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The yeast mitotic cyclin Clb2 cannot substitute for S phase cyclins in replication origin firing

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Cyclin-dependent kinases (CDKs) drive the cell cycle, central to which is the accurate control of chromosome replication. In *Saccharomyces cerevisiae*, six closely related B-type cyclins (Clb1–6) drive the events of S phase and mitosis. Either Clb5 or Clb6 can activate early-firing replication origins, whereas only Clb5 can activate late origins. Clb1–4 are expressed later in the cell cycle. Whether Clb cyclins differ only in timing of expression, or else impart different kinase specificities is under ongoing investigation. This study shows that the expression of Clb2 during S phase in cells lacking Clb5 failed to rescue late origin activation. Early expression of Clb2 in cells lacking both Clb5 and Clb6 did not activate early origins on schedule to restore the correct S phase entry time. Therefore, Clb2 cannot drive timely activation of either early or late replication origins, demonstrating that Clb2-directed CDK has a specificity distinct from that driven by Clb5 and Clb6.

INTRODUCTION

Nine cyclins are expressed in an ordered sequence and associate with the cyclin-dependent kinase (CDK) Cdc28 to drive the yeast cell cycle. The three G_1 (Cln) cyclins initially switch on a series of six B-type (Clb) cyclins, beginning with the expression of Clb5 and Clb6 during S phase, followed by Clb3 and Clb4 in early G_2, and finally Clb1 and Clb2 later in G_2 (reviewed in Nasmyth, 1996). Clb5 and Clb6 drive chromosome replication (Figure 1A) (Epstein and Cross, 1992; Kühne and Linder, 1993; Schwob and Nasmyth, 1993). Either Clb5 or Clb6 is able to activate early-firing replication origins but only Clb5 can activate late replication origins, so that S phase in *clb5* mutant cells is prolonged due to the failure of late origin activation (Figure 1B) (Donaldson *et al.*, 1998). Clb1,2,3 and 4 normally promote mitotic events. However, in the absence of Clb5 and Clb6, Clb1,2,3 and 4 can jointly take over the role of activating S phase by firing both early and late origins (Figure 1C). Such redundancy has led to questions concerning the extent to which the various B-type cyclin proteins are functionally interchangeable. This study addresses whether cells that express the mitotic cyclin Clb2 in place of one or both S phase cyclins can fire their replication origins correctly.

RESULTS AND DISCUSSION

Early expression of Clb2 cannot activate late origins

Cross *et al.* (1999) recently constructed a strain in which the *CLB5* open reading frame (ORF) is precisely replaced with that of *CLB2*. The resulting *clb5::CLB2* construct expresses *CLB2* message under the control of the *CLB5* promoter, causing artificially early accumulation of Clb2–Cdc28 kinase activity instead of, and at the normal time of Clb5–Cdc28 activity. The ability of Clb2–Cdc28 activity to fire late replication origins can therefore be tested by analysing late origin firing in the *clb5::CLB2* strain (Figure 1D).

Late origin firing was examined in the *clb5::CLB2* strain (Figure 2) using two-dimensional agarose gel electrophoresis (Brewer and Fangman, 1987; Friedman and Brewer, 1995). This technique separates replication intermediates so that DNA fragments containing bubble structures (and therefore active replication origins) form a distinctive 'bubble arc' (see *ARS1* panel in Figure 2). Replication of the fragment by forks originated elsewhere instead results in a 'Y arc'.

