Cyclin Regulation by the S Phase Checkpoint*

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In eukaryotic cells a surveillance mechanism, the S phase checkpoint, detects and responds to DNA damage and replication stress, protecting DNA replication and arresting cell cycle progression. We show here that the S phase cyclins Clb5 and Clb6 are regulated in response to genotoxic stress in the budding yeast Saccharomyces cerevisiae. Clb5 and Clb6 are responsible for the activation of the specific Cdc28 cyclin-dependent kinase activity that drives the onset and progression of the S phase. Intriguingly, Clb5 and Clb6 are regulated by different mechanisms. Thus, the presence of Clb6, which is eliminated early in an unperturbed S phase, is stabilized when replication is compromised by replication stress or DNA damage. Such stabilization depends on the checkpoint kinases Mec1 and Rad53. The stabilization of Clb6 levels is a dynamic process that requires continued de novo protein synthesis, because the cyclin remains subject to degradation. It also requires the activity of the G1 transcription factor Mlu1 cell cycle box-binding factor (MBF) in the S phase, whereas Dun1, the checkpoint kinase characteristically responsible for the transcriptional response to genotoxic stress, is dispensable in this case. On the other hand, two subpopulations of endogenous Clb5 can be distinguished according to turnover in an unperturbed S phase. In the presence of replication stress, the unstable Clb5 pool is stabilized, and such stabilization requires neither MBF transcriptional activity nor de novo protein synthesis.

Genotoxic stress poses a threat to the genomic integrity of the dividing cell. In eukaryotic cells a surveillance mechanism, the S phase checkpoint, detects and responds to genotoxic stress, protecting DNA replication and blocking cell cycle progression (1-3). An impaired checkpoint response results in genomic instability and promotes cancer in metazoan organisms (4, 5).

The checkpoint elements and pathways are highly conserved. In the budding yeast Saccharomyces cerevisiae, DNA damage or replication stress sensed in the S phase results in the activation of the central transducer protein kinase Mec1 (6), which in turn activates the effector protein kinase Rad53 (7). Rad53 is required in all aspects of the checkpoint response: to stabilize essential replisome proteins in stalled forks, avoiding irreversible, lethal fork collapse (8-11); to block origin firing (12); to suppress undesirable recombination events (13, 14); and to activate a transcriptional response, mediated by its downstream paralog kinase Dun1 (15, 16).

To identify new Rad53 targets, we have assayed a number of proteins involved in the control of the cell cycle for in vitro phosphorylation by Rad53. Identification of Clb6 as a Rad53 substrate prompted us to examine the regulation of Clb5 and Clb6, the two S phase cyclins in budding yeast, in response to genotoxic stress. The nine budding yeast cyclins can bind to and activate Cdc28/Cdk1, the only cyclin-dependent kinase catalytic subunit in budding yeast. The different cyclins confer different specificity and promote progression through the different phases of the mitotic cycle (17-25). Clb5,6-Cdc28 (S-CDK)2 together with Dbf4-Cdc7 (Dbf4-dependent kinase) are the two kinase activities responsible for the initiation of DNA replication and progression through the S phase (17, 26-28). Transcription of the CLB5 and CLB6 genes in late G1 has been attributed to the G1-specific transcription factor MBF (17, 26-28). Mlu1 cell cycle box-binding factor (MBF) is a heteromeric transcription factor composed of an Mlu1 cell cycle box-binding subunit, Mbp1, and the transactivation subunit Swi6 (29-31). MBF is activated at Start and characteristically regulates the expression of genes involved in DNA replication and DNA repair (32).

Clb5 protein levels remain stable through the S phase, and Clb5 is targeted for degradation in metaphase by the anaphase-promoting complex bound to Cdc20, together with mitotic cyclins (33, 34). Despite the fact that the levels of Clb5 remain constant during this window of time, overexpression and shut-off experiments suggest that Clb5 is an unstable protein throughout the cell cycle (35-37), and the cyclin half-life has been estimated to range from 8 to 10 min when the anaphase-promoting complex is inactive (37). However, to the best of our knowledge, the stability of endogenous Clb5 has not been examined.

