho*I *CLB5*, *clb5::CLB2*, or *clb5::clb2Δdb* fragments from plasmids HAdR1, C5C2-3NF, or C5C2-DB1, respectively (Cross *et al.* 1999) into the integrating vector pRS306 (Sikorski and Hieter 1989). The *clb2Δdb* mutation contains the P55Q change and a deletion of amino acids

56–64, which removes the destruction box and prevents Clb2p from being destroyed by the Hct1/Cdh1 complex (Cross *et al.* 1999). All three plasmids were targeted to integrate at the *ura3-52* locus by digestion with *Nco*I, subsequent transformation into *ura3*− yeast, and selection for Ura+. pRAS440 (2μ) was produced from pWS12933 (ARS1 CEN4) by ligation of the 2.2-kb *Hin*dIII Gal-CLB5-HA fragment into the pRS426 vector (Sikorski and Hieter 1989).

The Cdc45-3XHA tag was added to strains 311 and 728 (Hardy *et al.* 1997) by targeting it to integrate at the *CDC45* locus by *Cla*I digestion of plasmid p306cdc45-HA/C (Aparicio *et al.* 1999) and subsequent transformation and selection for Ura+. In this case, the insertions were verified by immunoblot with anti-HA antibody (Oshiro *et al.* 1999) and PCR analysis (Aparicio *et al.* 1999).

Fluorescence-activated cell sorter analysis: Cells were grown in synthetic defined media or YPD medium to a density of 2–4 × 10^6 cells/ml or 1–2 × 10^7 cells/ml (midlogarithmic phase), respectively, and then processed for fluorescence-activated cell sorter (FACS) analysis as previously described (Ostroff and Sclafani 1995). Cell numbers and sizes were determined with a Coulter Multisizer II using an aperture tube with a 100-μm orifice and latex beads as size standards.

Chromatin immunoprecipitation analysis: Chromatin immunoprecipitation (ChIP) assays followed the procedure described in Meluh and Koshland (1997), using Cdc45p, which was hemagglutinin (HA) tagged (Aparicio *et al.* 1997, 1999). Briefly, cells were fixed with formaldehyde and chromatin was isolated and immunoprecipitated with anti-HA antibody (12CA5; Boehringer Mannheim, Indianapolis). Anti-HA immunoprecipitates were then isolated with protein A-sepharose beads and then eluted from the beads. Cross-links were reversed by heat and protein was degraded by proteinase K digestion. Nucleic acids were then phenol extracted, isolated, and analyzed by PCR. PCR primers and conditions for ARS305, ARS1, ARS501, and ARS305/8 kb were as described (Aparicio *et al.* 1997, 1999). PCR products were subjected to electrophoresis in 2% agarose gels with 1× TAE buffer. Gels were stained with ethidium bromide to visualize the fluorescent DNA and photographed using a Bio-Rad (Richmond, CA) Fluor-Imager. Relative quantities of the fluorescent signal were estimated using ImageQuant software (version 1.11; Molecular Dynamics, Sunnyvale, CA) as described (Megee *et al.* 1999). The PCR reaction was determined to be linear over a fivefold range using different amounts of template DNA in the reaction.

Cell synchrony: Cells in midlogarithmic phase were synchronized with synthetic α-factor (200 nm or 20 μm for *bar1* or *BAR1*+ strains, respectively) and released in prewarmed YPD medium containing pronase (Ostroff and Sclafani 1995) at the appropriate temperature. Synchrony was monitored by phase-contrast microscopy at 400× magnification (90–95% unbudded cells indicated α-factor arrest). Arrest and released samples were analyzed by flow cytometry to determine the degree of DNA replication and cell cycle position.

RESULTS

Cdc45p can be loaded onto early origins in G1 phase in the *mcm5-bob1* mutant in the absence of *CLB*s: It has been shown that certain structural alterations present in chromatin at ARS1 in S phase are dependent on active Cdc7/Dbf4 kinase. In the *mcm5-bob1* mutant, these alterations are present constitutively in G1 phase, when both Cdc7p and Cdk1p kinases are inactive (Geraghty *et al.* 2000). It is not known what proteins are responsible for these changes. One hypothesis to explain these results is that phosphorylation by both kinases is needed to produce the alteration and the *mcm5-bob1* mutation mimics this alteration. Because Cdc45p loading at origins requires both kinases (Zou and Stillman 2000), we measured Cdc45p loading onto several origins by the ChIP method (Aparicio *et al.* 1999; Zou and Stillman 2000). An HA-tagged *CDC45* was integrated into both wild-type (311) and *mcm5-bob1* mutant (728) strains (Table 1), to produce strains 923 and 924, respectively (materials and methods). The tagged Cdc45p is the only Cdc45p produced by these strains due to the integration (Aparicio *et al.* 1999). Strains 923 and 924 were arrested in G1 phase with α-factor and released for a short time to monitor early events such as Cdc45p loading at ARS305 and ARS1 (Aparicio *et al.* 1999). It has been shown that Cdc45p loading is low (Aparicio *et al.* 1999) or even undetectable (Zou and Stillman 2000) in G1-arrested cells. Similar results are seen at both ARS305 and ARS1 (Figure 1). In contrast, a fourfold increase in Cdc45p bound to ARS305 and ARS1 is seen in the *mcm5-bob1* mutant in G1 phase (Figure 1). In wild-type cells, Cdc45p loading at both ARS1 and ARS305 occurs 20–40 min after the release just when DNA synthesis begins (Figure 1), as previously described (Aparicio *et al.* 1999; Zou and Stillman 2000). Cdc45p binding is specific to origin DNA, as it is not found at non-origin DNA (8 kb from ARS305). Interestingly, the increase in Cdc45p loading seen in *mcm5-bob1* is not found at a late origin, ARS501. In all cases, the PCR reaction was shown to be in the linear range (materials and methods). This result is not due to increased levels of Cdc45p as the level of Cdc45p in both wild-type and *mcm5-bob1* mutant strains is similar (data not shown). We conclude that *mcm5-bob1* allows for constitutive loading of Cdc45p at early origins. Cdc45p loading may be responsible for the structural changes seen at ARS1 in this mutant (Geraghty *et al.* 2000).