Early replication origin firing appeared normal in *CLB* (wild type), *clb5* and *clb5::CLB2* strains, as illustrated in Figure 2 for the early origins *ARS305* and *ARS1*. *ARS603* best exemplifies the results obtained for late origins. Clear bubble arcs confirmed late origin activity in the *CLB* strain, but as described previously (Donaldson *et al.*, 1998), late origin firing was greatly dimin-

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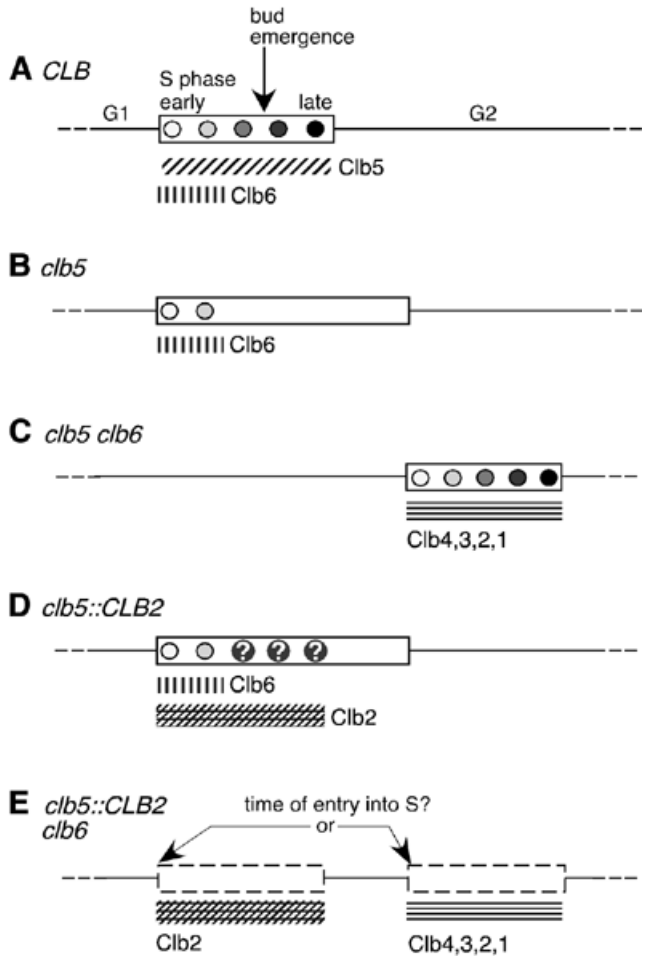

Fig. 1. Summary of known roles of B-type cyclins in origin activation and outline of the experiments described. (A–E) show time lines representing the cell cycle in various strains. S phase is shown as a rectangle; shaded circles represent replication origin activation events, with light circles as early-firing origins and darker circles as later-firing origins. Hatched bars refer to the function of various cyclins in activating replication origins. (**A**) Wild-type (*CLB*) cell cycle. Clb5 can activate both early and late origins whereas Clb6 is only capable of activating early origins. (**B**) In *clb5* cells late origins fail to fire normally and late replicons are instead replicated 'passively' by forks from early origins, with the result that S phase is prolonged. (**C**) *clb5 clb6* cells are delayed in entering S phase, because replication can only begin once Clb1–4 activity has accumulated. (**D**) This study addresses whether late origins fire (question marks) in a strain that expresses Clb2 in place of and at the normal time of Clb5 (the *clb5::CLB2* strain). (**E**) In the second part of this study, I investigate whether early expression of Clb2 in the absence of Clb5 and Clb6 fires early origins to allow cells to enter S phase on time.

ished in the *clb5* strain. Bubble arcs were also very faint in the *clb5::CLB2* strain, indicating that, in general, late origins were not activated by premature Clb2 activity replacing that of Clb5. Expression of Clb2 during S phase, therefore, did not rescue the late origin-firing defect of the *clb5* mutant.

To allow quantitative estimation of the activation of a late origin in the *clb5::CLB2* strain, the direction of replication fork movement was analysed at a locus to the right of the late replication origin *ARS603* using the modification to the 2D-gel procedure described previously (Friedman and Brewer, 1995; Friedman *et al.*, 1997). The chromosomal location of *ARS603* makes it convenient for this analysis; it is the leftmost efficient origin on chromosome VI so that replication forks progressing rightward through sequences to the right of *ARS603* must have been initiated at that origin (Figure 3A). If *ARS603* is not activated this chromosome region is instead replicated 'passively' by a leftward-moving replication fork initiated at a more centromere-proximal (presumably early) origin (Figure 3A). The cartoon in Figure 3B illustrates the 2D gel arcs expected if *ARS603* is active or inactive. Results of the analysis in *CLB*, *clb5* and *clb5::CLB2* strains are shown in Figure 3C. As in the *clb5* strain, in the *clb5::CLB2* strain the predominant arc corresponded to a leftward-moving fork. *ARS603* was therefore inactive and the region was instead replicated passively by a leftward-moving replication fork in the majority of *clb5::CLB2* cells.

