MOLECULAR AND CELLULAR BIOLOGY, June 1993, p. 3744–3755
0270-7306/93/063744-12$02.00/0

Vol. 13, No. 6

Full Activation of p34^{CDC28} Histone H1 Kinase Activity Is Unable To Promote Entry into Mitosis in Checkpoint-Arrested Cells of the Yeast *Saccharomyces cerevisiae*

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Received 2 November 1992/Returned for modification 12 January 1993/Accepted 23 March 1993

In most cells, mitosis is dependent upon completion of DNA replication. The feedback mechanisms that prevent entry into mitosis by cells with damaged or incompletely replicated DNA have been termed checkpoint controls. Studies with the fission yeast *Schizosaccharomyces pombe* and *Xenopus* egg extracts have shown that checkpoint controls prevent activation of the master regulatory protein kinase, p34^{cdc2}, that normally triggers entry into mitosis. This is achieved through inhibitory phosphorylation of the Tyr-15 residue of p34^{cdc2}. However, studies with the budding yeast *Saccharomyces cerevisiae* have shown that phosphorylation of this residue is not essential for checkpoint controls to prevent mitosis. We have investigated the basis for checkpoint controls in this organism and show that these controls can prevent entry into mitosis even in cells which have fully activated the cyclin B (Clb)-associated forms of the budding yeast homolog of p34^{cdc2}, p34^{CDC28}, as assayed by histone H1 kinase activity. However, the active complexes in checkpoint-arrested cells are smaller than those in cycling cells, suggesting that assembly of mitosis-inducing complexes requires additional steps following histone H1 kinase activation.

Successful duplication and segregation of cellular constituents during cell proliferation requires that several complex processes occur in a defined order. For example, the genome must be fully and accurately replicated before the microtubule spindle separates sister chromatids in mitosis. This order of events can be subverted by mutations or drug treatments, such that mitosis begins before the chromosomes are ready, resulting in "mitotic catastrophe" with missegregation or fragmentation of the genetic material (11). These observations suggest that a control mechanism (termed "checkpoint control") exists to ensure the normal order of events by preventing mitosis until completion of DNA replication. The mutations and drug treatments leading to mitotic catastrophe are presumed to interrupt such checkpoint control (11). Our goal is to elucidate the molecular mechanisms governing this checkpoint in the budding yeast *Saccharomyces cerevisiae*.

Entry into mitosis is controlled by the master regulatory kinase, p34^{cdc2}, in all eukaryotes studied to date (4, 18, 21). Activation of p34^{cdc2} requires its association with a regulatory subunit, known as cyclin, as well as dephosphorylation of inhibitory tyrosine (and, in some species, threonine) residues of p34^{cdc2} (12, 18, 19). Upon activation, the cyclin-p34^{cdc2} complex phosphorylates a variety of both structural and regulatory substrates, initiating the many changes that occur in mitosis (20). The cyclin-p34^{cdc2} regulatory system is an attractive candidate for the target of checkpoint control, as delaying activation of p34^{cdc2} until DNA replication is completed would ensure that chromosome separation was not attempted prematurely.

For the fission yeast *Schizosaccharomyces pombe*, analysis of mutations that accelerate or delay entry into mitosis has revealed a regulatory pathway based on inhibitory phosphorylation of the Tyr-15 residue of p34^{cdc2} (8, 21). When DNA replication is blocked in *S. pombe* cells by treatment with the drug hydroxyurea, cyclin-p34^{cdc2} complexes accumulate but remain inactive as a result of p34^{cdc2} Tyr-15 phosphorylation. Strikingly, cells expressing a Tyr-15→Phe mutant allele of *cdc2* experience mitotic catastrophe when exposed to hydroxyurea (28). Furthermore, reduction of p34^{cdc2} phosphorylation by mutation of regulatory genes also causes mitotic catastrophe in cells treated with hydroxyurea (5, 17). These observations suggest that checkpoint controls in this organism operate by stimulating or maintaining the inhibitory phosphorylation of p34^{cdc2} at Tyr-15, thus preventing p34^{cdc2} activation and entry into mitosis. Biochemical studies using extracts from *Xenopus* eggs support a similar conclusion regarding the mechanism of checkpoint control (3, 13, 27).

Prevention of p34^{cdc2} activation in the cases above is achieved through inhibitory phosphorylation of p34^{cdc2}. Cyclin accumulation and binding to p34^{cdc2} are unaffected. In contrast, checkpoint controls in *S. cerevisiae* do not require the inhibitory phosphorylation of p34^{CDC28} (the *S. cerevisiae* homolog of p34^{cdc2}) (1, 28). Although a small amount of p34^{CDC28} becomes phosphorylated at Tyr-19 (homologous to Tyr-15 in p34^{cdc2}) upon arrest by hydroxyurea, cells expressing a mutant p34^{CDC28} protein that cannot be phosphorylated at this site do not enter into mitosis until DNA replication has been completed (1, 28). Thus, another mode of checkpoint control is required in *S. cerevisiae*. One possibility is that mitotic activation of p34^{CDC28} might be regulated at the level of accumulation or activity of cyclins.

In *S. cerevisiae*, four B-type cyclins termed Clb1, Clb2, Clb3, and Clb4 have been implicated in the control of entry into mitosis (6, 7, 23, 29). The abundance of the Clbs and their association with p34^{CDC28} vary through the cell cycle, peaking in mitosis. In this study, we have investigated the effects of checkpoint control on the activation of Clb-p34^{CDC28} complexes and the effects of hyperactivating Clb-p34^{CDC28} complexes on checkpoint control. Our results

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TABLE 1. Strains

Strain	Relevant genotype
DLY005	*MATa/MATα*
DLY333	*MATa/MATα GAL1::CLB1(LEU2)*
DLY385	*MATa/MATα GAL1::CLB2(LEU2)*
DLY338	*MATa/MATα GAL1::CLB3(LEU2)*
DLY328	*MATa/MATα GAL1::CLB1Δ152(LEU2)*
DLY001	*MATa bar1*
DLY344	*MATa bar1 GAL1::CLB1(LEU2)*
DLY345	*MATa bar1 GAL1::CLB3(LEU2)*
DLY343	*MATa bar1 GAL1::CLB1Δ152(LEU2)*
CSY324	*MATa bar1 GAL1::cdc2^{+}(URA3)*
CSY325	*MATa bar1 GAL1::cdc2^{+}(URA3) GAL1::CLB1(LEU2)*
CSY326	*MATa bar1 GAL1::cdc2^{+}(URA3) GAL1::CLB2(LEU2)*
CSY327	*MATa bar1 GAL1::cdc2^{+}(URA3) GAL1::CLB3(LEU2)*
CSY328	*MATa bar1 GAL1::cdc2^{+}(URA3) GAL1::CLB1Δ152(LEU2)*
CSY304	*MATa bar1 GAL1::cdc2$^{Y\rightarrow F}$(URA3)*
CSY305	*MATa bar1 GAL1::cdc2$^{Y\rightarrow F}$(URA3) GAL1::CLB1(LEU2)*
CSY306	*MATa bar1 GAL1::cdc2$^{Y\rightarrow F}$(URA3) GAL1::CLB2(LEU2)*
CSY307	*MATa bar1 GAL1::cdc2$^{Y\rightarrow F}$(URA3) GAL1::CLB3(LEU2)*
CSY308	*MATa bar1 GAL1::cdc2$^{Y\rightarrow F}$(URA3) GAL1::CLB1Δ152(LEU2)*
CSY102	*MATa cdc9 leu2 ura3*
CSY103	*MATa cdc9 leu2 ura3 GAL1::CLB1(LEU2)*
CSY104	*MATa cdc9 leu2 ura3 GAL1::CLB2(LEU2)*
CSY105	*MATa cdc9 leu2 ura3 GAL1::CLB3(LEU2)*
CSY106	*MATa cdc9 leu2 ura3 GAL1::CLB1Δ152(LEU2)*

show that checkpoint controls in *S. cerevisiae* are capable of preventing entry into mitosis even if Clb-p34^{CDC28} complexes are fully activated, as assayed in vitro with histone H1 as a substrate. Despite this apparent activation, complexes from checkpoint-arrested cells were distinguishable from those in cycling cells by their behavior during gel filtration chromatography. We suggest that activation of Clb-p34^{CDC28} complexes in vivo requires further assembly steps that are not detectable by the standard in vitro assay and that these are the target for checkpoint controls in this organism.

