Recovery of the Yeast Cell Cycle from Heat Shock-induced G₁ Arrest
Involves a Positive Regulation of G₁ Cyclin Expression by the S
Phase Cyclin Clb5*

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In the yeast Saccharomyces cerevisiae, heat shock stress induces a variety of cellular responses including a transient cell cycle arrest before G₁/S transition. Previous studies have suggested that this G₁ delay is probably attributable to a reduced level of the G₁ cyclin gene (CLN1 and CLN2) transcripts. Here we report our finding that the G₁ cyclin Cln3 and the S cyclin Clb5 are the key factors required for recovery from heat shock-induced G₁ arrest. Heat shock treatment of G₁ cells lacking either CLN3 or CLB5/CLB6 functions leads to prolonged cell cycle arrest before the initiation of DNA synthesis, concomitant with a severe deficiency in bud formation. The inability of the clb5 clb6 mutant to resume normal budding after heat shock treatment is unanticipated, since the S phase cyclins are generally thought to be required mainly for initiation of DNA synthesis and have no significant roles in bud formation in the presence of functional G₁ cyclins. Further studies reveal that the accumulation of G₁ cyclin transcripts is markedly delayed in the clb5 clb6 mutant following heat shock treatment, indicating that the CLN gene expression may require Clb5/Clb6 to attain a threshold level for driving the cell cycle through G₁/S transition. Consistent with this assumption, overproduction of Clb5 greatly enhances the transcription of at least two G₁ cyclin genes (CLN1 and CLN2) in heat-shocked G₁ cells. These results suggest that Clb5 may positively regulate the expression of G₁ cyclins during cellular recovery from heat shock-induced G₁ arrest. Additional evidence is presented to support a role for Clb5 in maintaining the synchrony between budding and DNA synthesis during normal cell division as well.

Cells from various organisms are equipped through evolution with the ability to react to an abrupt elevation of temperature in their environment with what has been collectively termed heat shock response to increase their chance of survival in such an environment (1–4). Heat shock treatment of yeast cells, for instance, induces several observable responses including a reprogramming of gene expression, acquisition of thermostolerance, and a transient cell cycle arrest at G₁ (1, 5–7). The heat shock-induced G₁ arrest in yeast lasts for a period of approximately 1 h (7). Once the heat shock proteins are induced and thermostolerance is acquired, the normal cell cycle resumes. Normal cell cycle progression in yeast relies on sequential activation of the cyclin-dependent kinase Cdc28 by the cell cycle stage-specific cyclins (16–19). G₁/S transition, often referred to as START, is dependent on three G₁ cyclins: Cln1, Cln2, and Cln3 (16–19). Cln3 is particularly important, since it controls the expression of other G₁ cyclins, as well as Clb5 and Clb6, two S phase cyclins required for initiation of DNA synthesis (20, 21). By a yet unknown mechanism, Cln3 activates the transcription factor SBF, which is composed of the Swi4 and Swi6 proteins (22–24). SBF in turn drives the transcription of a set of genes including CLN1 and CLN2, leading to execution of START and budding (22–24). In a parallel fashion, Cln3 is also thought to activate another transcription factor Mbf, consisting of Mbp1 and Swi6, which then stimulates expression of the genes involved in DNA synthesis including CLB5 and CLB6 (22, 23).

Thus, the cyclin cascade initiated by Cln3 sets in motion two parallel cell cycle events downstream of START, i.e. budding and DNA replication, each controlled by a pair of functionally overlapping cyclins. Although G₁ cyclin functions are sufficient for passage of START and bud formation, the initiation of DNA replication cannot properly take place without the functions of Clb5/Clb6. Loss of Clb5/Clb6 functions results in a delay in initiation of DNA synthesis but has no effects on the timing of bud emergence (25), suggesting that Clb5/Clb6 are principally required for DNA synthesis control. Under special conditions, however, Clb5 has been shown to be more versatile, capable of performing some overlapping functions with the G₁ cyclins. Overproduction of Clb5, for example, can rescue the lethality of

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the cln1 cln2 cln3 triple mutant (26). Similarly, elevation of the Clb5 activity through inactivation of Sic1, an inhibitor of the Clb5-Cdc2 kinase, also suppresses the START deficiency in the cln1 cln2 cln3 triple mutant (27, 28). These observations suggest that, although Clb5 normally is not required for execution of START and budding, it is able to provide these functions if its activity is sufficiently increased.