The arc corresponding to the rightward replication fork from *ARS603* did, however, appear slightly increased in the *clb5::CLB2* when compared with the *clb5* strain. To estimate origin firing of *ARS603* in the three strains, the proportion of forks moving in either direction was quantitated as described in Friedman and Brewer (1995) (see also Figure 3 legend). The results indicated that *ARS603* origin firing was slightly restored in the *clb5::CLB2* cells when compared with *clb5* (firing in ~23% of *clb5* and ~32% of *clb5::CLB2* cells). Results from the fork-direction gel analysis of *ARS603* firing in the *clb5::CLB2* strain, therefore, confirmed those from the conventional 2D gels.

Overexpression of Clb2 has been found to reduce the message levels of a number of S phase genes including *RNR1*, which encodes ribonucleotide reductase (Spellman *et al.*, 1998). To investigate whether this effect might cause the failure of late origin activation in the *clb5::CLB2* strain, firing of *ARS603* was tested in *clb5::CLB2*/p*CLB5* cells, which contain a plasmid-borne copy of wild-type *CLB5* under its own promoter. Late origin firing was restored in this strain (Figure 4), ruling out the possibility that the late origin firing defect in the *clb5::CLB2* strain was the result of such a mechanism.

The 2D gel analyses indicated that late origin firing remains deeply defective when Clb2 is expressed in place of Clb5, and that premature expression of Clb2, therefore, cannot fulfil the late origin-firing function of Clb5. Quantitative estimation using fork-direction gel analysis showed that early Clb2 expression results in only slight rescue of the late origin defect of a *clb5* mutant. These results are also in close agreement with those described by Cross *et al.* (1999). The lengthened S phase of *clb5* deletion mutants (Figure 1B) is manifest in asynchronous cultures as an accumulation of cells with DNA content intermediate between that of G_1 and G_2. Cross *et al.* used flow cytometry analysis to show that asynchronously growing *clb5::CLB2* cultures have a similar accumulation of S phase cells, indicating that S phase is also lengthened in the *clb5::CLB2* strain. Both the analysis in Cross *et al.* (1999) and the results presented here show that Clb2 is unable to fulfil the S phase function of Clb5.

Early expression of Clb2 does not activate early origins on time

Next, I tested whether prematurely expressed Clb2 can activate early replication origins. *clb5* cells begin S phase at the normal time because Clb6 alone is sufficient to activate early replication