More recently it has emerged that Clb6 is degraded ahead of Clb5 and the mitotic cyclins, early in the S phase, in a S-CDK-dependent manner (38). This is an intriguing observation, because it is not known at this point why cells must limit the presence of Clb6 to such a short window of time in the cell cycle.

Because S-CDK drives cells into and through the S phase, it would be an attractive checkpoint target. In this respect, it is a
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TABLE 1

| Strain | Genotype | Source |
|--------|----------|--------|
| W303-1a | MATa ade2-1 ura3-1 his3-11,15 trpl-1 leu2-3,112 can1-100 | Ref. 41 |
| W303-1a CLB6-13myc(KanMX6) bar1Δ::URA3 | This study |
| YGP17 | W303-1a CLB6-13myc(KanMX6) bar1Δ::URA3 | This study |
| W303-1a sml1Δ rad53Δ::LEU2 CLB6-13myc bar1Δ::URA3 | This study |
| YGP18 | W303-1a sml1Δ CLB6-13myc bar1Δ::URA3 | This study |
| W303-1a bar1Δ::URA3 | This study |
| YGP20 | W303-1a bar1Δ::URA3 | This study |
| YGP23 | W303-1a CLB6-13myc(KanMX6) bar1Δ::URA3 | This study |
| YGP26 | W303-1a CLB6-13myc(KanMX6) bar1Δ::URA3 | This study |
| YGP27 | W303-1a CLB6-13myc(KanMX6) bar1Δ | This study |
| YGP37 | W303-1a rad53-21 CLB6-13myc(KanMX6) bar1Δ::URA3 | This study |
| YGP39 | W303-1a SWI6-13myc(HIS3) bar1Δ::URA3 | This study |
| YGP40 | W303-1a CLB6-3HA(KanMX6) bar1Δ::URA3 | This study |
| YGP48 | W303-1a SWI6-3HA(HIS3) bar1Δ::URA3 | This study |
| YGP54 | W303-1a dun1Δ::LEU2 CLB6-13myc(KanMX6) bar1Δ::URA3 | This study |
| YGP70 | W303-1a CLB6-13myc(KanMX6) TRP1::GAL-NRM1ΔN-13myc(URA3) bar1Δ | This study |

well established fact that Dbf4, the activating subunit of Dbf4-dependent kinase, is phosphorylated by Rad53 in response to replication stress (39), although the role of this modification remains unclear. Less is known about the possible regulation of replication stress (39), although the role of this modification might be modified in a way that the stability of the cyclin in an unperturbed S phase. The unstable Clb5 pool is stabilized in response to genotoxic stress, requiring neither MBF activity nor de novo protein synthesis.

EXPERIMENTAL PROCEDURES

Strains—The strains used in this study are listed in Table 1. All of the strains are derived from S. cerevisiae W303-1a (45). To reduce the concentration of α-factor required to synchronize cells in the G1 phase, strains were made bar1Δ by integration of the EcoRI-Sall fragment of pGst1 (46). The rest of the gene deletion strains were made by integration of PCR-generated selectable marker cassettes amplified from pRS vectors (47), with oligonucleotides complementary to the sequences flanking the gene of interest. In all cases the full open reading frame was deleted. Transformants were selected in the adequate selection medium and confirmed by genomic PCR with primers flanking the deleted locus. In addition, the rad53Δ and dun1Δ mutants were confirmed to be sensitive to the genotoxic reagents hydroxyurea (HU) and methylmethane sulfonate. Clb6 was C-terminally tagged at its genomic locus by integration of PCR-generated cassettes amplified from either the pFA6a-3HA-KanMX6 (3HA tag) or the pFA6a-13Myc-KanMX6 (13Myc tag) vectors, using oligonucleotides complementary to the sequences flanking the stop codon (48). The GAL-NRM1ΔN strain was generated as described (49). Oligonucleotide sequences will be provided upon request.