Complete bypass of Cdc7p/Dbf4p is dependent on both Clb5p and Clb2p, but not on other *CLB*s: Both Cdc7p and Cdk1p kinases are needed for the initiation of DNA replication (Kelly and Brown 2000; Sclafani 2000; Lei and Tye 2001). The *mcm5-bob1* mutation allows for Cdc45p loading without both kinases (Figure 1), yet DNA replication still does not occur. Therefore, we investigated the role of Cdk1p kinase in *mcm5-bob1* bypass by performing genetic experiments using *cdc7ts mcm5-bob1* strains and strains with deletions of the *CLB* genes. In this type of analysis, we test if genetic interactions such as synthetic lethality or suppression occur. Initially, we crossed a *mcm5-bob1 cdc7ts* strain (747) with a *clb5*Δ strain (755; Table 2). Because *MCM5* (chromosome XII) and *CDC7* (chromosome IV) are unlinked, the predominant ascus will display 3+:1− temperature-sensitive mutant (Tsm) segregation as indicative of an extragenic suppressor (Hardy *et al.* 1997). However, we see a reduction of 3+:1− asci and an increase of

TABLE 1

Strains and plasmids used in this study

Strain	Genotype
299	*MATα his3Δ1 leu2-3,112 trp1-289 ura3-52 can1 cyh2*
451	*MAT***a** *ura3-52 trp1-289 his3Δ1 cyh2*
302	*MATα cdc7-7 leu2-3,112 trp1-289 his3Δ1 ura3-52*
303	*MAT***a** *cdc7-7 leu2-3,112 ura1 his3Δ1*
P253	*MAT***a** *cdc7-1 mcm5-bob1 ura3-52 can1 trp1-289 his3Δ1*
743	*MAT***a** *leu2-3,112 trp1-289 ade1 arg4 his7 his3Δ1 cyh2*
747	*MATα cdc7-7 mcm5-bob1 ade1 arg4 his7 ura1 lys2*
755	*MAT***a** *clb5::ARG4 trp1-289 leu2-3,112 ade1 arg4 his3Δ1 his7 cyh2*
775	*MATα cdc7-1 clb5::ARG4 mcm5-bob1 arg4 ura3-52 can1 cyh2 his3Δ1 ade1*
772	*MATα cdc7-7 mcm5-bob1 clb5::ARG4 clb6::ADE1 arg4 ade1 his7 leu2 ura1 cyh2*
773	*MATα cdc7-7 mcm5-bob1 clb5::ARG4 ade1 arg4 his7 ura1 lys2*
816	*MATα cdc7-1 mcm5-bob1 clb2::LEU2 leu2-3,112 ura3-52*
947	*MAT***a** *cdc7-1 mcm5-bob1 clb1::URA3 ura3-52 can1 trp1-289 his3Δ1*
820	*MAT***a** *cdc7-1 mcm5-bob1 clb2::LEU2 leu2-3,112 ura3-52 can1 cyh2*
805	*MAT***a** *cdc7-1 mcm5-bob1 clb3::TRP1 ura3-52 can1 trp1-289 his3Δ1*
807	*MAT***a** *cdc7-1 mcm5-bob1 clb4::HIS3 ura3-52 can1 trp1-289 his3Δ1*
762	*MAT***a** *cdc7-7 mcm5-bob1 clb6::ADE1 leu2 ade1 arg4 his7 his3Δ1*
875	*MATα cdc7-1 clb5::ARG4 mcm5-bob1 arg4 ura3-52 can1 cyh2 his3Δ1 ade1 trp1::hisG*
876	*MAT***a** *cdc7-1 mcm5-bob1 clb2::LEU2 leu2-3,112 ura3-52 can1 cyh2 trp1::hisG*
935	*MAT***a** *bar1 ura3-52 trp1-289 leu2-3,112 clb2::LEU2 can1 his6*
913	*MAT***a** *cdc7-1 mcm5-bob1 clb2::LEU2 leu2-3,112 ura3-52 can1 cyh2 trp1::hisG::Gal-CLB2 TRP1*
914	*MAT***a** *cdc7-1 mcm5-bob1 clb2::LEU2 leu2-3,112 ura3-52 can1 cyh2 trp1::hisG::Gal-CLB2 TRP1*
778	*MATα cdc7-1 mcm5-bob1 clb5::ARG4 ura3-52 leu2-3,112 can1 sic1Δ::URA3*
685	*MATα cdc7-1 mcm5-bob1 sic1Δ::URA3 leu2-3,112 his7 ura3-52 can1 cyh2 lys2*
311	*MAT***a** *bar1 ura3-52 trp1-289 leu2-3,112 can1 his6*
923	*MAT***a** *bar1 ura3-52 trp1-289 leu2-3,112 can1 his6 cdc45::p306CDC45-3XHA URA3+*
728	*MAT***a** *bar1 his6 mcm5-bob1 ura3-52 trp1-289 leu2-3,112 lys2 his3Δ1*
924	*MAT***a** *bar1 his6 mcm5-bob1 ura3-52 trp1-289 leu2-3,112 lys2 his3Δ1 cdc45::p306CDC45-3XHA URA3+*
810	*MAT***a** *ura1 his7 cdc4-1 mcm5-bob1*
947	*MAT***a** *cdc7-1 mcm5-bob1 ura3-52 can1 trp1-289 his3Δ1 clb1::URA3*