In the present study, the roles of various cyclins in the recovery of cell cycle from heat shock-induced G1 arrest have been examined. Our results suggest that Clb5 is one of the key factors required for this process. Clb5 facilitates the cell cycle recovery following heat shock by promoting initiation of DNA replication on one hand and positively regulating the expression of the G1 cyclin genes to promote budding on the other.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and General Methods—**All yeast strains used in this study were derived from the wild type strain W303 and are listed in Table I. The rad24 and cln1 cln3 mutants were originally derived from U. Surana and I. Herskowitz, respectively. YUS454 (cln1 cln2 cln3::TRP1 cln1 cln2 cln3) contains the CLN3 gene under control of the methionine-repressible MET3 promoter for viability. Yeast extract-yeast peptone, synthetic complete, and dropout media were prepared as described by Rose et al. (29). Recombinant DNA methodology was performed as described by Sambrook et al. (30). PCR was performed with Vent polymerase (New England Biolabs) as recommended by the manufacturer. Genetic manipulations were performed according to standard methods (29). Cell morphology and DNA content analysis by FACScan were performed as described previously (31).

**Plasmid Construction—**Plasmids used in this study are listed in Table II. To generate the plasmids pMC229–233, polymerase chain reaction-amplified coding regions of CLN2, CLN3, CLB3, CLB5, and CLB6 were cloned individually into a centromere vector under control of the GAL1 promoter containing URA3 as a selectable marker. The plasmids pMC234 and pMC235 were generated by fusing the coding regions of CLN2 and CLN3 with GAL1 in a centromere vector containing the LEU2 gene.

**Gene Disruption—**Gene disruptions were performed using the one-step replacement method (32). The CLN2 gene was disrupted by replacing its 1.3-kb XhoI–NcoI fragment by URA3 in wild type and a cln1 mutant to generate YMC432 and YMC434, respectively. The CLB3 gene was disrupted by replacing the 1-kb PsvI–BglII fragment by HIS3, and the CLB4 gene was disrupted by replacing the 0.5-kb StuI–SpeI fragment by TRP1. YMC404 (clb3Δ::HIS3 clb4Δ::TRP1) was generated by two consecutive disruptions. Similarly, YMC406 (clb5Δ::URA3 clb6Δ::TRP1) was made by consecutive disruptions of CLB5, whose 1-kb PvuII–FokI fragment was replaced by URA3, and CLB6, whose 0.9-kb BstXI–XbaI fragment was replaced by TRP1.

**Synchronization and Heat Shock Treatment—** Overnight cultures were diluted to an A600 of 0.1 and were allowed to grow at 25 °C to an A600 of 3.0. At this point, α-factor was added to a final concentration of 5 μg/ml. When greater than 95% cells had been arrested in G1, the cultures were divided into two halves, with one shifted to 42 °C for 30 min and another remaining at 25 °C. The cells were then washed by filtration and resuspended in fresh medium at 25 °C. Samples taken at intervals were used for analysis of cell morphology, DNA content, and Northern blotting.

**TABLE I**

| Strain | Relevant genotype |
|--------|------------------|
| W303a  | MATa ade2 leu2 trp1 his3 |
| YMC309a| MATa rad8Δ TRP1 ade2 ura3 leu2 his3 |
| YMC431 | MATa rad24 ade2 ura3 leu2 trp1 his3 |
| YMC406 | MATa clb5Δ::URA3 clb6Δ::TRP1 ade2 leu2 his3 |
| YMC404 | MATa clb3Δ::HIS3 clb4Δ::TRP1 ade2 ura3 leu2 |
| YMC432 | MATa cln1Δ::TRP1 cln2Δ::URA3 ade2 leu2 his3 |
| YMC433 | MATa cln1Δ::URA3 cln2Δ::TRP1 cln3Δ::ura3 |
| YMC434 | MATa cln1Δ::TRP1 cln2Δ::URA3 ade2 leu2 his3 |
| YMC435 | MATa cln1Δ::URA3 cln2Δ::TRA1 ade2 leu2 his3 |
| YMC436 | MATa cln1Δ::TRA1 cln3Δ::URA3 ade2 leu2 his3 |
| YUS454 | MATa cln1Δ::TRA1 cln2Δ::URA3 ade2 ura3 (pMET3-CLN3) |

**TABLE II**

| Plasmids | Construct |
|----------|-----------|
| pMC229   | GAL1-CLN2, URA3, yeast centromere vector |
| pMC230   | GAL1-CLN3, URA3, yeast centromere vector |
| pMC231   | GAL1-CLB5, URA3, yeast centromere vector |
| pMC232   | GAL1-CLB6, URA3, yeast centromere vector |
| pMC233   | GAL1-CLB3, URA3, yeast centromere vector |
| pMC234   | GAL1-CLN2, LEU2, yeast centromere vector |
| pMC235   | GAL1-CLN3, LEU2, yeast centromere vector |

**FIG. 1.** Heat shock induces a transient G1 arrest in wild type and DNA checkpoint mutants. Cells were synchronized in G1 by α-factor and divided into two halves with one shifted to 42 °C for 30 min and another remaining at 25 °C. After washing, the cells were resuspended in fresh medium and incubated at 25 °C. Samples taken at intervals were analyzed for DNA content by FACScan and budding profiles. The heat-treated cultures are shown on the right and the untreated ones on the left. The strains were W303 (wild type), YMC309α (rad9), and YMC431 (rad24). The percentage of unbudded cells is presented with S.D. values. At least 200 cells were counted for each sample.