Culture Media, Cell Synchronization, Generation of Genotoxic Stress, and Inhibition of Protein Synthesis—The cultures were grown at 24 °C, under orbital rotation, in YP medium (1% yeast extract, 2% Bacto-Peptone), supplemented with 2% sugar (dextrose, raffinose, or galactose). To synchronize cells in the G1 phase, the mating pheromone α-factor was added at 50 ng/ml to cultures in midexponential growth phase. After one generation time, the cells were inspected under the microscope to confirm that the budding index was lower than 5%. To release cells into the S phase, synchronized cultures were washed three times in the corresponding release medium. Genotoxic stress was generated essentially as described (50); replication stress was generated with 200 μM hydroxyurea, a reversible inhibitor of the ribonucleotide reductase activity that catalyzes the rate-limiting step in dNTP synthesis; DNA methylation damage was generated with 0.033% methylmethane sulfonate. Where indicated, protein synthesis was inhibited by adding 100 μg/ml cycloheximide (CHX) to the medium.

Flow Cytometry and Immunofluorescence Microscopy—Samples for propidium iodide fluorescence-activated cell sorting were processed as described (51) and analyzed using a Becton Dickinson FACScan. For immunofluorescence microscopy Swi6–13Myc and untagged control cells were fixed with 3.7% formaldehyde and then spheroplasted and processed essentially as described (52). To visualize Swi6, 9E10 anti-Myc monoclonal antibody (Roche Applied Science) was used at 5 μg/ml as a primary antibody, and Alexa 488 coupled antimouse antibody (Invitrogen) was used as a secondary antibody.
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We next explored whether Clb6 is indeed regulated in vivo in response to conditions that activate the S phase checkpoint kinase Rad53. The cells synchronized in G1, with the pheromone α-factor, were released into the S phase, either in the presence or in the absence of HU, a reagent that generates replication stress by depleting the pool of dNTPs. As shown in Fig. 2, Clb6 disappears soon after cells enter an unperturbed S phase, as had been described before (38). However, when cells enter the S phase in the presence of HU, Clb6 remains stable, long after the time Clb6 is eliminated in an unperturbed S phase. The budding index progresses normally, discounting the possibility that
Clb6 stabilization is the result of a defect in cell cycle progression. Identical results were obtained with Clb6–3HA (strain YGP40; data not shown).

Clb6 is equally stabilized when cells enter the S phase in the presence of methylmethane sulfonate, a reagent that generates DNA methylation damage (not shown). Therefore Clb6 stabilization occurs at least in response to two different types of genotoxic stress known to activate the S phase checkpoint.

In addition, Clb6 stabilization correlates with Rad53 activity.

FIGURE 3. The stabilization of Clb6 in response to replication stress depends on Rad53. A log phase culture (strain YGP17) was synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase in the presence of hydroxyurea (1h HU). After 1h of incubation, HU was washed away, and the cells were allowed to recover from replication stress in rich medium (YPD). The samples were taken at the indicated time points. B, rad53 mutants fail to stabilize Clb6 in the presence of replication stress. The same experiment described in the legend to Fig. 2 was carried out with rad53-21 mutant (73) cells (strain YGP37). Upper panels, fluorescence-activated cell sorting analysis of DNA content. Unreplicated (1C) and replicated (2C) haploid DNA contents are indicated. Budding indexes (BI) are shown as measures of synchronicity and cell cycle progression and to confirm that cells exposed to replication stress progress into the cell cycle despite the absence of DNA replication. Lower panels, whole cell extracts were analyzed by immunoblot with anti-Myc (Clb6) antibodies or anti-Rad53 (Rad53) antibodies where indicated. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