Plasmid	Genotype	Source/reference
pWS12933	ARS1 CEN4 URA3 Gal-CLB5-HA	B. Futcher
pRAS440	2μ URA3 Gal-CLB5-HA	This work
pGal-CLB2	Yip lac204 Gal-CLB2 TRP1	Fitch *et al.* (1992)
pRAS531	Yip 3X-HA CLB5	This work
pRAS532	YipClb5p-3X-HA CLB2	This work
pRAS533	YipClb5p-3X-HA clb2Δdb	This work
p306cdc45-HA/C	Yip URA3 C terminus of Cdc45 fused to 3X-HA	Aparicio *et al.* (1999)
pMDM203	sic1::URA3	Schwob *et al.* (1994)
pYQ122	2μ URA3 Gal-HA-6Xhis-CLN2	Shellman *et al.* (1999)
YDL155WY	2μ URA3 Gal-HA-6Xhis-CLB3	Invitrogen
YLR103CY	2μ URA3 Gal-HA-6Xhis-Cdc45	Invitrogen
clb5::ARG4ΔBspE1	ARS CEN TRP1 clb5::ARG4	Epstein and Cross (1992)
clb6::ADE1	pBLUEScript KS clb6::ADE1	Basco *et al.* (1995)
pCD3	pUC119 clb3::TRP1	Fitch *et al.* (1992)
pCD4	pUC119 clb4::HIS3	Fitch *et al.* (1992)
scb1::URA3	pT7T319U clb1::URA3	Ghiara *et al.* (1991)

2+:2− asci. The result is statistically significant to $P < 0.01$. This implies that the *mcm5-bob1* suppressor is being suppressed in some of the Tsm− segregants, resulting in an abundance of Tsm− spores. We hypothesize that the *cdc7ts mcm5-bob1 clb5* strains had a Tsm− phenotype because the *clb5* mutation is suppressing the *mcm5-bob1* suppressor. This was confirmed by a complementation test. Ten Arg+ Tsm− (*clb5::ARG4 cdc7ts*) colonies were selected. We would expect 50% to contain *mcm5-bob1*. When these strains are mated to *cdc7ts* tester strains of either mating type (strains 302 or 303), the resultant diploid cells will be Tsm− because *mcm5-bob1* either is not present or is inactive due to *clb5* mutation in the original parent. If the recessive *mcm5-bob1* is present,

then the homozygous *cdc7ts* diploid will papillate to Tsm+ due to the high rate of mitotic recombination at the rDNA locus, which is proximal and produces *mcm5-bob1/mcm5-bob1* homozygotes at high frequency (5–10%; Hardy *et al.* 1997). As expected, 50% (6/12) of the Arg+ Tsm− strains tested papillated to Tsm+. This indicates that *mcm5-bob1* was present in 50% of these strains even though they had a Tsm− phenotype. To directly confirm this conclusion, an *arg4− cdc7ts mcm5-bob1* strain (747) was transformed with a linear DNA containing the *clb5::ARG4* disruption (Epstein and Cross 1992). All (50/50) *clb5::ARG4* transformants analyzed now became Tsm−. Similar transformation procedures were used to disrupt the *CLB1*, *CLB2*, *CLB3*, *CLB4*, or *CLB6* genes (materials and methods). The results are summarized in Table 3. Only deletion of *CLB5* or *CLB2* suppressed *mcm5-bob1*. In addition, deletion of *SIC1*, which is an inhibitor of Clb5p (Schwob *et al.* 1994), resulted in an increase of *mcm5-bob1* suppression, as the *sic1 mcm5-bob1 cdc7ts* colonies grew faster at the restrictive temperature. However, this increase of *mcm5-bob1* suppression could not compensate for the loss of *CLB5*, as the *mcm5-bob1 cdc7ts clb5 sic1* strain remained Tsm−. We conclude that Clb5p and Clb2p are required for the *mcm5-bob1* mutation to suppress *cdc7ts* mutations.

Both *clb5* and *clb2 mcm5-bob1 cdc7ts* cells arrest at the G1/S boundary: In the case of Clb5p, our results were consistent with the role of Cdk1p/Clb5p at the G1/S boundary (Schwob and Nasmyth 1993; Nasmyth 1996b). However, our results with Clb2p are surprising as Clb2p has its major function in mitosis (Fitch *et al.* 1992), although there is much overlap in the function and role of all six *CLB* genes (Nasmyth 1996a). Therefore we tested if the *cdc7ts mcm5-bob1 clb5* or *clb2* strains had a similar phenotype to *cdc7ts* strains at the restrictive temperature; that is, cells with a 1C DNA content arrested at the G1/S boundary (Sclafani 2000). Both the *clb5 mcm5-bob1 cdc7ts* strain (773) and the *clb5 clb6 mcm5-bob1 cdc7ts* strain (772) arrested with a 1C DNA content, indicative of a G1 arrest after 1.5 hr at the restrictive temperature (Figure 2A). As *clb5* mutants have a wider S phase peak (Epstein and Cross 1992; Schwob and Nasmyth 1993), it is easier to distinguish the G1 peak in the *clb5 clb6 mcm5-bob1 cdc7ts* strain (772) rather than in the corresponding *clb5* strain (773). Of these cells, 90–95% displayed the large-budded Cdc phenotype as expected of an arrested *cdc7ts* mutant (Hartwell *et al.* 1973). In contrast, the isogenic wild-type strain (743), the *clb6 mcm5-bob1 cdc7ts* strain (762), or the *CLB+ mcm5-bob1 cdc7ts* strain (747; data not shown) continue to cycle and display both 1C and 2C peaks. We conclude that loss of Clb5p completely suppresses *mcm5-bob1* and prevents S phase entry of cells without Cdc7p function.