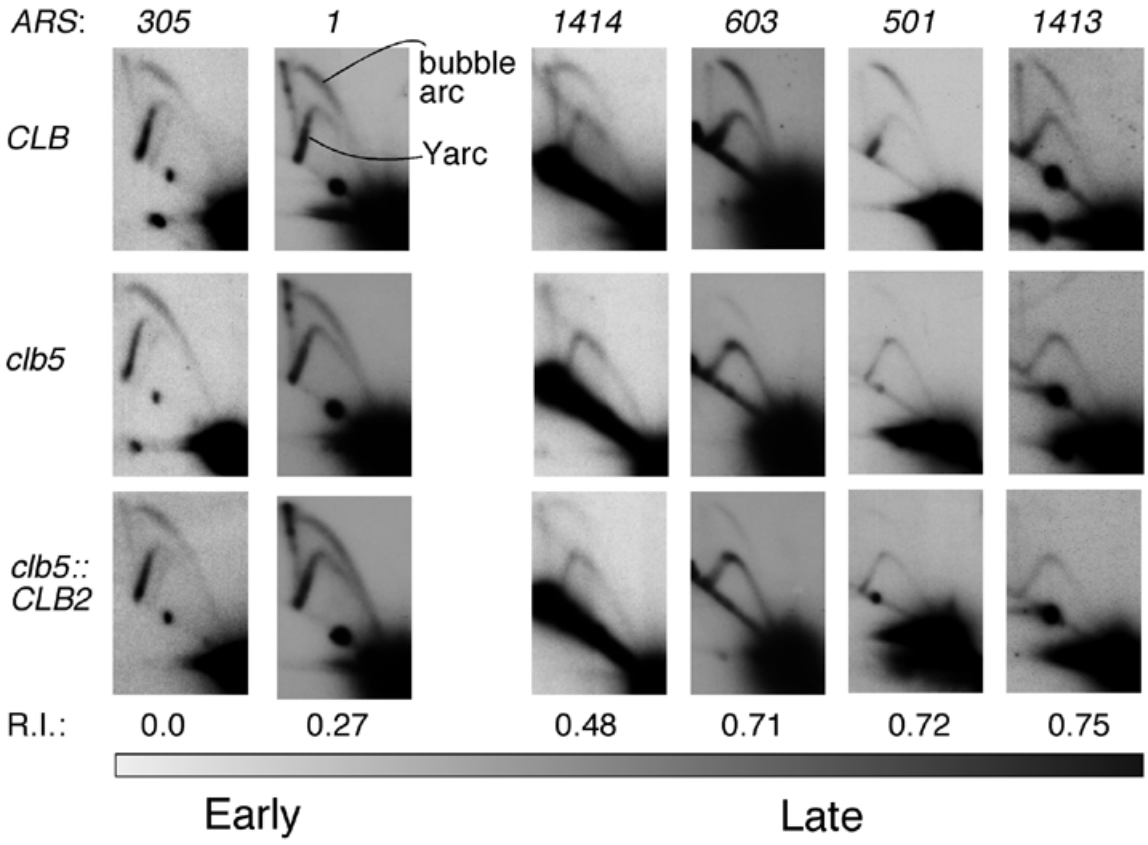

Fig. 2. 2D gel analysis of early and late replication origin use. Origin use was examined in *CLB*, *clb5* and *clb5::CLB2* strains (top, middle and bottom panels, respectively). The presence of a bubble arc indicates that the origin is activated, while the Y arc represents the proportion of fragments replicated passively. Early origin use appears normal in all three strains. Late origin firing is greatly diminished in *clb5* strains. Late origin firing in *clb5::CLB2* strains appears defective as in the *clb5* mutant. Names of the origins are given above the panels. R.I. value is a measure of origin activation time, with 0.0 representing a very early origin and 0.75 one of the latest known origins (Friedman *et al.*, 1996).

origins (Figure 1B). *clb5 clb6* mutants are dependent on one or more of cyclins Clb1–4 to promote S phase, and are therefore delayed in entering S phase (Figure 1C) (Donaldson *et al.*, 1998). Whether prematurely expressed Clb2 activates early replication origins can therefore be investigated (Figure 1E) by testing whether a *clb5::CLB2 clb6* mutant begins S phase on time (like the *clb5* strain) or is instead delayed in S phase entry (like *clb5 clb6*).

Because of their delayed S phase entry and consequent shorter G_2 phase, asynchronously growing cultures of *clb5 clb6* cells have a reduced proportion of cells with a G_2 DNA content (Figure 5A, left hand panels). *clb5::CLB2 clb6* cultures showed a similar depressed G_2 peak in their flow cytometry profile, suggesting that they share the delayed S phase entry found in the *clb5 clb6* mutant.