MATERIALS AND METHODS

Yeast strains and media. All yeast strains used in this study (see Table 1) were derivatives of BF264-15DU (*MATa ade1 his2 leu2-3,112 trp1-1 ura3Dns*) (24). The *GAL1::CLB* integrative *LEU2*-marked plasmids were constructed as previously described for Clb1 (7). Polymerase chain reaction products containing the *CLB*-coding regions were generated with oligonucleotides containing *Bam*HI or *Bgl*II sites and cloned into the *Bam*HI site of YIpG2. YIpG2::CLB plasmids were linearized at the unique *Bst*EII site for integration at the *LEU2* locus. Plasmid YIpG3 was constructed as follows. (i) The *Sma*I-*Pst*I sites in the pUC19 polylinker were eliminated by digestion, blunting, and religation. (ii) A 1.1-kb *Hin*dIII fragment containing the *URA3* gene was cloned into the *Hin*dIII site. (iii) The *GAL1* promoter *Eco*RI-*Bam*HI fragment from YIpG2 was cloned into the corresponding sites in the polylinker. *GAL1::cdc2* and *GAL1::cdc2*$^{Y\text{-}15\rightarrow F}$ integrative *URA3*-marked plasmids were constructed by cloning the *cdc2*-containing *Bam*HI fragments (8) into the *Bam*HI site of YIpG3. YIpG3::cdc2 plasmids were linearized at the unique *Pst*I site for integration at the *URA3* locus. These plasmids were used to generate the strains listed in Table 1. The *cdc9-8* allele was crossed into BF264-15DU from strain 598-3 (*MATα trp1 ade2 ade3 leu1-2 ura3 can1 cyc2 sap3 cdc9-8*) (30) and then backcrossed once.

Yeast cultures were grown in YEP (1% yeast extract, 2% Bacto Peptone, 0.005% adenine, 0.005% uracil) supplemented with 2% sucrose, dextrose, or galactose as indicated.

Preparation of cell extracts, gel electrophoresis, and immunoblotting. Cells (10^7 to 10^8) were resuspended in 120 μl of lysis buffer (1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 7.5], 1 mM sodium pyrophosphate, and protease inhibitors [per milliliter] phenylmethylsulfonyl fluoride [80 μg], aprotinin [2 μg], leupeptin [2 μg], and pepstatin [2 μg]). An equal volume of glass beads was added, and the cells were disrupted by vortexing. Crude lysate was separated from glass beads by centrifugation through a pinhole in the bottom of the tube into a clean Microfuge tube. Cell debris was removed by centrifugation at 12,000 rpm in an Eppendorf microcentrifuge. Total protein in the supernatant was determined by UV A_{280} and normalized in each sample. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), each sample contained 0.2 A_{280} units of total protein in 1% SDS–10% glycerol–0.12 M Tris [pH 6.8]–2% β-mercaptoethanol. After standard SDS-PAGE, proteins were transferred to nitrocellulose and blocked with 2.5% instant milk and 2.5% bovine serum albumin in Tris-buffered saline for a minimum of 1 h. For a full characterization of antisera, see references 7 and 9. Blots were incubated with either affinity-purified anti-Clb1 (1/200 dilution), affinity-purified anti-Clb3 (1/300 dilution), or mouse monoclonal anti-PSTAIRE (1/5,000 dilution) for a minimum of 4 h at 4°C. They were then washed three times with Tris-buffered saline plus 0.05% Tween 20 for 15 min. Blots were incubated with a second antibody (either anti-rabbit or anti-mouse immunoglobulin G conjugated to horseradish peroxidase) for 60 min at room temperature, washed five times as described above, and developed by using enhanced chemiluminescence.

Kinase assays. Clb-Cdc28 complexes were immunoprecipitated from the same lysates used for immunoblotting as follows. Five microliters of affinity-purified anti-Clb3 antibodies was added to 0.1 A_{280} units of total protein. After incubating on ice for 1 h, 3 mg of protein A-Sepharose beads

resuspended in lysis buffer was added. After incubating on ice for 1 h with occasional mixing, the beads were washed twice with a radioimmunoprecipitation assay (RIPA) buffer (1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl [pH 7.5], 1 mM sodium pyrophosphate, 150 mM NaCl), once with 1% sodium deoxycholate–1% Triton X-100–50 mM Tris-HCl [pH 7.5], and twice with 20 mM Tris-HCl [pH 7.5]–7.5 mM $MgCl_2$. All washes were at 0°C. The histone H1 kinase activity in the immunoprecipitates was assayed in 20 mM Tris-HCl [pH 7.5]–7.5 mM $MgCl_2$–200 μM ATP plus 10 μCi of [γ-^{32}P]ATP (4,500 Ci/mmol) and 2 μg of calf thymus histone H1 (Boehringer Mannheim) per 20-μl assay mixture for 30 min at 37°C. Reactions were stopped by addition of the SDS loading buffer described above and boiling for 3 min. Samples were separated on 12% polyacrylamide gels and visualized by autoradiography.

Immunofluorescence and flow cytometry. For immunofluorescence, cells were fixed by the addition of 1/10 volume of 37% formaldehyde directly to the culture medium. After 1 to 2 h at room temperature, the cells were spheroplasted and processed as described previously (22). Monoclonal rat anti-α-tubulin antibody (YOL1) was used at a 1/200 dilution. Affinity-purified rhodamine-conjugated goat anti-rat immunoglobulin G second antibody was used at a 1/100 dilution. 4′,6-Diamidino-2-phenylindole was added to the mounting medium to stain DNA. Fluorescence photomicroscopy of stained cells was performed with a Zeiss Axiophot photomicroscope fitted with a ×100 objective. Flow-cytometric analysis of cellular DNA content was performed as described previously (15).

Viability assays. Cells were sonicated to disrupt aggregates, counted with a hemacytometer, and spread onto YEP-dextrose agar plates. The number of colonies on each plate (indicating the number of viable cells plated) was scored after incubation at 30°C (or 25°C for the *cdc9* strains).

Determination of *cdc9* leak-through. To determine how tight the *cdc9* arrest was at the restrictive temperature, we used the vital stain fluorescein isothiocyanate-concanavalin A, which binds to cell wall mannan. Cells arrested at 36°C for 3.5 h were stained for 10 min in 0.1 mg of fluorescein isothiocyanate-concanavalin A per ml in Tris-buffered saline, washed, and incubated for a further 2, 3, or 4 h in growth medium. Cells that leaked through the arrest in this period produced buds with unlabelled cell walls, so the percent leak-through could be assessed by scoring the number of unlabelled buds or cells. Leak-through in different experiments varied from 5 to 20%.

Column chromatography. Protein extracts were prepared from 1×10^9 to 2×10^9 cells (cycling or arrested with 0.2 M hydroxyurea for 4 h) as described previously (31). Protein concentrations were determined by using the Bio-Rad assay kit with bovine serum albumin as a standard. A 3.5-mg sample of extract was chromatographed on a Superose 12 10/30 column (Pharmacia) as described previously (31). Fractions (0.2 ml each) were collected. Forty microliters of each fraction was analyzed by SDS-PAGE and immunoblotting with anti-PSTAIRE antibody as described above. Thirty microliters of each fraction was immunoprecipitated with antiserum directed against the N terminus of p34^{CDC28} (32). Kinase assays were performed as described above, except that protein A-Sepharose was suspended in column buffer [50 mM Tris-HCl (pH 7), 50 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 mM dithiothreitol, 10% glycerol] prior to use. Activity was quantitated by excising histone H1 bands from dried SDS-polyacrylamide gels and Cerenkov counting.