With the *clb2* strains it was difficult to see an effect at the restrictive temperature because the cells have such a pronounced G2/M defect (Ghiara *et al.* 1991; Surana *et al.* 1991; Fitch *et al.* 1992). Essentially, even after 4 hr at the restrictive temperature, *cdc7ts mcm5-bob1 clb2* cells remain arrested with a 2C DNA content, indicative of a G2/M arrest (data not shown). Therefore, we first arrested a *clb2 mcm5-bob1 cdc7ts* strain (820) in G1 phase with α-factor at the permissive temperature and then released it to the restrictive temperature (Figure 2B; Ostroff and Sclafani 1995). If no bypass occurs, then S phase will not occur and the cells will remain at the G1/S boundary. As expected, after α-factor arrest this strain exhibits only a 1C DNA content indicative of a G1 arrest. After the release from α-factor arrest for 2 or 4 hr at the restrictive temperature, the cells retained a 1C DNA content. The cells released at the permissive temperature entered S phase and continued to cycle. In contrast, cells of control wild-type strain 311 or *clb2* strain 935 exhibited mainly a G2 peak after 2 hr at the restrictive temperature (data not shown) and therefore had completed replication.

As seen with the *clb5 mcm5-bob1 cdc7ts* strain, >90–95% of the cells displayed the large-budded Cdc phenotype. We conclude that both Clb5p and Clb2p are needed for *mcm5-bob1* to bypass the role of Cdc7p/Dbf4p in DNA replication.

Clb5p and Clb2p perform distinct roles in *mcm5-bob1* suppression of *cdc7ts*: Because Clb5p and Clb2p are major B-type cyclins in yeast (Nasmyth 1996a), it is possible that a reduction in total Cdk1p/Clbp activity by deletion of either *CLB5* or *CLB2* may be responsible for *mcm5-bob1* suppression of the *cdc7ts* mutation. Another hypothesis is that Clb5p and Clb2 perform distinct roles in the suppression and, therefore, both are required. We tested if overexpression of *CLB5* could complement the *clb2* defect and vice versa (Table 4). A *clb5 mcm5-bob1 cdc7ts* strain (775) and a *clb2 mcm5-bob1 cdc7ts* strain (820) were transformed with plasmids pGal-CLB2 or pGal-CLB5. Transformants were then tested for growth at the restrictive temperature. Growth under these conditions is indicative of suppression of *cdc7ts* by *mcm5-bob1*. As expected, the pGal-CLB5 plasmid complemented the *clb5* defect and the complementation was stronger in the presence of the galactose inducer, which increases the amount of Clb5 protein (Table 4). In contrast, overexpression of Clb5p was not able to complement the *clb2* defect. Similarly, the Clb2p overexpression could complement the *clb2* defect but not the *clb5* defect. We had to use the less restrictive temperature of 32° instead of 36° with Gal-CLB2 or no complementation was seen. This may be because constitutive expression of *CLB2* is known to have some detrimental effects (Fitch *et al.* 1992). Neither the *clb5* nor the *clb2* defect could be complemented by a Gal-CLN2 (Shellman *et al.* 1999) or a Gal-CLB3 expression plasmid. Therefore, our data support the latter hypothesis that Clb2p and Clb5p have distinct roles.

Our results are similar to those of Cross *et al.* (1999), who showed that expression of Clb2p in G1 phase via the *CLB5* promoter constructs failed to complement a