DNA replication was examined in synchronized cultures (Figure 5A, right hand panels). S phase in the *CLB* strain began ~30 min after release from α-factor. The *clb5::CLB2 clb6* culture began S phase ~45 min after release, corresponding to a delay of 15 min when compared with *CLB* cells. The difference in S phase entry time was not due to faster cell cycle progression of the *CLB* strain, because budding rates after release were similar for the two strains (Figure 5B). This delay in S phase entry of the *clb5::CLB2 clb6* strain showed that early Clb2 expression is inadequate to fire early origins on schedule and restore the normal S phase entry time.

As expected, DNA replication in *clb5 clb6* cells was also delayed, by ~30 min when compared with the *CLB* strain. The *clb5::CLB2 clb6* S phase entry was, therefore, somewhat advanced when compared with *clb5 clb6*. In other words, early Clb2 expression in a *clb5 clb6* background did have some effect on the time of replication initiation. Two possible mechanisms can be envisaged for this advancement of S phase entry time by the *clb5::CLB2* construct. Cdc28–Clb2 activity could have some competence for eventual activation of early origins, despite the fact that it is unable to activate them at the correct time. Alternatively, the advanced S phase entry time of *clb5::CLB2 clb6* when compared with *clb5 clb6* might be an indirect effect of early Clb2 expression—in particular, Clb2 might affect the expression time of other mitotic cyclins that could be involved in activating origins in the absence of S phase cyclins.

To ascertain whether early Clb2 expression affects expression of other cyclins, the kinetics of Clb4 accumulation were compared in *CLB*, *clb5 clb6* and *clb5::CLB2 clb6* strains.

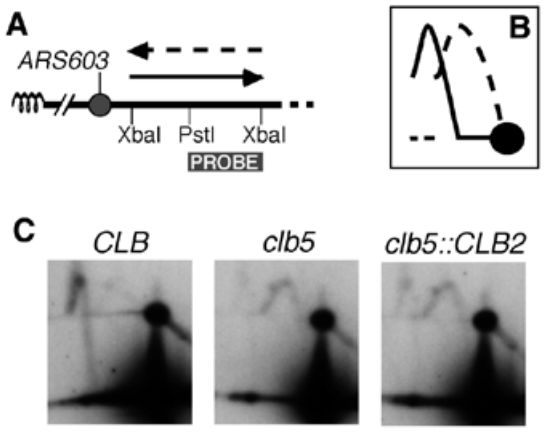

Fig. 3. Analysis of replication fork direction close to the late origin *ARS603*. (**A**) Chromosome configuration of the left arm of chromosome VI. If *ARS603* is activated, the *Xba*I fragment immediately to its right is replicated by the rightward-moving fork from *ARS603* (solid arrow). *ARS603* is the closest efficient origin to the left telomere (coiled line), so that if *ARS603* is not activated the *Xba*I fragment is instead replicated by a leftward-moving fork (dashed arrow). The direction of replication fork movement was analysed as described by Friedman and Brewer (1995), using *Pst*I for the in-gel digestion and probing for the larger *Pst*I–*Xba*I fragment. (**B**) The solid curve illustrates the Y arc expected if replication forks move rightward through the *Xba*I fragment (i.e. *ARS603* active). The dashed curve shows the expected Y arc if forks move leftward (i.e. *ARS603* inactive). (**C**) Fork-direction analysis within the *Xba*I fragment in *CLB*, *clb5* and *clb5::CLB2* strains. Quantitation of these blots (as described by Friedman *et al.*, 1997) allowed estimation that *ARS603* fires in 74% of *CLB* cell cycles, 23% of *clb5* cell cycles and 32% of *clb5::CLB2* cell cycles. The value obtained for *CLB* may be an underestimate of actual origin firing, because the minor arc was barely visible above background. Similarly, the value for *clb5* may represent an overestimation. Nevertheless, the values are generally consistent with those found previously (Friedman *et al.*, 1997; Donaldson *et al.*, 1998).