**Gene Overexpression—**In experiments where the expression of a gene was driven by the GAL1 promoter, the cells were first grown in medium containing raffinose as the sole carbon source. The cells were synchronized in G1, as described above. Galactose was then added to 2% to induce the gene expression for 15 min at 25 °C before being subjected to heat shock treatment. After α-factor was removed by filtration, the cells were resuspended in fresh medium containing raffinose and galactose and incubated at 25 °C. Samples were collected at intervals for further analysis.

In experiments showing that CLB5 overexpression overrides α-factor-induced G1 arrest, the G1-arrested cells were split into two parts with galactose added to one of them, and both cultures were continuously incubated with α-factor.
Northern Analysis—Total RNA was isolated as described by Cross and Tinkelenberg (33), and Northern blot analyses were performed as described by Price et al. (34). To show RNA signals of various genes on the same blot, the DNA probes used earlier on RNA blot were stripped by heating at 80 °C for 30 min in a solution containing 0.1% SDS, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.2). After washing briefly in H2O, the blot was hybridized with another 32P-labeled probe. The RNA levels were quantitated with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

RESULTS

Heat Shock Stress Causes a Transient Cell Cycle Arrest at G1—Previous studies on yeast cell cycle response to heat shock treatment were performed with asynchronous cell populations (12). To better assess the duration of the G1 arrest period and the kinetics of the recovery, we used mating pheromone (α-factor)-synchronized cells for examination of their response to heat shock treatment. Wild type cells were synchronized by α-factor and divided into two halves with one shifted to 42 °C for 30 min, while another remained at 25 °C, followed by washing and resuspension in fresh medium devoid of the mating pheromone. As shown in Fig. 1, cells that had been exposed to high temperature initiated DNA replication and budding at a later time point than those that were not treated with heat shock. The delay was about 60 min (Fig. 1). This result confirms the previous finding that the cell cycle arrest induced by heat shock is prior to START, since budding and DNA replication were both delayed (12).

It is well known that another type of stress, DNA damage, also induces cell cycle arrest in yeast, which is mediated through a control mechanism termed “checkpoint” (35, 36). The checkpoint genes such as RAD9 and RAD24 have been shown to be required for the transient cell cycle arrest at G1 caused by DNA damage (37). To test whether the DNA damage checkpoint also functions in the G1 arrest caused by heat shock treatment, we examined the cell cycle response to heat shock stress in the rad9 and rad24 mutants. As shown in Fig. 1, both mutants exhibited a G1 arrest that lasted about 60 min, similar to the wild type cells, suggesting that the cell cycle arrest after heat shock is not mediated through DNA damage checkpoint genes.

Cln3 and Clb5/Clb6 Are Essential for Recovery from Heat Shock-induced G1 Arrest—Decrease in the CLN1 and CLN2 transcript levels has been suggested as a possible cause of the cell cycle arrest in heat shock-treated cells (12). We therefore examined various cyclin mutants for their response to heat shock treatment. Cells lacking CLN2 responded to heat shock treatment with a G1 arrest and recovered 60 min later, as did wild type cells (Fig. 2). The cln1 mutant behaved similarly (data not shown). Deletion of both CLN1 and CLN2 genes caused a further delay of 30 min (Fig. 2). Nevertheless, the double mutant resumed cell cycle and divided normally after the 90-min delay (Fig. 2 and data not shown).

Remarkably, the cln3 mutant failed to recover from the heat shock-induced G1 arrest for at least 5 h (Fig. 2). The mutant cells showed no sign of DNA replication and virtually no bud formation during the entire period of the experiment (Fig. 2). The cln1 mutant behaved similarly (data not shown). Deletion of both CLN1 and CLN2 genes caused a further delay of 30 min (Fig. 2). Nevertheless, the double mutant resumed cell cycle and divided normally after the 90-min delay (Fig. 2 and data not shown).

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for example, only 26% of the double mutant cells were budded, compared with over 50% in the populations of either the wild type or any of the cln1, cln2, clb5, and clb6 single mutants (Fig. 2, and data not shown). Furthermore, the budded cell population of the double mutant was limited to about 50% for at least 5 h (Fig. 2). It is evident, therefore, that the clb5 clb6 mutant was unable to recover from the heat shock-induced G1 arrest for a rather prolonged period. Both cln3 and clb5 clb6 mutants eventually resumed normal division without showing significant reductions in their viability (data not shown). In comparison, the clb3 clb4 double mutant, defective in another pair of S phase cyclins, Clb3/Clb4, (25, 38, 39), exhibited no defect in recovery from the G1 arrest (Fig. 2).