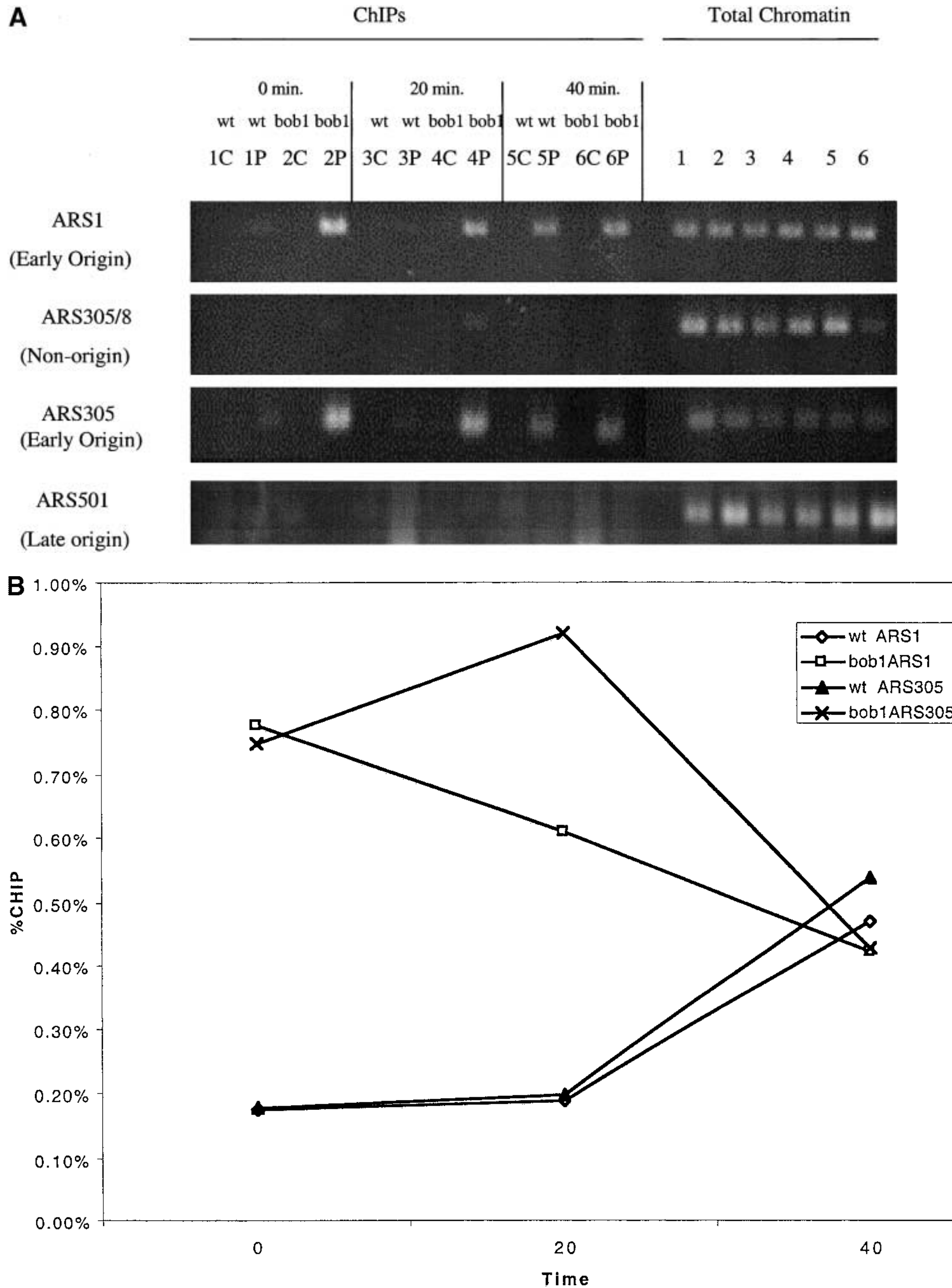

FIGURE 1.—Cdc45p binding to origins is higher in the *mcm5-bob1* mutant strain in G1 phase. (A) Agarose gel electrophoresis of PCR products from chromatin immunoprecipitates of wt (strain 923, lanes labeled 1, 3, or 5) and *mcm5-bob1* (strain 924, lanes labeled 2, 4, or 6) mutant cells with a tagged Cdc45-HA protein. The cells were synchronized in G1 with α-factor (0 min, lanes labeled 1 or 2) and released for 20 min (lanes labeled 3 or 4) or 40 min (lanes labeled 5 or 6). Chromatin was immunoprecipitated with anti-HA antibody and subjected to PCR to detect ARS1, ARS305, ARS305 + 8 kb, or ARS501 sequences. Lanes labeled "P" are from the anti-HA immunoprecipitates, while those labeled "C" are controls that did not contain any antibody. Total chromatin (1/250 dilution relative to immunoprecipitates) used was also subjected to PCR and is shown on the right. (B) ARS1 and ARS305 PCR products from A were estimated (MATERIALS AND METHODS) and normalized to the total amounts (% ChIP is amount immunoprecipitated). (C) Flow cytometry of cells used in A stained with propidium iodide.

clb5 defect. These same constructs were also used to show that Clb2p cannot completely substitute for Clb5p in the firing of origins in S phase (DONALDSON 2000). Therefore, we used these constructs to further test our hypothesis and make the case above (Table 5). We found that even when Clb2p is expressed in G1 phase from the *CLB5* promoter, it cannot complement the *clb5* defect. This is true even if the *clb2Δdb* mutant is used in which the destruction box is deleted. When this Clb2 mutant protein is produced, it is resistant to the effects of Hct1/Cdh1-regulated proteolysis by the APC (for a review, see PETERS 1998) and can accumulate earlier in the cell cycle like Clb5p (CROSS *et al.* 1999). Nevertheless, it fails to complement the *clb5* defect. However, all *clb5::clb2* constructs can complement the *clb2* defect. We conclude, as did CROSS *et al.* (1999), that the timing of *CLB* expression is not as important as the type of Clb protein that is produced.

DISCUSSION

In this report, we provide both genetic and molecular evidence that Cdc7p/Dbf4p and Cdk1p/Clbp kinases interact to regulate DNA replication. We have demonstrated that the bypass of Cdc7p/Dbf4p function by the *mcm5-bob1* suppressor requires both Clb5p and Clb2p (Table 3). The roles of these two forms of the Cdk1 protein kinase in bypass are distinct and cannot be sub-

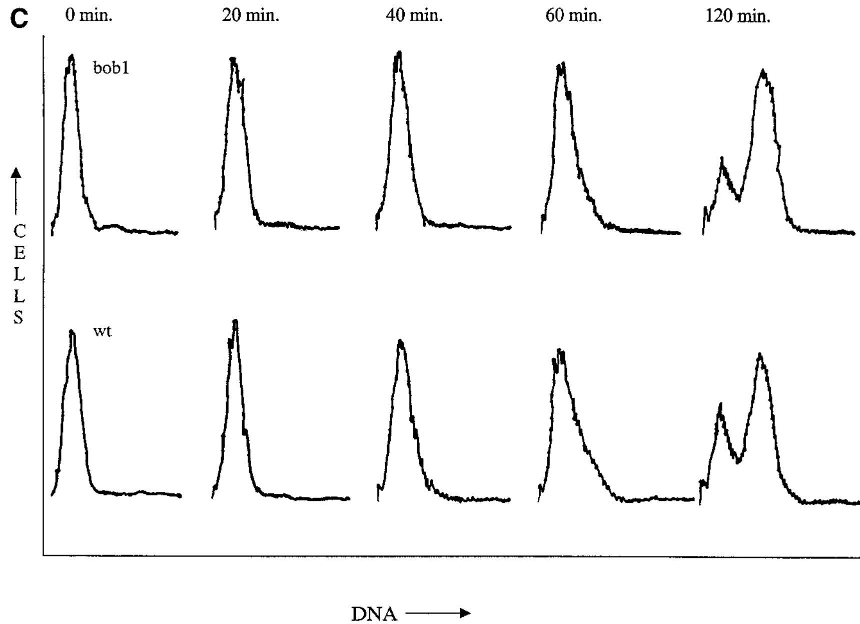

FIGURE 1.—*Continued.*

stituted by overexpression of other Clbs (Table 4). Similarly, the role of Clb5p in DNA replication cannot be substituted by the expression of the mitotic Clb2p in G1 phase (Table 5). At least with regard to the S phase role for Clb5p, others have reached a similar conclusion (CROSS *et al.* 1999; DONALDSON 2000). These latter two studies have shown that Clb5p is needed for origin activation in DNA replication and that Clb2p cannot substitute efficiently in this function.