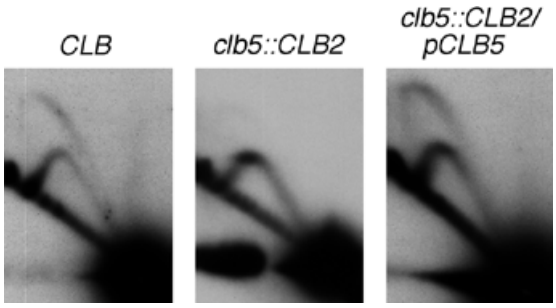

Fig. 4. The late origin *ARS603* is active in a *clb5::CLB2*/p*CLB5* strain. 2D gel analysis of replication intermediates in asynchronously growing *CLB* (left), *clb5::CLB2* (centre) and *clb5::CLB2*/p*CLB5* (right) strains.

Western blot analysis of synchronized cultures showed that Clb4 levels are maximal by ~60 min after release from α-factor in *CLB* and in *clb5 clb6* cells (Figure 5C), whereas in the *clb5::CLB2 clb6* strain, Clb4 levels peaked ~15 min earlier. The data obtained from flow cytometry and western analyses were consistent with the notion that Clb4 (and/or Clb3) might activate S phase in the *clb5::CLB2 clb6* and *clb5 clb6* strains. To test the competence of Clb4 in activating S phase, I constructed a *clb5::CLB4 clb6* strain. This strain lacks both S phase cyclins and has the *CLB5* ORF precisely replaced by that of *CLB4*, so that Clb4 is expressed from the *CLB5* promoter. Flow cytometry analysis of asynchronously growing *clb5::CLB4 clb6* cells showed a reduced peak of cells with G_2 DNA content, very similar to that in the *clb5::CLB2 clb6* culture (Figure 5D). This result implied that S phase in the *clb5::CLB4 clb6* strain is delayed as in *clb5::CLB2 clb6*, and S phase entry time is not significantly rescued by early expression of Clb4 protein.

The result obtained from the *clb5::CLB4 clb6* strain argues against a substantially better proficiency of Clb4 than Clb2 in promoting timely S phase entry. The advancement of S phase in *clb5::CLB2 clb6* cells when compared with a *clb5 clb6* strain (Figure 4A) is, therefore, most likely due to a direct effect of early Clb2 expression than to the observed advancement of Clb4 expression in response to early Clb2. These results cannot exclude the possibility that either Clb3- or Clb4-directed Cdc28 does contribute directly to origin firing in *clb5::CLB2 clb6* cells—however, a more probable interpretation of the data in Figure 5A is that, despite its incompetence for timely early origin activation, Clb2 can contribute to eventual activation of at least some replication origins in the absence of S phase cyclins.

The experiments described in this study examined the extent to which a mitotic cyclin (Clb2) that is artificially expressed at the normal time of S phase can fire early and late replication origins, and so substitute for the S phase cyclins. The results indicate that premature Clb2 is unable to activate either class of origins at the correct time and efficiency. Late origin firing was almost as defective in the *clb5::CLB2* strain as in a *clb5* mutant. The marginal improvement in late origin firing efficiency in *clb5::CLB2* probably represents a slight competence of Clb2–Cdc28 to fire late replication origins directly. Clb2–Cdc28 also failed to fire early replication origins at the correct time. The *clb5::CLB2* construct promotes Clb2–Cdc28 kinase activity at the same time and to similar levels as normal Clb5–Cdc28 activity (Cross *et al.*, 1999). Nevertheless, S phase entry was significantly delayed in the *clb5::CLB2 clb6* strain, showing that early Clb2 expression cannot substitute for Clb6 or Clb5 to restore timely early origin firing. Although Clb4 expression was advanced in the *clb5::CLB2 clb6* strain, this effect did not appear to cause the advancement in S phase onset when compared with *clb5 clb6*. The current data instead suggests that Clb2, possibly together with other mitotic cyclins, is unable to fire early origins on time but can eventually activate some replication origins.