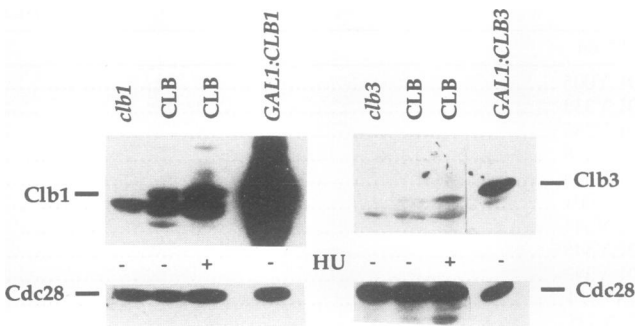

FIG. 1. Clb levels in hydroxyurea-treated cultures. Wild-type (CLB), *clb1*, and *clb3* cells were grown in YEP-dextrose and incubated in the presence (+) or absence (−) of 0.2 M hydroxyurea (HU) as indicated. *GAL1*::*CLB1* and *GAL1*::*CLB3* cells were grown in YEP-galactose. Lysates were prepared from the cells and analyzed by immunoblotting as described in Materials and Methods. At the mobility corresponding to the 45-kDa molecular mass marker, the blot was divided. The upper portion was incubated with rabbit anti-Clb antisera as indicated, while the lower portion was incubated with mouse monoclonal anti-PSTAIRE and served as a loading control. There is a background band recognized by the anti-Clb1 antibody running below the full-length protein (compare the first two lanes), and a Clb1 degradation product that ran just below this band is apparent in the third lane.

RESULTS

Clb proteins accumulate to intermediate levels in cells arrested by hydroxyurea. The Clb proteins fall into two classes on the basis of sequence relatedness and timing of expression during the cell cycle. Clb1 and Clb2 accumulate during G_2 and peak in mitosis, while Clb3 and Clb4 begin to accumulate at the beginning of S phase and are maintained at a high level until mitosis (6, 7, 23, 29). This cell cycle-dependent accumulation is largely the result of regulated transcription. The activity of Clb-p34^{CDC28} complexes (assayed in vitro with histone H1 as a substrate) roughly parallels the abundance of the individual Clbs through the cell cycle (9). No single *CLB* gene is essential, suggesting that Clbs overlap in their function(s). However, *clb1 clb2* and *clb2 clb3* mutants are inviable and unable to enter mitosis, suggesting that at least the Clb1, Clb2, and Clb3 proteins may be important for mitotic induction (6, 23).

To assess the effects of activating checkpoint controls on the Clb proteins, we treated wild-type cells with hydroxyurea to block DNA replication and measured the abundance of a representative of each class of Clbs by Western blotting (immunoblotting) (Fig. 1). Compared with an asynchronous population (in which the Clb level reflects an average of the levels at various stages of the cell cycle), hydroxyurea-arrested cells displayed a two- to fourfold increase in Clb levels. A similar increase was observed for Clb-associated histone H1 kinase activity (not shown, but see Fig. 5). It is not clear how this level compares with the levels of histone H1 kinase activity normally attained during mitosis, because it is not technically feasible to obtain a homogeneously synchronized mitotic cell population. One frequently employed estimate for mitotic kinase levels is the histone H1 kinase activity obtained in cells arrested by the antimicrotubule agent nocodazole or benomyl, which is

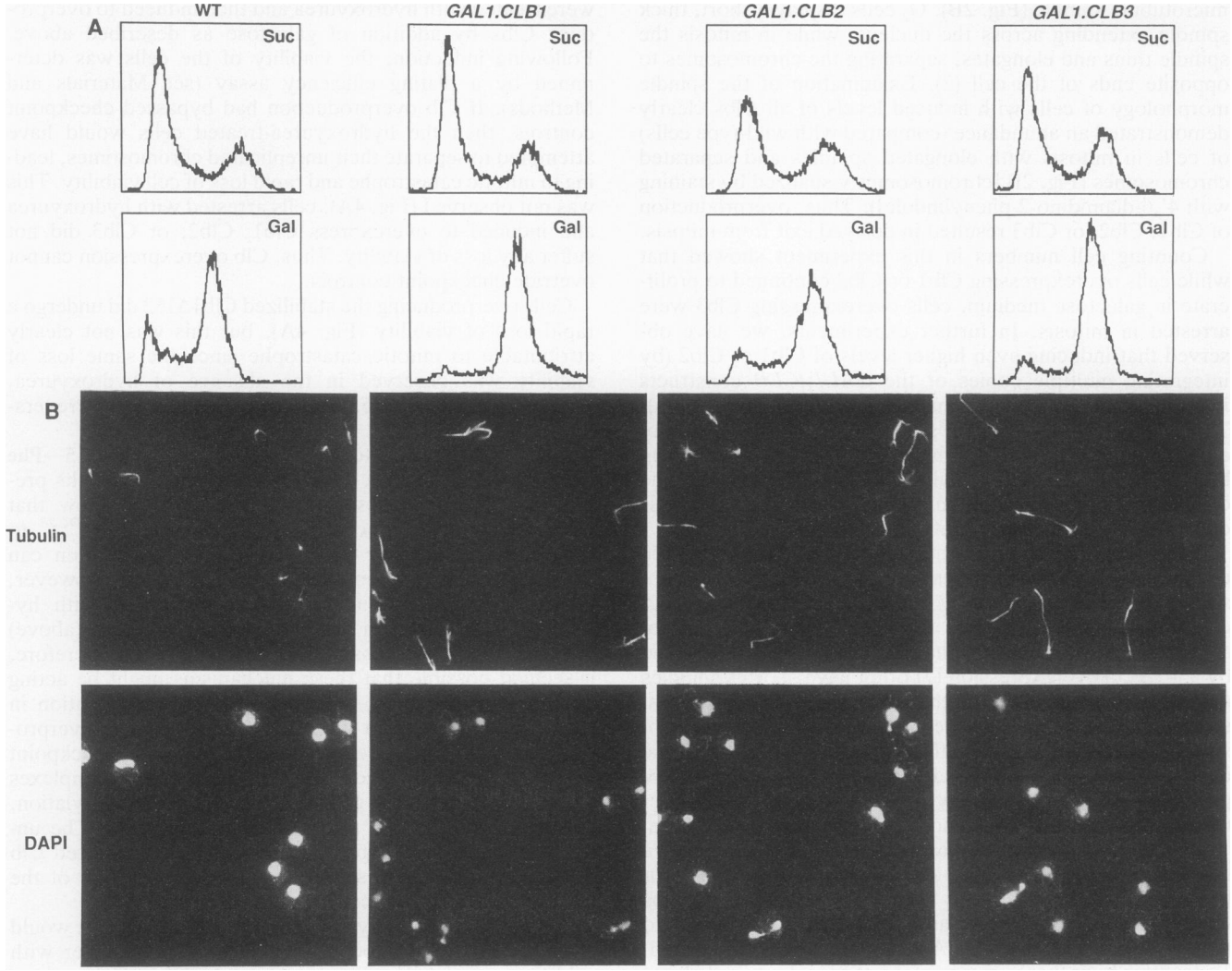

FIG. 2. Clb overexpression delays or prevents exit from mitosis. (A) Cells containing the indicated *GAL1*::*CLB* genes were grown in YEP-sucrose (Suc) and transferred to YEP-galactose (Gal) for 3 h. DNA content of propidium iodide-stained cells was determined by flow cytometry. Cell number (*y* axis) is plotted against fluorescence (*x* axis). Left peak, G_1 cells; right peak, G_2 and M phase cells. WT, wild type. (B) Tubulin distribution (top) and DNA distribution (bottom) in the same cells following transfer to YEP-galactose. DAPI, 4′,6-diamidino-2-phenylindole.

about two- to fourfold higher than that in hydroxyurea-arrested cells (e.g., see Fig. 4 in reference 28). This suggests that Clb accumulation is antagonized by checkpoint controls. However, the nature of the arrest obtained with such agents (G_2 arrest, mitotic arrest, or an aberrant state not corresponding to a particular stage of the cell cycle) is unclear, since microtubule morphology provides the only way to distinguish between these options in *S. cerevisiae* (2). In addition, it is not clear that mitotic levels of Clb proteins are actually essential for mitosis, since individual *CLB* genes are dispensable. Thus, this experiment is insufficient to indicate whether regulation of Clb accumulation plays an important part in checkpoint control.