**Transcription of the CLN Genes Is Impaired in the clb5 clb6 Mutant after Heat Shock**—The severe defect in recovery from heat shock-induced G1 arrest exhibited by the cln3 mutant is, by and large, within expectations, since this cyclin is the crucial factor in promoting the initiation of bud formation and DNA replication. However, the inability of the clb5 clb6 double mutant to resume normal budding was unexpected, because the Clb5/Clb6 cyclins are thought to be required mainly for DNA synthesis and not for bud formation when functional G1 cyclins are present (25). To seek an explanation for this finding, we tested whether the CLN transcript levels were affected in the double mutant. As shown in Fig. 3a, the CLN1 and CLN2 transcripts in the untreated wild type cells accumulated to a peak level 30 min after release from synchronization, followed by periodic oscillations. In the heat shock-treated wild type cells, the CLN1 and CLN2 transcripts lagged 30–60 min before reaching the peak levels (Fig. 3a). Similarly, the accumulation of the CLB5 transcript was also delayed, albeit to a lesser extent compared with that of CLN1 and CLN2 (Fig. 3a). The CLN3 transcript was present in α-factor-treated cells and appeared to be the least affected by heat shock treatment (Fig. 3a). In contrast, the clb5 clb6 mutant displayed a marked reduction in all G1 cyclin transcripts after heat shock treatment (Fig. 3b). The CLN1 and CLN2 transcripts in clb5 clb6 cells did not reach levels comparable with those in the untreated cells until 180 min after release from G1 synchronization (Fig. 3b). The level of the CLN3 transcript was also decreased significantly in the mutant after heat exposure (Fig. 3b). The clb5 clb6 mutant, nevertheless, produced the ACT1
and SSA4 transcripts in a manner similar to wild type cells after heat exposure, indicating that the mutant was not defective in the general and heat shock-induced transcriptions (Fig. 3). These results together suggest that the Clb5/Clb6 cyclins are involved in the recovery of CLN transcript abundance in cells that have undergone heat shock treatment. After washing, the cells were resuspended in fresh medium containing raffinose (for the -GAL cultures) or raffinose plus galactose (for the +GAL culture) and incubated at 25 °C. Samples taken at intervals were analyzed for DNA content and budding profiles. The percentage of unbudded cells is presented with the S.D. values. HS, heat shock. GAL, galactose.

![Fig. 4](image)

**Fig. 4.** Overexpression of CLN and CLB5 eliminates heat-induced G1 arrest. Wild type cells containing each of the GAL1-CLN2, GAL1-CLN3, GAL1-CLB5, or GAL1-CLB3 constructs were grown in raffinose and synchronized by α-factor. The cells were proportioned equally into three parts with galactose added to one of them. The three cultures were incubated for another 15 min before subjecting two of them including the one in galactose to heat shock treatment. After washing, the cells were resuspended in fresh medium containing raffinose (for the -GAL culture) or raffinose plus galactose (for the +GAL culture) and incubated at 25 °C. Samples taken at intervals were analyzed for DNA content and budding profiles. The percentage of unbudded cells is presented with the S.D. values. HS, heat shock. GAL, galactose.

Overexpression of CLN and CLB5 Eliminates the Heat Shock-induced G1 Arrest—The observation that the CLN transcript accumulation was delayed by heat shock treatment supports the notion that the cell cycle arrest following heat shock is attributable to the suboptimal level of G1 cyclin expression. Indeed, overexpression of CLN2 is able to abolish the G1 arrest induced by heat shock treatment in asynchronous cells (12). To better understand the mechanisms of the heat shock-induced G1 arrest, we examined the effects of overexpression of other cyclin genes on cell cycle response to heat shock. The CLN2, CLN3, CLB5, and CLB3 genes were each placed under control of pGAL1, a strong inducible promoter, and introduced into wild type cells. After synchronization in G1 by α-factor in a raffinose-containing medium, galactose was added for 15 min to induce the cyclin gene expression, followed by heat shock and release to fresh medium containing galactose. As shown in Fig. 4, overexpression of each CLN2, CLN3, and CLB5 completely eliminated the G1 arrest. The cyclin-overproducing cells displayed cell cycle kinetics after heat shock treatment comparable with those of untreated wild type cells, as judged by the FACScan pattern and budding profiles (Fig. 4). In contrast, overexpression of two other S phase cyclin genes, CLB3 and CLB4, generated no effect on the G1 arrest after heat shock (Fig. 4, and data not shown), an observation in agreement with the results presented in Fig. 2.