In contrast to some previous studies (for example, Amon *et al.*, 1994), the results described here address the capability of cyclins expressed at normal levels to fire origins. Haase and Reed (1999) have succeeded in constructing a strain that is deleted for all six B-type cyclins and maintained by overexpression of *CLB1* under galactose promoter control. Time of S phase entry of this *clb1,2,3,4,5,6 GAL1-CLB1* strain has not been measured; however, its viability implies that, when overexpressed, the mitotic cyclin Clb1 can drive activation of at least the minimum number of origins required to complete chromosome replication. Overexpression is, however, likely to be essential for Clb1 to

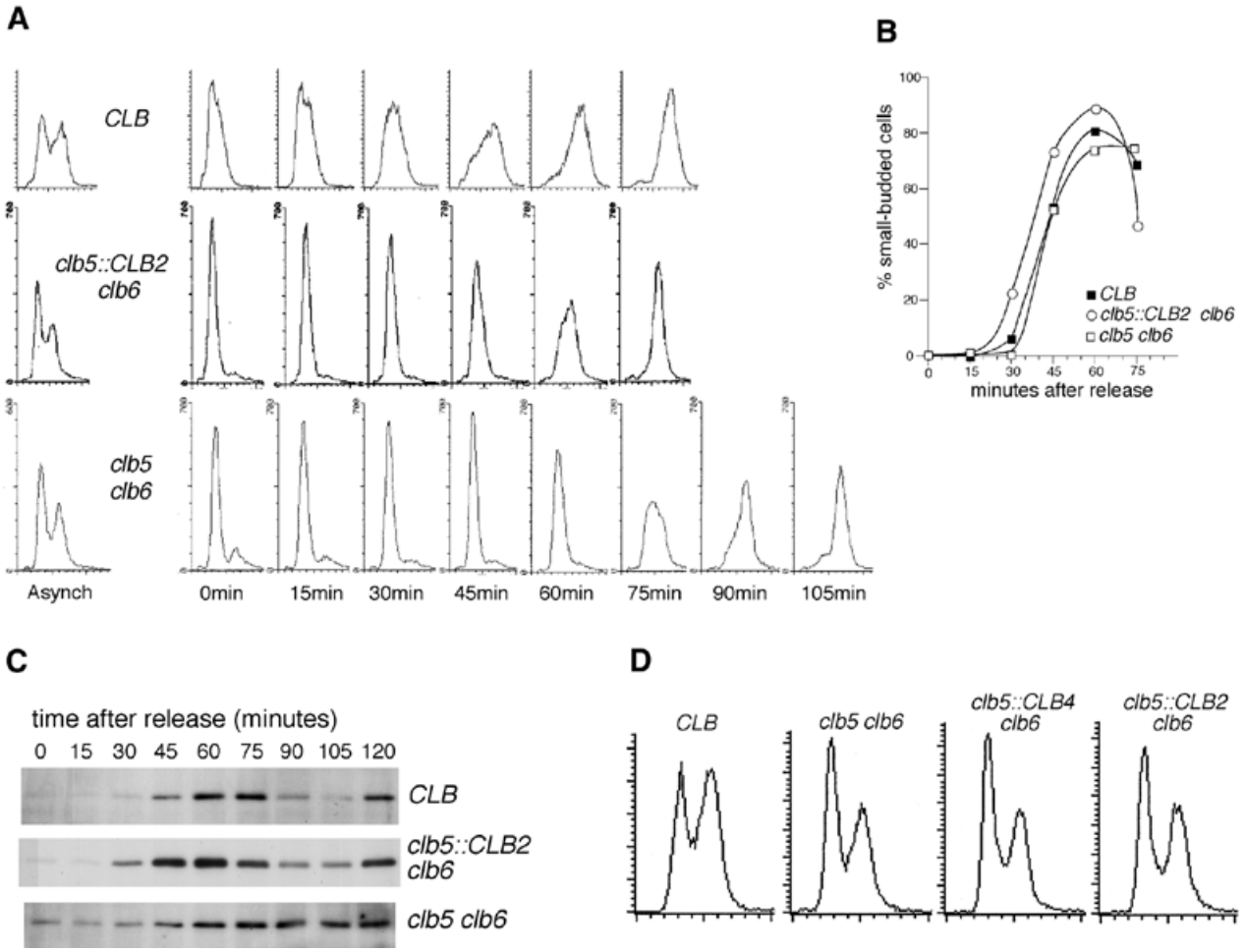

Fig. 5. Time of S phase and Clb4 accumulation in *CLB*, *clb5::CLB2 clb6* and *clb5 clb6* strains. (**A**) Flow cytometry analysis of DNA content in asynchronously growing cultures (left panels) and in cultures synchronized by release from α-factor (right panels). Time is minutes after release. (**B**) Kinetics of bud emergence in (A). (**C**) Western blot analysis of Clb4-HA accumulation in *CLB*, *clb5::CLB2 clb6* and *clb5 clb6* strains, in α-factor release experiments on strains with epitope-tagged Clb4. Bud emergence kinetics were very similar to those in (B). (**D**) Flow cytometry analysis of asynchronously growing *CLB*, *clb5 clb6*, *clb5::CLB4 clb6* and *clb5::CLB2 clb6* strains.

fulfil this task, since a *clb3,4,5,6* strain is inviable. Clb1 could well share the origin-firing ability shown here for Clb2—that is, defective for timely origin firing but able to promote some eventual chromosome replication.

In general, the presence of multiple B-type cyclins is a complicating factor in experiments aimed at investigating the competence of individual Clb–Cdc28 activities to fire replication origins. Future experiments will test origin activation when single cyclins are expressed in cells lacking all other B-type cyclin activities. While such experiments will be necessary to fully understand cyclin specificity in origin firing, it is already clear from the results presented here that substitution of Clb5 and Clb6 by Clb2 cannot restore a normal S phase.