Clb overexpression delays or prevents exist from mitosis. In order to determine whether reduced Clb accumulation is important for checkpoint control, we sought to artificially increase Clb levels by overexpression from a heterologous promoter. We constructed chimeric genes linking the galactokinase (*GAL1*) promoter to the *CLB1*, *CLB2*, or *CLB3*-coding region (see Materials and Methods). The *GAL1* promoter is silent when cells are grown in sucrose-based medium but is rapidly activated upon transfer to galactose-based medium. The *GAL1*::*CLB* constructs were integrated into the genome of diploid wild-type cells, and the effects of transferring these cells to galactose-containing medium were analyzed by Western blotting, flow cytometry, and immunofluorescence to determine the effects of Clb overproduction on cell cycle progression (Fig. 1 and 2).

Analysis of Clb levels in these strains showed that transfer to galactose induced a massive accumulation of the regulated Clb, achieving levels 20- to 50-fold higher than those in cycling cells (Fig. 1; also, data not shown). Cell cycle analysis of populations shifted to galactose for 3 h showed that Clb induction resulted in accumulation of cells with a 4 N DNA content (characteristic of G_2 and M phase cells) in all cases (Fig. 2A). To determine whether this reflected an enrichment of cells in G_2 or in mitosis, the cells were fixed and stained with antitubulin antibodies to visualize the

microtubule spindle (Fig. 2B). G_2 cells display a short, thick spindle extending across the nucleus, while in mitosis the spindle thins and elongates, separating the chromosomes to opposite ends of the cell (2). Examination of the spindle morphology of cells with induced levels of all Clbs clearly demonstrated an abundance (compared with wild-type cells) of cells in mitosis with elongated spindles and separated chromosomes (Fig. 2B [chromosomes visualized by staining with 4′,6-diamidino-2-phenylindole]). Thus, overproduction of Clb1, Clb2, or Clb3 resulted in delayed exit from mitosis.

Counting cell numbers in this experiment showed that while cells overexpressing Clb1 or Clb2 continued to proliferate in galactose medium, cells overexpressing Clb3 were arrested in mitosis. In further experiments, we have observed that inducing even higher levels of Clb1 or Clb2 (by integrating multiple copies of the *GAL1::CLB* constructs into the genome) resulted in mitotic arrest (data not shown). This is reminiscent of the arrest resulting from expression of a truncated Clb, Clb1Δ152, which lacks a mitotic destruction box (7). We presume by analogy to that situation that the delay or failure to exit from mitosis seen with our strains is due to persistent activation of the Clb-p34^{CDC28} kinase.

It is unclear from these experiments whether the mitotic cells accumulate in metaphase or anaphase. We have observed that cells arrested by Clb3 overexpression (Fig. 2) contain long, thin spindles and well-separated chromosomes, suggestive of an anaphase arrest, while cells arrested by Clb1Δ152 overexpression (7) often have shorter spindles and DNA bridges between the two sets of chromosomes, suggestive of a metaphase arrest. However, interpretation of such subtle morphological differences is uncertain since the degree of chromosome separation may be an artifact of prolonged arrest in a nonphysiological mitotic state rather than truly representative of normal metaphase or anaphase.

Clb overproduction cannot override checkpoint control. We reasoned that if reduced Clb accumulation is an important facet of checkpoint control, then overproduction of Clbs would override this control and cells would be driven into mitosis even though their DNA had not been fully replicated. To test this hypothesis, we grew the strains described above in sucrose medium and arrested the cells by addition of hydroxyurea for 5 to 6 h. Following arrest, galactose was added to the cultures for a further 4 h in the continued presence of hydroxyurea. The cells were then fixed and stained with antitubulin antibodies to determine whether they had entered mitosis. Figure 3 shows one such experiment using the *GAL1::CLB3* strain. Cells arrested with hydroxyurea displayed short, thick microtubule spindles extending across undivided nuclei (Fig. 3A and B) characteristic of G_2 cells. Induction of Clb3 in cells not treated with hydroxyurea drove the cells to arrest in mitosis with elongated spindles and separated chromosomes (Fig. 3C and D). However, induction of Clb3 in cells arrested by hydroxyurea failed to drive them into mitosis: the cells retained a short spindle and undivided nucleus (Fig. 3E and F). Similar results were obtained with the Clb1- and Clb2-overexpressing strains (data not shown). In all cases, >90% of the hydroxyurea-treated cells arrested with a scorable large-bud morphology, and >97% of these displayed short G_2 spindles even after induction of Clb synthesis with galactose. Overexpression of Clbs in the hydroxyurea-arrested cells was confirmed by Western blotting (see below). Thus, checkpoint controls are able to prevent mitotic induction even when Clbs are overproduced.

We confirmed this surprising result by an independent method. Strains carrying the various *GAL1::CLB* constructs were treated with hydroxyurea and then induced to overproduce Clbs by addition of galactose as described above. Following induction, the viability of the cells was determined by a plating efficiency assay (see Materials and Methods). If Clb overproduction had bypassed checkpoint controls, then the hydroxyurea-treated cells would have attempted to separate their unreplicated chromosomes, leading to mitotic catastrophe and rapid loss of cell viability. This was not observed (Fig. 4A): cells arrested with hydroxyurea and induced to overexpress Clb1, Clb2, or Clb3 did not suffer any loss of viability. Thus, Clb overexpression cannot override checkpoint controls.

Cells overproducing the stabilized Clb1Δ152 did undergo a rapid loss of viability (Fig. 4A), but this was not clearly attributable to mitotic catastrophe since the same loss of viability was observed in the absence of hydroxyurea, suggesting that Clb1Δ152 overexpression had toxic irreversible effects.

Clb overproduction together with a p34^{cdc2} Tyr-15→Phe mutant cannot override checkpoint control. The results presented above and those published previously show that neither Clb overproduction nor mutation of p34^{CDC28} to render it unresponsive to inhibitory phosphorylation can override checkpoint control in *S. cerevisiae*. However, activation of checkpoint control by treatment with hydroxyurea did result in reduced Clb accumulation (above) and some p34^{CDC28} tyrosine phosphorylation (1). Therefore, it seemed possible that these mechanisms might be acting redundantly in parallel to ensure p34^{CDC28} inactivation in response to checkpoint control. In this scenario, overproduction of Clbs would be ineffective at overriding checkpoint control because the accumulated Clb-p34^{CDC28} complexes would be inhibited by p34^{CDC28} tyrosine phosphorylation. Similarly, the p34^{CDC28} Tyr-19→Phe mutant would be unable to override checkpoint controls because reduced Clb accumulation would result in insufficient activation of the relevant Clb-p34^{CDC28} complexes.

To test this possibility, we constructed strains that would simultaneously overproduce individual Clbs together with wild-type or Tyr-15→Phe mutant p34^{cdc2} proteins upon addition of galactose (see Materials and Methods). Previous studies have shown that both wild-type and Tyr-15→Phe mutant *cdc2* from *S. pombe* are interchangeable with *CDC28* from *S. cerevisiae* (28). We arrested *GAL1::CLB GAL1::cdc2* cells with hydroxyurea and then added galactose to induce expression of the *GAL1*-regulated genes. To assess the level of cdc2 overexpression we performed Western blots using an anti-PSTAIRE monoclonal antibody, which recognizes an epitope conserved between the Cdc28 and cdc2 proteins. Assuming that the antibody recognizes both proteins efficiently, the cdc2 proteins were expressed at more than 10-fold-higher levels than the endogenous Cdc28 protein upon galactose induction (data not shown). This should provide enough cdc2 to compete effectively for cyclin binding. As shown in Fig. 4B, viability assays of these cells indicated no loss of viability upon incubation in galactose, showing that, even in the presence of the nonphosphorylatable p34^{cdc2} mutant, overexpression of Clbs was unable to override checkpoint control.