Overexpression of CLB5 Enhances CLN Transcriptions—It has been suggested that the G1 cyclins are the rate-limiting factors in G1 cells for passage of START (16). The finding that heat shock stress failed to result in cell cycle arrest in CLN overexpression cells suggests the same for the heat shock-
induced G1 arrest. Since the Clb5/Clb6 cyclins are not rate-limiting factors for START (25), the ability of overexpressed CLB5 to negate the heat shock-induced G1 arrest is indicative of an indirect role for Clb5/Clb6 in START promotion, possibly as stimulators of the CLN gene transcription. This possibility was investigated by analyzing the CLN transcript abundance in cells overexpressing CLB5. As shown in Fig. 5a, CLB5 overexpression reversed the delay of CLN1 and CLN2 transcripts caused by heat shock, making them appear at the same time as in the wild type cells that experienced no heat shock stress (Fig. 5a). CLN3 transcript levels were not significantly affected by CLB5 overexpression (Fig. 5a). Overexpression of another B-type cyclin gene (CLB3) had no effect on the CLN1 transcription at all (Fig. 5b), consistent with the results described above, namely that neither the mutations nor the overexpression of the CLB3/CLB4 genes conferred any impact on heat shock-induced G1 arrest. It is noteworthy that both CLN1 and CLN2 transcripts retained a periodic expression pattern throughout the cell cycle in the CLB5 overexpression cells.

CLB5 Overexpression Stimulates CLN1/CLN2 Transcription in the cln3 and swi4 Mutants—The finding that CLB5 overexpression stimulates CLN1/CLN2 gene transcription led us to test whether an increase in Clb5 activity will bring about G1/S transition in cln3 cells that had endured heat shock stress. In the absence of heat shock treatment, CLB5 overexpression resulted in slight advancement in cell cycle progression in cln3 cells (Fig. 6a). Heat shock treatment of cln3 cells led to persistent cell cycle arrest at G1, which could be effectively negated by CLB5 overexpression (Fig. 6a), suggesting that the defect of heat-treated cln3 mutant in carrying out G1/S transition is likely to be derived from insufficient CLN and CLB5 transcriptions. This was ascertained by examining the levels of CLN1, CLN2, and CLB5 transcripts in heat-treated cln3 cells. As shown in Fig. 6b, heat shock treatment strongly prevented the accumulation of CLN1/CLN2/CLB5 transcripts, and no transcripts were detected for these three cyclins until 180 min after release from synchronization. Induction of CLB5 overexpression allowed the appearance of the CLN1/CLN2 transcripts to be advanced by 90 min in the heat-treated cln3 cells (Fig. 6b). This suggests that CLB5 overexpression can stimulate CLN1/CLN2 transcription in the absence of the CLN3 function.

Since CLN1/CLN2 expression is mainly dependent on the transcription factor SBF (22, 24), we therefore examined the CLN1/CLN2 levels in the swi4 mutant, which is defective in the DNA binding component of SBF (24). The swi4 cells exhibited

\[ \text{Fig. 5. Overexpression of CLB5 enhances transcription of CLN genes.} \]

Wild type cells containing either the GAL-CLB5 or GAL-CLB3 plasmids were processed by the procedure described in the legend to Fig. 4. The transcript levels are shown in the left panel. The same blot was probed, stripped and reprobed with labeled DNA fragments of indicated genes. Quantitative presentation of the RNA signals is shown in the right panel using a PhosphorImager. a, the cells containing GAL-CLB5. b, the cells containing GAL-CLB3. HS, heat shock. GAL, galactose.
no defect in recovery from heat shock treatment, since they responded to heat shock with a G1 arrest that lasted for about 60 min (Fig. 6c). CLB5 overexpression in swi4 cells completely eliminated this G1 arrest (Fig. 6c). The appearance of CLN1/CLN2 transcripts in swi4 cells was delayed by heat shock treatment until 90 min after release from synchronization, which was, again, greatly advanced by the overexpression of CLB5 (Fig. 6d). These results indicate that the stimulation of CLN1/CLN2 transcription by Clb5 was achieved independently of SBF functions.