Clb6 Stabilization Requires Continued de Novo Protein Synthesis—Clb6 could be stabilized through different mechanisms. Rad53 might block the S phase degradation of Clb6 by the ubiquitin ligase SCFCdc4. Alternatively, Clb6 steady state levels might be stabilized through a balance of continued degradation and de novo protein synthesis. To distinguish between these two scenarios, we examined the levels of Clb6 when protein synthesis is inhibited by the presence of CHX in a compromised S phase. Wild type cells were synchronized in the G1 phase with α-factor and released into the S phase in the presence of hydroxyurea. After 1h of incubation, HU was washed away, and the cells were allowed to recover from replication stress in rich medium (YPD). The samples were taken at the indicated time points. B, rad53 mutants fail to stabilize Clb6 in the presence of replication stress. The same experiment described in the legend to Fig. 2 was carried out with rad53-21 mutant (73) cells (strain YGP37). Upper panels, fluorescence-activated cell sorting analysis of DNA content. Unreplicated (1C) and replicated (2C) haploid DNA contents are indicated. Budding indexes (BI) are shown as measures of synchronicity and cell cycle progression and to confirm that cells exposed to replication stress progress into the cell cycle despite the absence of DNA replication. Lower panels, whole cell extracts were analyzed by immunoblot with anti-Myc (Clb6) antibodies or anti-Rad53 (Rad53) antibodies where indicated. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

CLB6 mRNA Decays in S Phase but Persists in the Presence of Replication Stress—CLB5 and CLB6 mRNA accumulate at the G1-S transition and then decay as cells progress through the cell cycle (17). However, if cells undergoing replication stress retain the ability to restore the presence of Clb6 while the cyclin remains highly unstable, the CLB6 transcript should be present under these conditions. We analyzed the levels of CLB6 mRNA...
YGP17) was synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase in the presence of hydroxyurea (1hHU). The culture was then split in two. One half was further incubated under the same conditions (HU) (A), whereas cycloheximide was added to the other half (B), keeping the presence of hydroxyurea (HU + CHX). The samples were taken at the indicated time points, and whole cell extracts were analyzed by immunoblot with anti-Myc antibodies (Clb6). A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control. Budding indexes (Bi) are shown on top as measures of synchronicity and cell cycle progression and to confirm that cells progress into the cell cycle despite the absence of DNA replication or protein synthesis. While this work was underway, de Bruin et al. (56) reported that in the fission yeast Schizosaccharomyces pombe MBF reactivation occurs in response to replication stress in a checkpoint-dependent manner. To explore whether the stabilization of Clb6 protein levels in response to replication stress depends on MBF function, we released cells into the S phase in the presence of HU and then induced Nrm1ΔN, an hyperstable form of Nrm1 that efficiently blocks MBF activity (49). As shown in Fig. 7, Clb6 remains present 1 h after release from α-factor in the presence of HU. However, the cyclin rapidly disappears in cells overexpressing Nrm1ΔN, despite the continued presence of HU, whereas Clb6 levels remain stable in the control cells under identical conditions. In the same samples the levels of Clb5 are not decreased upon MBF inhibition. These observations indicate that MBF transcription is crucial to maintain the presence of Clb6 in a compromised S phase but not to keep the levels of Clb5 stable (see Clb5 results below).

As part of the inactivation control of MBF upon entry into the S phase, the transactivator subunit Swi6 is excluded from the nucleus (55, 57). If the stabilization of Clb6 levels in a compromised S phase requires MBF activity, a prediction would be that the checkpoint response must override the nuclear exclusion of Swi6. To examine the cellular sublocalization of Swi6, we carried out immunofluorescence microscopy on a strain expressing Myc-tagged Swi6 from its original locus. The cells were synchronized in G1, with the pheromone α-factor and synchronously released into the S phase, either in the presence or in the absence of HU. Samples were taken at different times, fixed with formaldehyde, and stained with anti-Myc antibodies to localize Swi6 by fluorescence microscopy. As shown in Fig. 8, the cells allowed to enter an unperturbed S phase present a diffuse fluorescence signal similar to what has been described previously (57, 58). In steep contrast, the cells undergoing replication stress show a strong, well defined Swi6-Myc nuclear signal, a required condition for MBF-driven transcription. The observed signals are specific to Swi6, because they are absent in untagged cells collected and processed under identical conditions; also, identical results were obtained with Swi6-3HA-tagged cells (strain YGP48, data not shown).