Why does *mcm5-bob1* depend on both Clb5p and Clb2p to bypass Cdc7p/Dbf4p? Normally Clb5p and Clb2p are not essential for DNA replication, presumably because other Clb proteins can substitute (SCHWOB and NASMYTH 1993; NASMYTH 1996b). The absence of Cdc7p/Dbf4p in the *mcm5-bob1* mutant may uncover the otherwise nonessential but distinctive roles of Clb5p and Clb2p in DNA replication. One hypothesis is that the *mcm5-bob1* suppressor is inefficient and a further reduction of efficiency by removing either Clb5p or Clb2p from this pathway is lethal. Evidence for *mcm5-bob1* inefficiency in DNA replication is seen in that *cdc7*Δ *mcm5-bob1* strains grow slower (JACKSON *et al.* 1993; HARDY *et al.* 1997; WEINREICH and STILLMAN 1999), are sensitive to hydroxyurea (HU; WEINREICH and STILLMAN 1999), and fail to support replication of some ARS plasmids (MATERIALS AND METHODS). Efficient DNA replication would occur only if one attains a threshold level of modification due to the combined action of Cdc7p/Dbf4p, Cdk1p/Clb5p, and Cdk1p/Clb2p. In this model, Cdk1p/Clb2p, Cdk1p/Clb5p, and Cdc7p/Dbf4p would act independently to modify the DNA replication complex. The sum of these modifications assures efficient replication.

The requirement for Clb5p in DNA replication is

TABLE 2

Results of crosses of *clb5* or *CLB5*+ × *mcm5-bob1 cdc7ts*

Tsm+:Tsm−[a] (Ascus type)	Observed (× *clb5*)	Expected[b]	Observed (× *CLB5*+)
2:2 (NPD)	12	2	1
3:1 (T)	1	8	7
4:0 (PD)	0	2	0
P value[c]	<0.01	NA	0.2

NA, not applicable.

[a] Tsm is the phenotype of a temperature-sensitive mutant.

[b] Calculated from the 1:1:4 ratio of parental ditype:nonparental ditype:tetratype (PD:NPD:T) asci expected for unlinked genes (SHERMAN *et al.* 1979).

[c] Calculated by χ^2 test.

TABLE 3

Suppression of *mcm5-bob1* by *clb5* and *clb2* deletions

Strain	Relevant genotype[a]	36°[b]	Conclusion
747	*CLB*+	+	Bypass
775	*clb5*	−	No bypass
772	*clb5 clb6*	−	No bypass
820	*clb2*	−	No bypass
947	*clb1*	+	Bypass
805	*clb3*	+	Bypass
807	*clb4*	+	Bypass
762	*clb6*	+	Bypass
685	*CLB*+ *sic1*	++	Bypass
778	*clb5 sic1*	−	No bypass

[a] All strains are *mcm5-bob1 cdc7ts* (Table 1).

[b] Strains were streaked for single colonies on YPD plates and incubated at the restrictive temperature of 36° for 2 days. +, colony formation; +/+, the formation of larger colonies; −, a failure to form colonies.