Histone H1 kinase activity is fully activated in checkpoint-arrested cells. One way to account for the results presented above would be that checkpoint controls prevent p34^{CDC28} activation through a route other than reduced Clb transcription or increased p34^{CDC28} tyrosine phosphorylation. For example, degradation or posttranslational modification of Clbs might prevent accumulation of active Clb-p34^{CDC28}

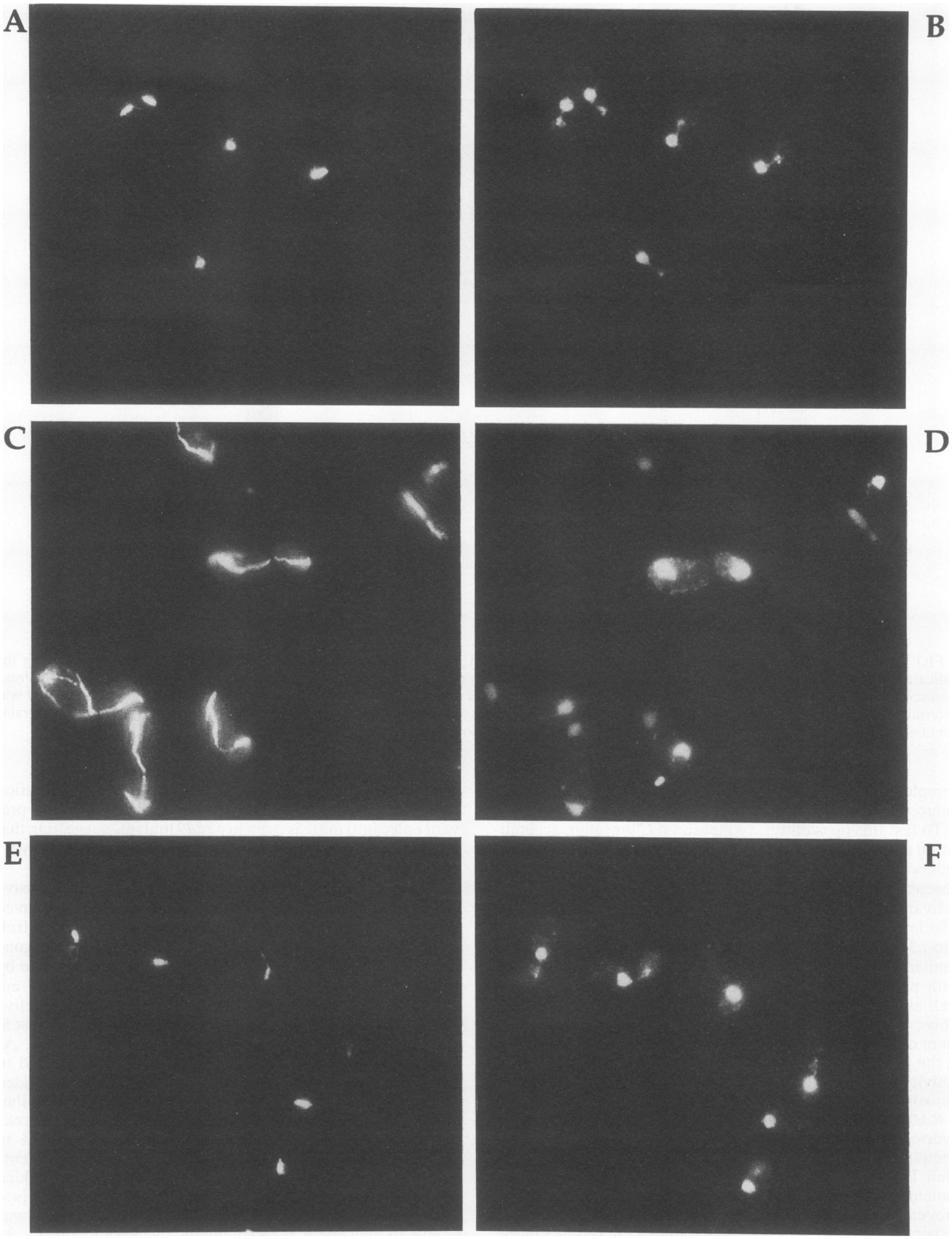

FIG. 3. Clb3 overexpression does not override hydroxyurea-induced S-phase arrest. Cells containing the *GAL1*::*CLB3* gene were grown in YEP-sucrose and treated as follows: (A and B) 0.2 M hydroxyurea in YEP-sucrose for 9 h; (C and D) YEP-galactose for 4 h; (E and F) 0.2 M hydroxyurea in YEP-sucrose for 5 h followed by 0.2 M hydroxyurea in YEP-galactose for 4 h. (A, C, and E) Tubulin distribution; (B, D, and F) DNA distribution.

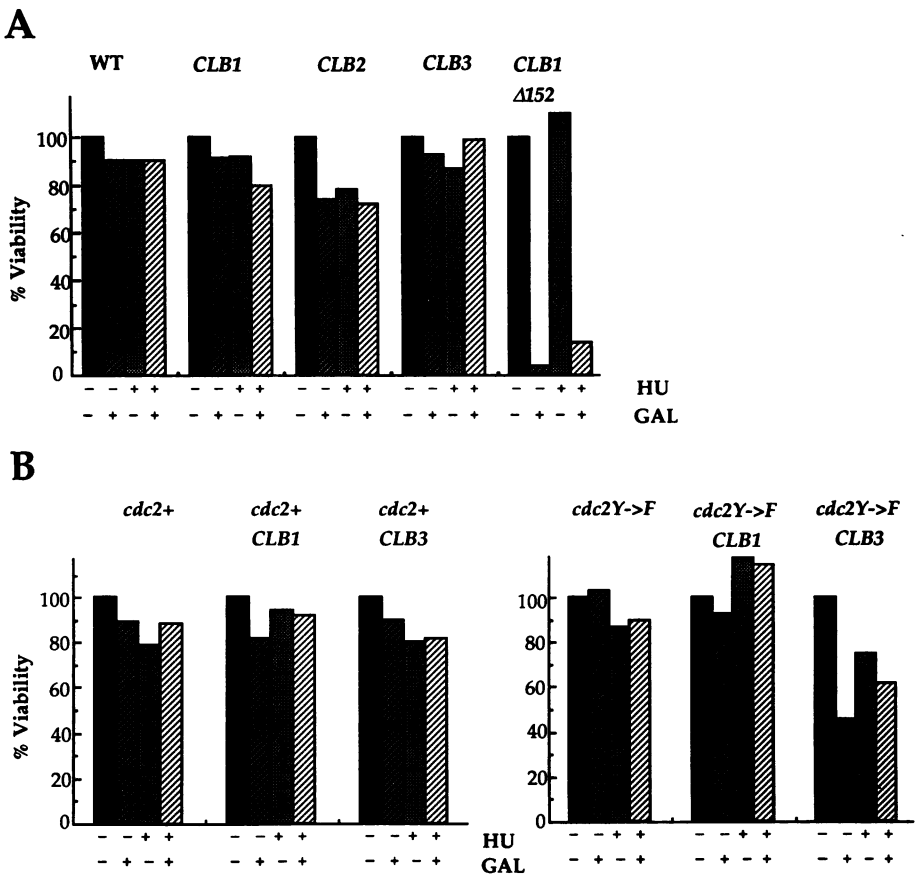

FIG. 4. Clb overexpression does not cause mitotic catastrophe in hydroxyurea-arrested cells. (A) Cultures of strains containing the indicated *GAL1*::*CLB* genes were treated (+) with 0.2 M hydroxyurea (HU) for approximately 5 h. Cells were then transferred to fresh medium with (+) or without (−) galactose (GAL) and/or 0.2 M HU as indicated and incubated for an additional 4 h. Percent viability was determined as described in Materials and Methods. (B) Viability assays were performed as for the results shown in panel A with strains containing *GAL1*::*cdc2* (left) or *GAL1*::*cdc2*$^{Y\rightarrow F}$ (right) genes in addition to the *GAL1*::*CLB* genes.

complexes even when transcription of Clbs is massively induced by the *GAL1* promoter.