Overexpression of Clb5 Stimulates CLN1/CLN2 Transcription in the Presence of Mating Factor—The CLN1/CLN2 gene transcription is known to be inhibited by a-factor (40). The data presented in Fig. 6 already showed that CLB5 overexpression could override the a-factor-dependent inhibition of CLN1/CLN2 transcription. To further demonstrate this finding, the CLN1/CLN2 transcription was analyzed using samples derived from the cultures in which a-factor was constantly present. In agreement with the previous report (40), the transcription of CLN1 and CLN2, but not CLN3, was strongly repressed in the presence of a-factor in wild type cells (Fig. 7a). No significant accumulation of the CLN1/CLN2 transcripts was observed until the end of the time course (240 min, Fig. 7a). In a sharp contrast, the CLN1/CLN2 transcription was markedly induced in the CLB5 overexpression cells shortly after the addition of galactose (Fig. 7a). This result demonstrates that CLB5 overexpression can strongly stimulate the CLN1/CLN2 transcription despite the presence of a-factor.

CLB5 overexpression in wild type cells not only stimulated the CLN1/CLN2 expression, but also promoted budding and DNA replication, in the presence of a-factor (Fig. 7b). Similar results were also observed in a-factor-treated cells of the cln1 cln2, cln1 cln3, and swi4 mutants (Fig. 7b).

CLB5 May Coordinate between Budding and DNA Replica-
It is important to note that the CLB5-promoted cell cycle progression in the presence of α-factor retained a good synchrony between budding and DNA replication (Fig. 7b). This raised an interesting possibility that the Clb5 regulation of CLN gene expression may not be limited to cell cycle response to heat shock stress and could conceivably be important in coordination of budding and DNA synthesis. To examine this hypothesis, we followed the timing of budding relative to DNA synthesis in the Clb5-overproducing cells in the absence of functional Cln cyclins. If Clb5 is important for coordination between DNA synthesis and budding by positively regulating G1 cyclin gene expression, an increase in Clb5 levels will promote the two processes simultaneously only in the presence of at least one CLN gene, as is the case in the experiment presented in Fig. 7b. Wild type cells, as shown in Fig. 8, exhibited good coordination of budding and DNA synthesis during the cell cycle after release from α-factor synchronization. At the time, for example, when nearly half of the population had replicated DNA, as judged by the size of the 2N peak in FACScan (93 min after release), 52% of the cells remained unbudded. Wild type cells overexpressing CLB5 initiated DNA synthesis earlier, generating half of the population in S phase at 79 min after release. Importantly, the budding process was similarly advanced in these cells, showing 53% of the unbudded population at this time point. This suggests, therefore, that, although Clb5 is thought to be much more efficient in promoting DNA synthesis than in promoting bud formation (16, 17, 25, 41, 42), its overproduction in the presence of one or more functional G1 cyclin genes maintains the coordination between budding and DNA synthesis. In contrast, in cells lacking all three G1 cyclin genes (the cln1 cln2 cln3 triple mutant), an uncoupling of the two processes was observed. While CLB5 overexpression allowed the triple mutant to bud as well as to replicate DNA after release from α-factor synchronization, the budding process was significantly delayed in relation to DNA synthesis. At the time when half of the population had replicated DNA (79 min), 94% of the population still remained unbudded (Fig. 8). These results confirm the anticipation that an increase in Clb5 activity will advance both processes of DNA synthesis and budding simultaneously only in the presence of functional G1 cyclin genes.

**DISCUSSION**

Heat Shock-induced G1 Arrest Is Unlikely to Be Caused by Checkpoint-like Mechanisms—Like many other organisms, yeast cells react to heat shock stress with a reprogramming of cellular activities to increase their ability to survive in the stressful environment. One of the consequences of this reaction is the transient cell cycle arrest at G1. Since another type of stress signal, DNA damage, causes a similar G1 delay, which is
dependent on the so-called checkpoint functions (37), it is reasonable to ask whether the heat shock-induced G1 delay is also a result of active inhibition of the cell cycle by a mechanism similar to the DNA damage checkpoint. It has been reported that a putative transcriptional repressor, Xbp1, is induced by various stress signals including heat shock (13). Overexpression of XBP1 leads to down-regulation of transcription of an extensive array of genes including CLN1/CLN2/CLN3 (13). Our present study shows that the heat shock-induced G1 delay does not involve the functions of some of the DNA damage checkpoint genes. In addition, we also found that elimination of the G1 delay in heat-treated cells by artificially forcing the passage of START has no discernible effect on cell viability (data not shown). Together with the earlier observation that the heat stress-induced cell cycle arrest is not required for acquisition of thermotolerance (5), these results suggest that the G1 arrest after heat shock is unlikely to be a manifestation of a checkpoint-like control mechanism. Instead, it may be a result of insufficient START-promoting G1 cyclins caused by the selective gene expression employed by the cell to gain thermotolerance.