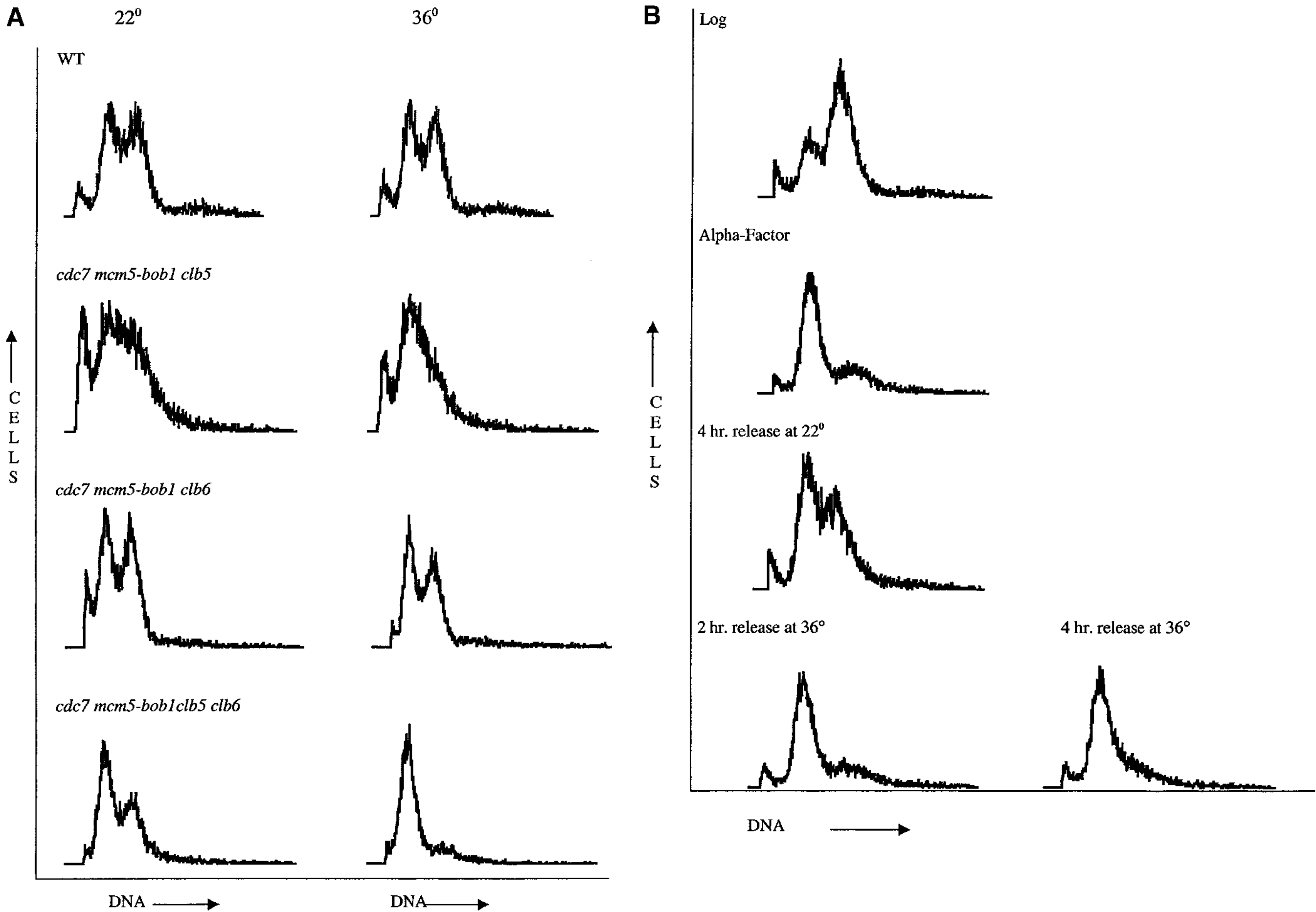

Figure 2.—Both *clb5 clb6 mcm5-bob1 cdc7ts* and *clb2 mcm5-bob1 cdc7ts* mutant cells arrest in G1 phase. Flow cytometry was used to measure the cell cycle position and DNA content of (A) cells of wt strain 743, *mcm5-bob1 cdc7ts clb5* strain 773, *clb6 mcm5-bob1 cdc7ts* strain 762, or *clb5 clb6 mcm5-bob1 cdc7ts* strain 772 grown at 22° and after being shifted to 36° for 1.5 hr. The profile for the *CLB+ mcm5-bob1 cdc7ts* strain (747) was similar to strains 743 and 762 (data not shown) or (B) cells of *clb2 mcm5-bob1 cdc7ts* strain 820 before (log) or after being arrested with α-factor for 3 hr and then released at 22° or 36° for 2 or 4 hr. Cells of control wild-type strain 311 or *clb2* strain 935 exhibited mainly a G2 peak after 2 hr at 36° (data not shown) and therefore had completed replication.

expected as it is known to have a role in the process (Donaldson *et al.* 1998b). However, the requirement for Clb2p is surprising as its major role is believed to be in mitosis (Nasmyth 1996a,b). The question is whether the role of Clb2p in *mcm5-bob1* bypass is executed in G1 phase like Cdc7p function (Hartwell *et al.* 1973; Sclafani 2000) or in mitosis like Clb2p function (Nasmyth 1996a,b). Our flow cytometry data indicate that a *mcm5-bob1 cdc7ts clb2* strain will arrest before S phase like a *cdc7ts* strain using cultures synchronized in G1 (Figure 2B). However, asynchronous *mcm5-bob1 cdc7ts clb2* cultures remain at G2/M after being shifted to the restrictive temperature (data not shown). Thus, it remains a formal possibility that Clb2p may be needed for both S phase and mitosis in this strain. The best way to resolve this question is to perform execution-point analysis (Hartwell *et al.* 1973). We have tried unsuccessfully to map the execution point of Clb2p using strain 914 (Table 1), which is a *mcm5-bob1 cdc7ts clb2* strain with a conditional *Gal-CLB2* gene. This is because we have had to use 32° as the restrictive temperature for this strain (Table 4) and the *cdc7ts* mutation is sufficiently leaky at 32° to give several cycles of DNA replication before arrest occurs. Thus the determination of the *CLB2* execution point must await a different experimental strategy, which is in progress.

We cannot rule out the possibility that the effect of removing Clb5p or Clb2p is indirect and affects the expression of other gene products that are needed for *mcm5-bob1* bypass. In fact, Clb2p is known to regulate transcript levels of many genes that are cell-cycle regulated, including *MCM 2-7* and *CDC45* in mitosis (Spellman *et al.* 1998). However, the protein levels of these genes were not analyzed in that study. Although the transcript levels of *MCM* genes fluctuate during the cell cycle, there is no corresponding change in protein levels, which remain constant (for a review, see Kearsey and Labib 1998). For example, we have found that

TABLE 4

Effect of *CLB* or *CLN* overexpression on *mcm5-bob1* suppression of *cdc7*

Strain	Relevant genotype[a]	Plasmid	RT[b]	RT + Gal[b]
775	*clb5*	2μ Gal-CLB5	±	+
820	*clb2*	2μ Gal-CLB5	−	−
913	*clb5*	::Gal-CLB2[c]	−	−
914	*clb2*	::Gal-CLB2[c]	−	+
775	*clb5*	2μ Gal-CLB3	−	−
820	*clb2*	2μ Gal-CLB3	−	−
775	*clb5*	2μ Gal-CLN2	−	−
829	*clb2*	2μ Gal-CLN2	−	−

[a] All strains are *mcm5-bob1 cdc7ts* (Table 1).
[b] Strains were streaked for single colonies on YP raffinose or YP raffinose + galactose plates (+Gal) and incubated at the restrictive temperature (RT) for 2 days. +, colony formation; ±, the formation of small colonies; −, a failure to form colonies. Restrictive temperature was 36° or 32° for *clb5* strains, but 32° for *clb2* strains. All strains could grow on either growth medium at 22°.
[c] ::, plasmid was integrated.

TABLE 5

Effect of *CLB2* expression from the *CLB5* promoter on *mcm5-bob1* suppression

Strain	Relevant genotype[a]	Plasmid	36°[b]
875	*clb5*	::pRS306[c]	−
875	*clb5*	::pClb5p-CLB2[c]	−
875	*clb5*	::pClb5p-clb2Δdb[c]	−
876	*clb2*	::pRS306[c]	−
876	*clb2*	::pClb5p-CLB2[c]	+
876	*clb2*	::pClb5p-clb2Δdb[c]	+

[a] All strains are *mcm5-bob1 cdc7ts* (Table 1).
[b] Strains were streaked for single colonies on YPD plates and incubated at the restrictive temperature of 36° for 2 days. Plasmid pRS306 is the vector for both the pClb5p-CLB2 plasmid pRAS532 and the pClb5p-clb2Δdb plasmid pRAS533 (Table 1; materials and methods). +, colony formation; −, a failure to form colonies. All strains could grow at 22°.
[c] ::, plasmid was integrated.