To test this possibility, we measured Clb protein accumulation and Clb-p34^{CDC28} histone H1 kinase activation in cells induced to express Clbs by galactose in the presence or absence of hydroxyurea. As shown in Fig. 5A for Clb3, activation of checkpoint control by hydroxyurea had no effect on either the amount of Clb induction or the degree of Clb-p34^{CDC28} kinase activation upon addition of galactose. A similar result was obtained upon cooverexpression of Clb3 with p34^{cdc2} wild-type and mutant constructs (Fig. 5B) as well as overexpression of Clb1 and Clb2 (data not shown). Thus, checkpoint control activated by hydroxyurea treatment of the cells was able to prevent entry into mitosis even in the presence of a level of Clb-p34^{CDC28} histone H1 kinase activity which was otherwise sufficient to drive the cells into mitosis (Fig. 2).

***RAD9*-dependent checkpoint control behaves similarly to hydroxyurea-induced checkpoint control.** Cells that have completed DNA replication but have suffered damage to their DNA in G_2 (for example, as a result of exposure to ionizing radiation) are subject to a checkpoint control that prevents entry into mitosis until the DNA damage has been repaired. This checkpoint control requires the product of the *RAD9* gene of *S. cerevisiae*, without which mitosis is not delayed and cells with damaged DNA undergo mitotic catastrophe (30). In contrast, cells whose DNA replication has been blocked by treatment with hydroxyurea can prevent entry into mitosis even in a *rad9* mutant, suggesting that incomplete DNA replication and damaged DNA activate distinct checkpoint control pathways (11).

To determine whether the DNA damage-responsive (*RAD9*-dependent) checkpoint control pathway behaved similarly to the hydroxyurea-activated checkpoint control, we induced Clb overexpression in cells that had undergone arrest in G_2 with damaged DNA. This was accomplished by using the temperature-sensitive *cdc9* mutation. *CDC9* encodes the enzyme DNA ligase. Upon shift to the restrictive temperature, *cdc9* cells undergo a *RAD9*-dependent arrest in G_2 with unrepaired nicks in the chromosomal DNA. As shown in Fig. 6, *cdc9* cells arrested in G_2 and induced to overexpress Clb3 retained a short spindle and an undivided nucleus, characteristic of G_2 cells. A small and rather variable proportion (6 to 20%) of the scorable large-bud cells did display a mitotic configuration (compared with 3% in cells not overexpressing Clbs), but control experiments revealed that the same proportion of cells were leaking through the *cdc9* block at the temperature we employed (see Materials and Methods). Thus, Clb3 overexpression was unable to override *RAD9*-dependent checkpoint control. As with hydroxyurea-treated cells, a full mitotic level of Clb3-p34^{CDC28} histone H1 kinase was induced in *cdc9*-blocked

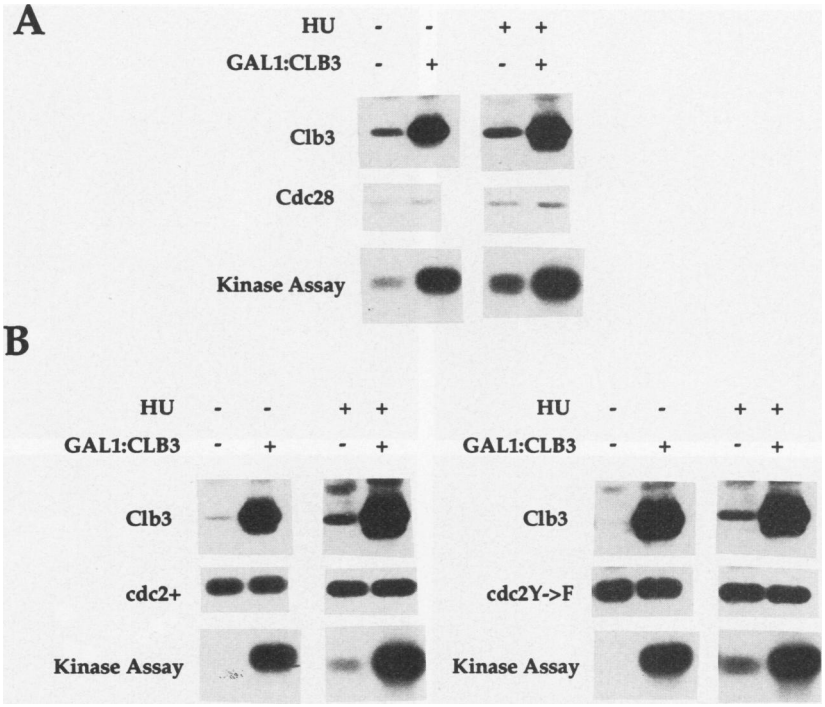

FIG. 5. Induction of Clb3-p34 kinase in hydroxyurea-arrested cells. (A) Wild-type and *GAL1::CLB3* cells (− and +, respectively for GAL1:CLB3) were incubated either in YEP-galactose for 4 h or with hydroxyurea (HU) for 5 h followed by 4 h in YEP-galactose plus HU (− and +, respectively for HU). Clb3 levels in cell lysates were determined by immunoblotting (top) by using Cdc28 levels (middle) as a control for protein loading. Clb3-associated kinase activity was assayed with the same lysates (bottom). (B) Similar assays were performed with cells containing additional *GAL1::cdc2*$^{+}$ (left) or *GAL1::cdc2*$^{Y\rightarrow F}$ (right) genes.

cells upon addition of galactose (data not shown). Similar results were obtained with Clb1- and Clb2-overexpressing strains and also by using the *cdc13* instead of the *cdc9* mutation to induce *RAD9*-dependent G_2 arrest (data not shown). Furthermore, Clb overexpression conferred no additional loss of viability on arrested *cdc9* cells, confirming the conclusions from the immunofluorescence experiments (data not shown). Thus, like hydroxyurea-activated checkpoint control, *RAD9*-dependent checkpoint control can prevent entry into mitosis even in cells that have fully activated their Clb-p34^{CDC28} histone H1 kinase.

Active Clb-p34^{CDC28} complexes are smaller in hydroxyurea-arrested cells. In unsynchronized growing cells, the p34^{CDC28}-associated histone H1 kinase activity is found in high-molecular-weight complexes which are eluted from a gel filtration column in a broad peak with an apparent molecular mass centered around 200 kDa (31). This is considerably larger than the size expected for the 1:1 complex between p34^{CDC28} (34 kDa) and cyclin (50 to 60 kDa), which has been demonstrated to be sufficient for histone H1 kinase activity (2a, 14). Thus, other proteins are likely to be present in the active complexes in vivo. One way to regulate p34^{CDC28} activity in response to checkpoints might therefore be to modify these putative other components, rendering the kinase unable to promote mitosis despite retaining the ability to phosphorylate histone H1 in vitro. To address this possibility, we separated extracts from cycling or hydroxyurea-arrested cells by gel filtration chromatography using a Superose 12 column (see Materials and Methods). Column fractions were assayed to determine the amount of p34^{CDC28}-associated histone H1 kinase activity (Fig. 7A) and p34^{CDC28} protein (Fig. 7B). In both cycling and hydroxyurea-arrested cells, the bulk of the p34^{CDC28} protein was eluted as a monomer (eluted between 15 and 16 ml [Fig. 7B]) with little or no histone H1 kinase activity, as previously described (31). The Western blots in Fig. 7B were probed with an anti-PSTAIRE monoclonal antibody which recognizes not only p34^{CDC28} but also a background band of slightly higher apparent molecular weight (see load lanes at left). Whereas extracts from cycling cells contained active complexes eluted in a broad peak between 10.6 and 12.6 ml, extracts from hydroxyurea-arrested cells displayed active complexes with significantly retarded mobility, being eluted between 12 and 13.6 ml (Fig. 7A). The p34^{CDC28} eluted with the complexes was similarly shifted from 10.8 to 12.4 ml in extracts from cycling cells to 12.6 to 13.6 ml in extracts from hydroxyurea-arrested cells (Fig. 7B; note that the immunoreactive band eluted between 13 and 14 ml in the asynchronous cell extract consists predominantly of the upper background band, not p34^{CDC28}). Thus, the active complexes in checkpoint-arrested cells were significantly smaller than those in cycling cells. For comparison, 1:1 complexes between purified p34^{CDC28} and bacterially expressed Clb3 were reconstituted (2a) and run on the same column. The fractions were assayed for histone H1 kinase activity, and the peak activity was eluted between 12.8 and 13.4 ml, very close to the complexes from hydroxyurea-arrested cells. This suggests that in hydroxyurea-arrested cells, the complexed p34^{CDC28} exists as a 1:1 heterodimer with Clbs, although on the basis of previous results (see Discussion) it seems likely that p18^{CKS1} is also present in these complexes (10).