Stabilization of Clb6 Levels in a Perturbed S Phase Does Not Require Dun1—It has been long established that the S phase checkpoint response involves the transcriptional induction of the ribonucleotide reductase genes mediated by the Rad53 downstream kinase Dun1 (15, 59, 60). In addition, the overall alteration in the transcriptional response to DNA damage of dun1 mutants is essentially identical to the alteration in mutants for the checkpoint upstream kinase Mec1, suggesting that the checkpoint transcriptional response is largely mediated by Dun1 (16). We asked whether Dun1 is also required to activate the MBF-dependent transcription of

**FIGURE 4. Clb6 stabilization requires continued de novo protein synthesis.** A log phase culture (strain YGP17) was synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase in the presence of hydroxyurea (1hHU). The culture was then split in two. One half was further incubated under the same conditions (HU) (A), whereas cycloheximide was added to the other half (B), keeping the presence of hydroxyurea (HU + CHX). The samples were taken at the indicated time points, and whole cell extracts were analyzed by immunoblot with anti-Myc antibodies (Clb6). A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control. Budding indexes (Bi) are shown on top as measures of synchronicity and cell cycle progression and to confirm that cells progress into the cell cycle despite the absence of DNA replication or protein synthesis.

**FIGURE 5. Cells retain the ability to restore the presence of Clb6 for as long as replication stress persists.** A log phase culture (strain YGP17) was synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase in the presence of hydroxyurea (1hHU). After 1h of incubation cycloheximide was added, keeping the presence of hydroxyurea (CHX + HU). After 30 min the cycloheximide was washed away while keeping the HU to allow protein synthesis to resume in the presence of replication stress. The samples were taken at the indicated time points, and the whole cell extracts were analyzed by immunoblot with anti-Myc antibodies (Clb6). A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control. Budding indexes (Bi) are shown on top as measures of synchronicity and cell cycle progression and to confirm that cells progress into the cell cycle despite the absence of DNA replication or protein synthesis.

in an unperturbed S phase and in S phase compromised by replication stress. As shown in Fig. 6 (YPD), the Clb6 transcript peaks 20 min after release from the α-factor pre-Start arrest. Clb6 mRNA then decreases in the S phase, and 60 min after cells have been released from the α-factor arrest, Clb6 mRNA levels are back to pre-Start levels, in agreement with previous reports. In contrast, the Clb6 transcript remains present when the S phase is challenged by replication stress (Fig. 6, right panel).

**CLB6 Expression in a Perturbed S Phase Depends on MBF Function**—The presence of Clb6 mRNA in a compromised S phase could result from transcriptional activity or from transcript stabilization. Expression of CLB6 in late G1 phase has been attributed to the Start transcription factor MBF (17). MBF is turned off upon entry into the S phase at least at two levels of control: the nuclear exclusion of the MBF transactivator subunit Swi6 (55) and binding of the MBF repressor Nrm1 (49).
CLB6 in response to replication stress. As shown in Fig. 9, a dun1Δ mutant strain proficiently stabilizes the levels of CLB6 in the S phase in the presence of HU. The samples were taken at the indicated time points. CLB6 mRNA levels were quantified by quantitative reverse transcription-PCR, using ACT1 mRNA as reference. The results are expressed as mean fold changes relative to α-factor arrested pre-Start cells (G1). The error bars are the standard deviations of three independent preparations. The results were checked to be reproducible in two independent time course sets of samples.

**FIGURE 6.** CLB6 mRNA is stabilized in response to replication stress. A log phase culture (strain YGP20) was synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase either in the absence (YPD) or in the presence of HU. The samples were taken at the indicated time points. CLB6 mRNA levels were quantified by quantitative reverse transcription-PCR, using ACT1 mRNA as reference. The results are expressed as mean fold changes relative to α-factor arrested pre-Start cells (G1). The error bars are the standard deviations of three independent preparations. The results were checked to be reproducible in two independent time course sets of samples.