Mcm2p levels are unaffected by a deletion of *CLB2* or *CLB5* (data not shown). Therefore, although it is unlikely that the loss of *CLB2* results in changes in expression of other critical proteins, it is still remains a possibility.

We have also shown that the *mcm5-bob1* mutation allows for the loading of Cdc45p at early origins in G1 phase-arrested cells (Figure 1), in which both Cdk1p and Cdc7p kinases are inactive (Kelly and Brown 2000; Sclafani 2000). Normally, the loading of Cdc45p is weak in G1 phase, increases as cells enter the S phase, and is dependent on both Cdk1p and Cdc7p kinases (Aparicio *et al.* 1999; Zou and Stillman 2000). In Xenopus, depletion of Cdc7p or inhibition of Cdk2 activity blocks the loading of Cdc45p (Jares and Blow 2000; Walter 2000). The interaction between Mcm2p, a known Cdc7p substrate (Sclafani 2000), and Cdc45p occurs after both kinases become active (Aparicio *et al.* 1999; Zou and Stillman 2000). Furthermore, both *CDC45* and *CDC7* have interdependent execution points (Owens *et al.* 1997), which suggests that they act at a common step in DNA replication. Therefore, Cdc7p and Cdk1p phosphorylation may stabilize the binding of Cdc45p to origins. This could occur by Mcm2 phosphorylation inducing a conformational change in the Mcm complex that stabilizes Cdc45p binding. The *mcm5-bob1* mutation may mimic this conformational change and allow for constitutive Cdc45p loading. Constitutive loading of Cdc45p in the *mcm5-bob1* mutant may explain why we observed that these cells advance into S phase faster than wild-type cells (Hardy *et al.* 1997).

The *mcm5-bob1* mutation results in a change of amino acid residue 83 from proline to leucine: P83L (Hardy *et al.* 1997). It is possible that this may produce a structural change in the Mcm2-7 protein complex. Indeed, changes in the chromatin structure of ARS1 that normally are dependent on Cdc7p can occur in G1 phase-arrested cells in *mcm5-bob1* mutant strains (Geraghty *et al.* 2000). These changes are consistent with unwinding of the origin at the ARS1 B2 element, which is important for stable Mcm2p and perhaps Cdc45p binding (Zou and Stillman 2000).

However, DNA replication does not occur in G1 in *mcm5-bob1* cells, but DNA replication must await activation of Cdk1p kinase. Therefore, additional events are required for complete replication bypass. We propose that these include modification(s) to the pre-RC by both Cdk1-Clb5 and Cdk1-Clb2 protein kinases. The result of these modifications could be the activation of Mcm helicase and the promotion of unwinding by Mcm helicase and Cdc45p. It has been suggested that both these proteins move with the replication fork (Jares *et al.* 2000; Walter and Newport 2000). Another possibility is that Mcm helicase is anchored to Mcm10p in the pre-RC and becomes disengaged at this point (Lei and Tye 2001). This is consistent with the failure of the *mcm5-bob1* mutation to bypass completely the requirement for Cdk1p/Clbp in G1 phase. This was demonstrated by us (Hardy *et al.* 1997) and others (Nougarede *et al.* 2000) by showing that *mcm5-bob1* could not bypass the DNA replication defect of a *cdc4ts* mutant, which is arrested in G1 phase with inactive Sic1p-Cdk1p-Clb5p complexes (Schwob *et al.* 1994).

Constitutive loading of Cdc45p at the late origin ARS501 does not occur in the *mcm5-bob1* mutant arrested in G1 phase (Figure 1), perhaps because Cdc45p loading at late origins occurs at late times in S phase (Aparicio *et al.* 1999) even though both kinases are

active at the beginning of S phase. In contrast, Mcm proteins load at both early and late origins in early G1 phase (Aparicio *et al.* 1999). The control of late replication at ARS501 is programmed in the preceding M phase by an unknown mechanism (Raghuraman *et al.* 1997). The *mcm5-bob1* mutation may have no effect on this unknown control mechanism and therefore cannot affect Cdc45p loading at ARS501. In *clb5* mutants, S phase is longer because late origins do not fire (Donaldson *et al.* 1998b). In *clb5 clb6* mutants, initiation is delayed and now late origins fire presumably using other Clb proteins. In contrast, we observe the same phenotype in both *clb5* and *clb5 clb6* mutants, that is, suppression of *mcm5-bob1* bypass (Table 3). Furthermore, loss of Cdc7p function just after early S phase still allows for replication of the entire genome without late origin firing (Bousset and Diffley 1998; Donaldson *et al.* 1998a). Therefore, we do not think that the suppression of the bypass phenotype by *clb5* and *clb2* mutations is related to the ability to fire late origins.

In summary, our data support the idea that Cdc7p kinase is needed for the loading of Cdc45p onto origins (Jares and Blow 2000; Walter 2000; Zou and Stillman 2000). We base this conclusion on the fact that the *mcm5-bob1* mutation, which bypasses Cdc7p/Dbf4p function, allows for constitutive loading of Cdc45p onto early origins in G1 phase of the cell cycle. Cdk1p kinase is also thought to be required for this step (Jares and Blow 2000; Walter 2000; Zou and Stillman 2000), but we show it can occur in *mcm5-bob1* mutant strains in a Cdk1p-independent manner. However, complete bypass of Cdc7p/Dbf4p is Cdk1p-dependent, requiring both Clb5p and Clb2p forms of the kinase for DNA replication. We have found that overexpression of Cdc45p with plasmid YLR103CY (Table 1) fails to suppress the phenotype of either *clb5* or *clb2 mcm5-bob1 cdc7ts* strains. Although it is a negative result, it is consistent with both kinases being required for other DNA replication functions in addition to Cdc45p loading.

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