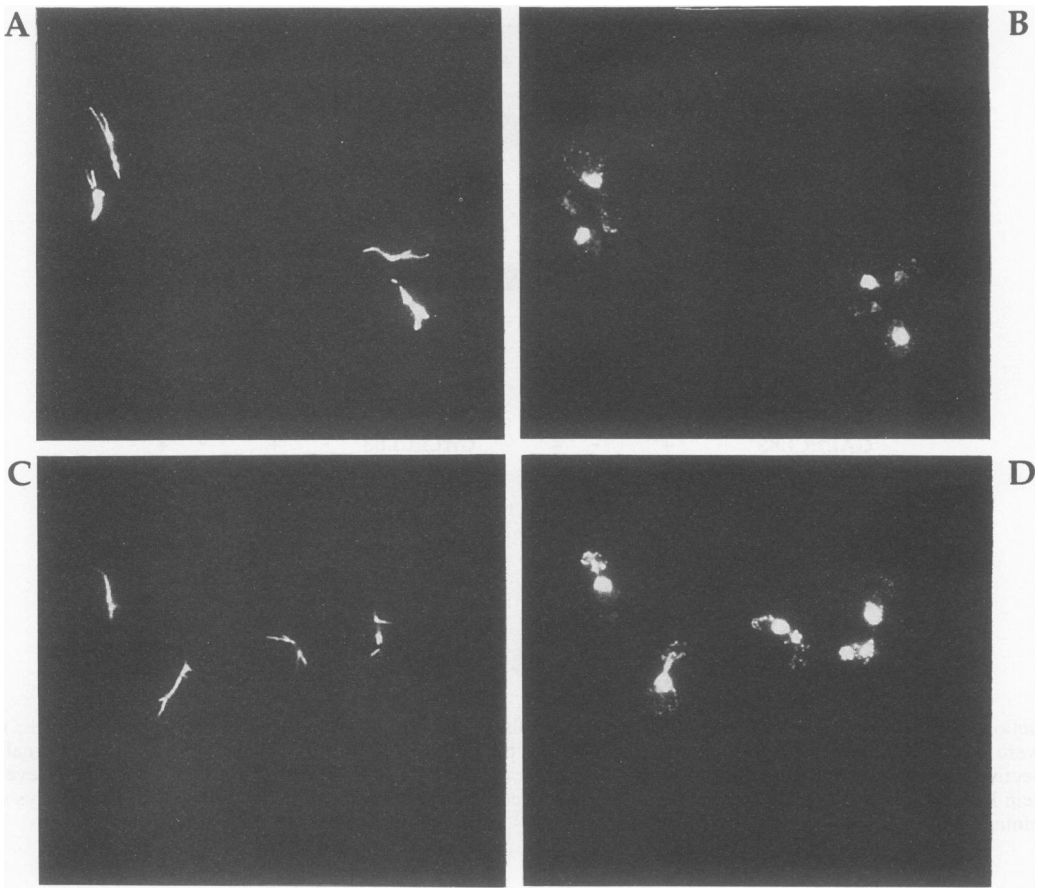

FIG. 6. Clb3 overexpression does not override *cdc9*-induced G_2 arrest. *cdc9-8* cells (A and B) or *cdc9-8 GAL1::CLB3* cells (C and D) were incubated in YEP-sucrose at 36°C (the restrictive temperature for this mutant) for 3.5 h and transferred to YEP-galactose for an additional 4 h at 36°C. The cells were fixed and processed to visualize the tubulin (A and C) and DNA (B and D) distributions.

DISCUSSION

Checkpoint controls in *S. cerevisiae* can prevent entry into mitosis even in the presence of fully activated Clb-p34^{CDC28} histone H1 kinase. Previous studies with both the fission yeast *S. pombe* and cell extracts from *Xenopus* eggs have shown that checkpoint controls in these systems prevent entry into mitosis by preventing the activation of p34^{cdc2}. This result is appealing, because the role of p34^{cdc2} as the key mitotic inducer makes it an ideal integration point for signals that control mitotic induction. In these experimental systems, inhibition of mitosis was correlated with an inhibition of p34^{cdc2}-associated histone H1 kinase activity measured in vitro. Thus, it is rather surprising that in *S. cerevisiae*, checkpoint controls appear to operate at a point downstream of p34^{CDC28} histone H1 kinase activation.

We have confirmed that hydroxyurea-treated cells contain a higher level of p34^{CDC28}-dependent histone H1 kinase activity than the average of an exponentially growing population. This result has been interpreted as an indication that checkpoint controls fail to restrict activation of the kinase (since a moderately high activity is observed) (28). However, this analysis can be rather misleading, since many different cyclins can associate with p34^{CDC28} and stimulate its kinase activity and it is not clear how much of each complex is actually required to be active in order to induce mitosis. For example, both *S. pombe* and *S. cerevisiae* cells can be arrested in G_2 by overexpression of the *wee1* gene (encoding a protein kinase that phosphorylates and inhibits p34^{cdc2}) and inactivation of the *cdc25* gene (encoding a protein phosphatase that dephosphorylates and activates p34^{cdc2}) (25). A considerable body of genetic evidence indicates that the G_2 block is the result of p34^{cdc2} inactivation. However, assays of total p34^{cdc2} or p34^{CDC28} histone H1 kinase activity in the G_2-arrested cells show quite a high activity from the supposedly inhibited kinase. In *S. cerevisiae*, this activity exceeds that observed with hydroxyurea-treated cells (data not shown; also, see reference 28). A possible resolution of this apparent paradox is that the activity observed in vitro derives from complexes of p34^{CDC28} with cyclins that are not relevant for mitotic induction.

To circumvent the ambiguities inherent in simple measurement of p34^{CDC28}-associated histone H1 kinase activity in checkpoint-arrested cells, we constructed strains that could be induced to produce large quantities of the three cyclins which have been shown genetically to play a role in mitotic induction, namely, Clb1, Clb2, and Clb3. Induction of these Clbs resulted in accumulation of Clb-p34^{CDC28} histone H1 kinase levels exceeding those normally attained during mitosis and caused arrest or delay in mitosis. Using these strains, we were able to show, by two independent techniques, that checkpoint controls could prevent entry into mitosis even in the presence of hyperactivated Clb-p34^{CDC28} complexes (the relevant complexes for mitotic induction). This was true both for the checkpoint control activated by