The Roles of G1 Cyclins in Recovery from Heat Shock-induced G1 Arrest—The G1 cyclins are probably the only proteins limiting for START execution, since cells overproducing G1 cyclins can pass through START immediately after cytokinesis (16). Rowley et al. (12) have attributed the heat shock-induced G1 arrest to low levels of CLN1 and CLN2, but not CLN3, transcripts. We have confirmed their observation using G1-synchronized cells. We show that in wild type cells, the accumulation of the CLN1/CLN2 transcripts is strongly suppressed by heat shock for at least 30 min, while that of CLN3 is less affected. However, a significant reduction in the CLN3 transcript level is observed in the clb5 clb6 mutant, suggesting that heat shock stress exerts an adverse impact on the expression of all three G1 cyclin genes, particularly in the absence of CLB5/CLB6 functions.
Fig. 8. Coordination of budding and DNA replication by CLB5. Wild type cells containing GAL-CLB5 were synchronized in G1 by α-factor in raffinose. The culture was divided into two parts, galactose was added to one part, and the culture incubated for another 15 min. After washing, the cells were released into fresh medium containing raffinose (left) or raffinose plus galactose (middle). The cln1 cln2 cln3 mutant (YUS454) containing GAL-CLB5 (right) was grown in raffinose in the absence of methionine and synchronized in G1 by α-factor in medium containing methionine. The cells were then released into fresh raffinose medium containing galactose and methionine. Aliquots of cells were taken at intervals for analysis of DNA content and budding profiles.

|          | Wild type | Wild type | cln1 cln2 cln3 |
|----------|-----------|-----------|----------------|
|          | pGALI-CLB5 | pGALI-CLB5 |               |
| IN | 1N | 2N | IN | 1N | 2N | IN | 1N | 2N | Time (min) |
| 38.5 ± 2.5 | 21.5 ± 3.5 | 49 ± 2 | 128 |
| 41 ± 4 | 19 ± 3 | 60 ± 3 | 114 |
| 46 ± 2 | 20.5 ± 2.5 | 66.5 ± 1.5 | 100 |
| 52.5 ± 1.5 | 26 ± 4 | 75.5 ± 2.5 | 93 |
| 63.5 ± 1.5 | 37 ± 2 | 87 ± 1 | 86 |
| 70 ± 3 | 53.5 ± 1.5 | 94.5 ± 1.5 | 79 |
| 90 ± 1 | 61.5 ± 2.5 | 90 ± 1 | 72 |
| 99 ± 0 | 72 ± 3 | 97 ± 0 | 65 |
| 99.5 ± 0.5 | 78 ± 2 | 99.5 ± 0.5 | 58 |
| 99.5 ± 1 | 88.5 ± 1.5 | 99.5 ± 1 | 51 |
| 99 ± 1 | 93 ± 1 | 101 ± 1 | 37 |
| 99.0 ± 0 | 96.5 ± 0.5 | 90 ± 1 | 30 |
| 99.5 ± 0.5 | 100 ± 1 | 99.5 ± 0.5 | 23 |
| 97 ± 1 | 98 ± 1 | 97 ± 1 | 0 |

Of the three G1 cyclins, Cln3 is the most important in that it determines the expression of the other two, as well as the expression of the Clb5 and Clb6 cyclins (16, 17, 19). Cln3 as such, however, is not believed to be directly involved in DNA replication and budding (25). Our studies indicate that Cln3 is required for cellular recovery from heat shock-induced G1 arrest. Neither the cln1 nor cln2 mutants exhibited a discernible defect in the recovery, and the cln1 cln2 double mutant only showed a 30-min delay in the recovery compared with the wild type (and either of the single mutants). In contrast, the cln3 mutant could not recover from the arrest during the entire 5-h observation. Therefore, although CLN1/CLN2 transcripts are most apparently inhibited from accumulation by heat shock stress among all the cyclin genes investigated, they are nonessential for the process of cell cycle recovery. This is probably due to the presence of Clb5 function, which is capable of carrying out START execution in place of G1 cyclins. In the absence of Cln3, the expression of all three cyclin genes (CLN1/CLN2 and CLB5) is greatly reduced after heat shock. This explains why cln3 mutant is unable to recover from the heat shock-induced G1 arrest for a prolonged time.

The Role of Clb5/Clb6 in Cell Cycle Recovery from Heat Shock-induced G1 Arrest—The Clb5/Clb6 cyclins are thought to be required mainly for normal S phase promotion. In the clb5 clb6 double mutant, initiation of S phase is greatly delayed (25). This has led to the conclusion that the CLN1/CLN2 transcripts are completely negated the delay of CLN1/CLN2 transcript accumulation. The fact that the transcription of CLN3 is not as dramatically enhanced as CLN1/CLN2 is probably due to the fact that it is already near an optimal level. Overexpression of CLB3 in the same way as CLB5 made no difference to CLN transcription, indicating that CLB3 plays no roles in cell cycle recovery from the G1 arrest. CLB5 overexpression also remarkably stimulates the CLN1/CLN2 transcription in the heat shock-treated cln3 and swi4 mutant cells. In the cln3 mutant, the appearance of CLN1/CLN2 transcripts is advanced by 90 min by Clb5 after heat shock treatment. There is at least 60-min advancement of CLN transcription in the heat shock-treated swi4 mutant as well. In addition, the transcription of the CLN1 and CLN2 genes, which is normally repressed by the mating pheromone α-factor, can be greatly stimulated by CLB5 overexpression in the presence of α-factor. Therefore, Clb5 is able to stimulate CLN1/CLN2 gene transcription when its activity is sufficiently elevated.