METHODS

Strain constructions. All strains were derived from BF264-15D (Richardson *et al.*, 1992). Strains 1768-12A and FC12-18 (both *CLB*), 1768-1A-1 (*clb5*), 1768-1A-2 (*clb5::CLB2*) and 1768-6C (*clb5 clb6*) were gifts from F.R. Cross. Other *clb6* derivatives were made from these strains by transformation with a *clb6::LEU2* deletion construct as described in Donaldson *et al.* (1998). p*CLB5* (used to make *clb5::CLB2*/p*CLB5*) was YCplac111 containing a 3.0 kb *Bst*XI–*Xba*I *CLB5* insert (Schwob and Nasmyth, 1993). The *CLB4* gene was HA-tagged at its 3′ terminus by the method of Longtine *et al.* (1998). A *clb5::CLB4* allele was PCR amplified using long primers that included the *Hin*dIII site 66 bp upstream of *CLB5* or the *Eco*RI site 22 bp from the *CLB5* 3′ end. The resulting *clb5::CLB4* PCR fragment was *Hin*dIII–*Eco*RI digested and ligated, together with the 812 bp *Eco*RI–*Xba*I fragment immediately 3′ to *CLB5*, into *Hin*dIII–*Xba*I-cut pBluescript KS$^-$. Resulting clones were sequenced and an error-free 2267 bp *Hin*dIII *clb5::CLB4* fragment was transformed into AW91 (*clb5::URA3 clb6*) and selected on 5-FOA. Correct *clb5::CLB4 clb6* integrants were identified by PCR analysis.

2D agarose gel electrophoresis and analysis of replication fork direction. These techniques were carried out as described in Donaldson *et al.* (1998).

Cell synchronization, western blotting and flow cytometry. All strains were *bar1*. Cells were synchronized with 200 nM α-factor and released by the addition of pronase (0.3 mg/ml) to the medium. Protein sample extraction and western blotting were based on Stirling *et al.* (1994) and standard techniques. Protein loading was checked by Ponceau S staining of blots, followed by detection of Clb4-HA using antibody 12CA5. Flow cytometry was as described by Haase and Lew (1997).

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