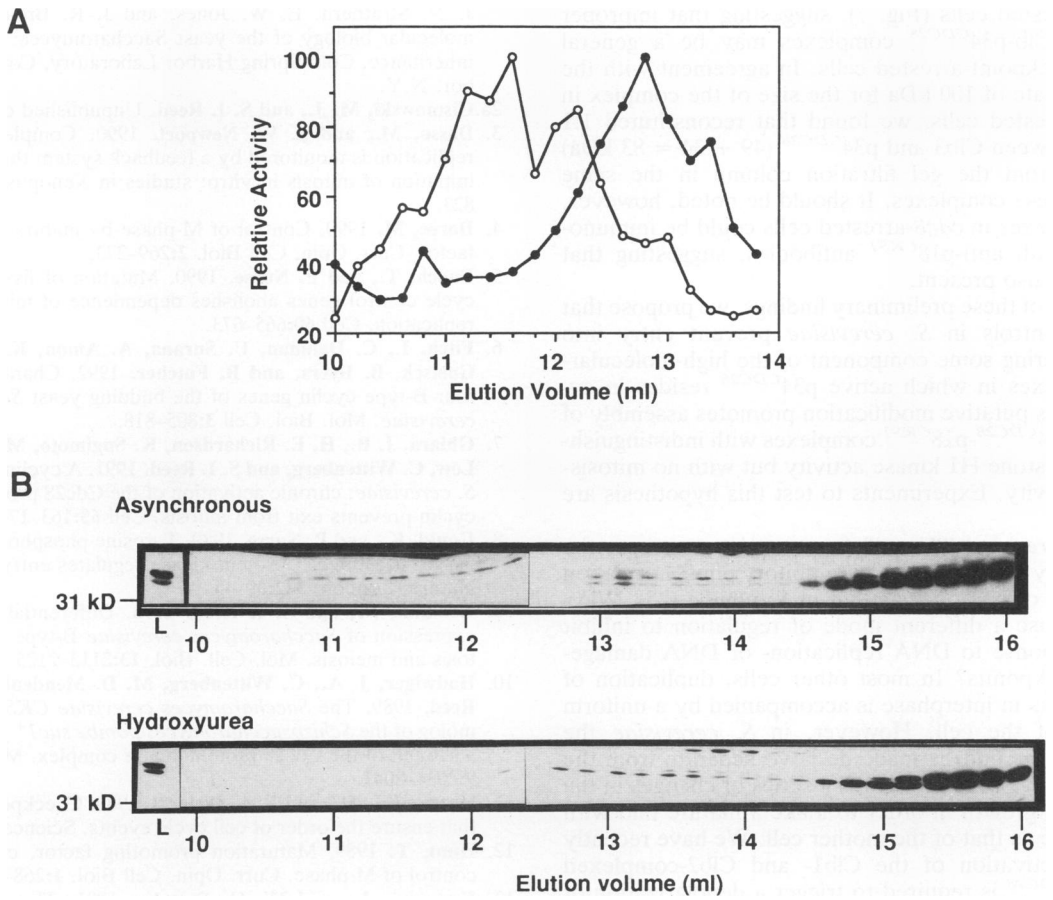

FIG. 7. The active p34^{CDC28} in hydroxyurea-arrested cells is assembled into smaller complexes than in cycling cells. Extracts from cycling cells or cells treated with 0.2 M hydroxyurea for 4 h were fractionated on a Superose 12 column. (A) Histone H1 kinase activity in anti-p34^{CDC28} immunoprecipitates from the indicated fractions. Assays were performed as for the results shown in other figures and quantitated by excising the histone H1 bands from the gel and counting the incorporated radioactivity. The counts are plotted as percent counts per minute in the peak fraction (peak counts were 5,300 cpm for the asynchronous samples and 9,500 cpm for the hydroxyurea-treated samples). Open and filled circles, asynchronous and hydroxyurea-treated samples, respectively. (B) Western blot of p34^{CDC28} in the indicated fractions. L, load. The upper band seen clearly in the load and some column fractions is a background band recognized by the antibody; the lower band is p34^{CDC28}.

incomplete DNA replication and for the *RAD9*-dependent checkpoint control activated by DNA damage.

How do checkpoint controls prevent mitosis in *S. cerevisiae*? The finding that checkpoint controls can block entry into mitosis downstream of p34^{CDC28} histone H1 kinase activation is surprising, because it is thought that triggering of mitosis by p34^{CDC28} involves phosphorylation of a large number of substrates, which then give rise to the different aspects of mitosis. There are three general ways to account for these findings. (i) Although active against the test substrate histone H1 in vitro, the Clb-p34^{CDC28} complexes in checkpoint-arrested cells are inactive against the substrates relevant to mitotic induction in vivo. (ii) The kinase is fully active against the relevant substrates in vivo but cannot induce mitosis because it does not have access to these substrates. (iii) The kinase is fully active and the substrates are available, but phosphorylation of these substrates is insufficient for mitotic induction.

The first explanation predicts that, in checkpoint-arrested cells, the kinase is altered in some way that changes its substrate specificity. The situation in the second explanation could potentially be achieved by mislocalization of the active p34^{CDC28} within the cell or by modification of the substrates so that they cannot be recognized. The third explanation might apply if, for example, checkpoint controls induce phosphatases that maintain the p34^{CDC28} substrates dephosphorylated despite the induced activity of the kinase.

The possibility that the kinase itself was altered in checkpoint-arrested cells, despite retaining in vitro activity, was first suggested by experiments with temperature-sensitive *cdc8* mutants of *S. cerevisiae*. *CDC8* encodes thymidylate kinase, which is required for DNA replication (26). Upon shift to the restrictive temperature, *cdc8* mutants undergo cell cycle arrest presumably as a result of checkpoint controls similar to those examined in this study by using hydroxyurea. In the arrested cells, p34^{CDC28}-associated histone H1 kinase activity was significantly retarded (relative to the kinase from cycling cells) in a gel filtration column, being eluted at an apparent molecular mass of about 100 kDa (see Fig. 7 in reference 10 and Fig. 6A in reference 31). This suggested that, although p34^{CDC28} in the checkpoint-arrested cells had considerable histone H1 kinase activity, it was not assembled into the normal high-molecular-weight complexes. We have reproduced this result by using hy-

droxyurea-arrested cells (Fig. 7), suggesting that improper assembly of Clb-p34^{CDC28} complexes may be a general feature of checkpoint-arrested cells. In agreement with the previous estimate of 100 kDa for the size of the complex in checkpoint-arrested cells, we found that reconstituted 1:1 complexes between Clb3 and p34^{CDC28} (49 + 34 = 83 kDa) were eluted from the gel filtration column in the same fractions as these complexes. It should be noted, however, that the complexes in *cdc8*-arrested cells could be immunoprecipitated with anti-p18^{CKS1} antibodies, suggesting that this protein is also present.

On the basis of these preliminary findings, we propose that checkpoint controls in *S. cerevisiae* prevent entry into mitosis by altering some component of the high-molecular-weight complexes in which active p34^{CDC28} resides in cycling cells. This putative modification promotes assembly of smaller Clb-p34^{CDC28}-p18^{CKS1} complexes with indistinguishable in vitro histone H1 kinase activity but with no mitosis-promoting activity. Experiments to test this hypothesis are in progress.

Why is *S. cerevisiae* different? *S. cerevisiae* possesses the same regulatory system based on inhibitory phosphorylation on tyrosine 19 of p34^{CDC28} as that in *S. pombe* (25). Why, then, does it use a different mode of regulation to inhibit mitosis in response to DNA replication- or DNA damage-activated checkpoints? In most other cells, duplication of cell constituents in interphase is accompanied by a uniform enlargement of the cell. However, in *S. cerevisiae* the daughter cell (the bud) is made de novo separate from the mother cell. This process requires a series of changes in the polarity of cell growth in order to make a mature bud with the same shape as that of the mother cell. We have recently shown that activation of the Clb1- and Clb2-complexed forms of p34^{CDC28} is required to trigger a depolarization of growth within the bud in G_2 (16). If these forms of the kinase are inhibited, either by elimination of the Clb or by inhibitory tyrosine phosphorylation of p34^{CDC28}, the buds become abnormally elongated (16). Thus, if checkpoint controls operated the same way as in *S. pombe* or *Xenopus* cells, an abnormally shaped cell would be formed while the cells awaited completion of DNA replication and/or repair. This is not what is observed with hydroxyurea-treated cells: the Clb-p34^{CDC28}-mediated maturation of the bud proceeds as usual, and the mother-bud pair then grow uniformly during the arrest. Perhaps the checkpoint control mechanism in *S. cerevisiae* has evolved to allow completion of Clb-p34^{CDC28}-mediated morphogenetic events while blocking nuclear division.

ACKNOWLEDGMENTS

We thank Sally Kornbluth and Curt Wittenberg for critically reading the manuscript and Nathalie Grandin for the Clb2 and Clb3 antibodies. The *S. pombe cdc2* Tyr-15→Phe mutant was a kind gift from K. Gould (Nashville, Tenn.).

C.S.S. is supported by a postdoctoral fellowship from the American Cancer Society. D.J.L. is supported by postdoctoral grant DRG1078 from the Damon Runyon/Walter Winchell Cancer Research Fund. M.J.C. is supported by postdoctoral grant GM14314-02 from the NIH. This work was supported by PHS grant GM38328 to S.I.R.

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