In fact, the effect of artificially elevated Clb5 activity on CLN gene expression has been investigated previously using small daughter cells isolated by centrifugal elutriation (25). Although the accumulation of the CLN1 transcript was noticeably advanced by Clb5 overproduction, it was not sufficient to prompt the authors to draw the conclusion that Clb5 is able to stimulate the CLN gene expression (25). The discrepancy in the degree of CLN activation by Clb5 in their observation and ours can be attributed to the fact that these authors used small early G1 cells, whereas we employed late G1 cells. It is likely that the accumulation of certain factors, such as those for gene transcription and mRNA translation, only reach a functional level.
for Clb5-stimulated CLN gene expression around late G1. Thus, overproduction of Clb5 alone in the early G1 cells is not expected to accomplish the task of activating CLN gene expression. Indeed, using late G1 cells, these authors observed strong induction of PCL1 and RNR1, two genes regulated by SBF and MBF, respectively, by Clb5 overproduction in the absence of three G1 cyclin genes (25).

In view of the previous findings that Clb5 overproduction can perform START functions and promote transcription of the SBF- and MBF-dependent genes in the cln1 cln2 cln3 triple mutant (25, 26), our new observation of CLN gene activation by Clb5 may be, after all, not completely unexpected. However, the important question remaining to be answered is whether Clb5 actually performs these functions under normal physiological conditions, as it did under those artificial ones. We now provide evidence to suggest that Clb5 is indeed required for optimal expression of CLN genes in vivo. First, the clb5 clb6 mutant is defective in resumption of normal budding after heat shock treatment despite the presence of all three CLN genes. Wild type G1 cells recover from heat shock-induced arrest after about 1 h. At this point, both budding and DNA replication have progressed approximately halfway (Fig. 1). Under the same conditions, however, the clb5 clb6 mutant is completely defective in initiation of DNA replication, and severely defective in bud formation, during the entire time course of 5 h (Fig. 2). Therefore, a physiological situation has been identified under which Clb5 and Clb6 are essential for proper bud formation. Second, transcription of all three CLN genes becomes impaired in the clb5 clb6 mutant after heat shock. Heat shock treatment of wild type cells delays appearance of the peak level CLN1/CLN2 transcripts for 30–60 min. However, attainment of peak levels of the CLN1/CLN2 transcripts is delayed for at least 150 min by heat shock stress in the double mutant. The accumulation of the CLN3 transcript is also significantly delayed in the double mutant. This indicates that the accumulation of the CLN transcripts to peak levels, crucial for promoting G1/S transition, requires the activity of the Clb5/Clb6 cyclins in heat shock-treated cells. These results obtained under physiological conditions clearly advocate a role for Clb5/Clb6 in positive regulation of CLN gene expression in vivo.

Clb5 is normally under the negative control by Sic1, which binds to, and inhibits the activity of the Clb5-Cdc28 kinase (42). Phosphorylation of Sic1 by Cln-associated Cdc28 kinase and its subsequent ubiquitination by the Cdc4-Skp1-Cdc53 complex triggers the proteolytic inactivation of Sic1 (43–46). The ability of Clb5 to promote expression of Cln1/Cln2 suggests that DNA replication and budding are promoted simultaneously by Clb5 in cells carrying one or more G1 cyclin genes (51). Nonetheless, the important point is that DNA replication and budding are promoted simultaneously by Clb5 in cells carrying one or more G1 cyclin genes (Fig. 7b). Examination of synchronized cell cycle progression further supports this notion. In the presence of CLN genes, initiation of DNA replication and bud formation are equally advanced by CLB5 overexpression (Fig. 8). However, in the absence of CLN genes, the synchrony between DNA replication and bud formation is lost (Fig. 8). Therefore, although Clb5 is intrinsically a poor promoter for bud emergence (16, 17, 25, 41, 42), its overproduction maintains the coordination between the two processes as long as at least one CLN gene is present. The dependence of Clb5-driven synchronous progression of cell cycle on the presence of CLN genes suggests that Clb5 promotes bud formation indirectly through increasing the activity of Cln cyclins, or the expression of the CLN genes.

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