**FIGURE 7.** MBF function is required for the stabilization of CLB6 levels in response to replication stress. Log phase cultures of GAL-NRM1ΔN (strain YGP70) or control cells (strain YGP27) grown in YP raffinose were synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase in the presence of hydroxyurea (HU). After 1 h of incubation, galactose was added to a final concentration of 0.05% while keeping the presence of hydroxyurea (HU). Upper panel, fluorescence-activated cell sorting analysis of DNA content. Unreplicated (1C) and replicated (2C) haploid DNA contents are indicated. Budding indexes (BI) are shown as measures of synchrony and cell cycle progression and to confirm that cells exposed to replication stress progress into the cell cycle despite the absence of DNA replication. Lower panel, whole cell extracts were analyzed by immunoblot with anti-Myc (CLb6 and Nrm1ΔN) or anti-CLb5 antibodies. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

**FIGURE 8.** Persistent nuclear localization of Swi6 in a compromised S phase. A log phase culture of Swi6–13Myc cells (strain YGP39) was synchronized in G1 phase with α-factor. The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase either in the presence of hydroxyurea (S-phase/HU) or in the absence of hydroxyurea (Unperturbed S-phase). The samples were taken after a 60-min incubation in HU or 30 min after the release into an unperturbed S phase. Epifluorescence microscopy was used to detect the immunofluorescence Swi6 signal and the 4′,6-diamidino-2-phenylindole-stained DNA signal. Cell morphology was visualized by Nomarski contrast microscopy.

CLB6 in response to replication stress. As shown in Fig. 9, a dun1Δ mutant strain proficiently stabilizes the levels of CLB6 in the S phase in the presence of HU. The dun1 deletion was confirmed by genomic PCR using primers flanking the deleted locus. The same result was confirmed using an independently generated dun1Δ strain. In addition, the dun1Δ strains are sensitive to HU and to methylmethane sulfonate (not shown), despite the fact that they present an intact upstream checkpoint response, as confirmed by normal Rad53 activation (see Rad53 immunoblot in Fig. 9).

Therefore, Dun1 is not universally required for the transcriptional response to replication stress. Work is currently in progress to determine whether Dun1 is generally dispensable for the activation of MBF-mediated transcription in response to genotoxic stress in the S phase.
An Unstable Pool of Clb5 Is Stabilized in Response to Replication Stress—We next explored whether the partner S phase cyclin, Clb5, is also regulated in response to genotoxic stress. Previous reports have shown that Clb5 is an unstable protein throughout the cell cycle (35–37). With respect to Clb5 stability in response to genotoxic stress, contradictory data are available, showing either stabilization (44) or a lack of response (37).

Because all previous studies were based on overexpressed Clb5, we chose to explore the stability of endogenous Clb5, both in an unperturbed S phase and in an S phase compromised by replication stress. As shown in Fig. 10A, Clb5 levels are comparable in both cases. However, if Clb5 levels are analyzed when protein synthesis is blocked with CHX, two Clb5 subpopulations become evident, according to their stability in an unperturbed S phase (Fig. 10B). A pool of the cyclin appears to be unstable, with steady state levels depending on continued de novo protein synthesis. A second pool remains stable throughout the time of the experiment. Under these conditions activation of the anaphase-promoting complex should be inhibited, because of the lack of mitotic cyclins promoting progression into mitosis (61); in any case, identical results were obtained when the experiment was repeated in the presence of 15 μg/ml nocodazole (not shown), a drug that interferes with microtubule polymerization, resulting in anaphase-promoting complex inhibition by the spindle checkpoint (62). To the best of our knowledge, it is the first time that such two subpopulations of this S phase cyclin have been described. The nature and function of each pool is at present not known.

Interestingly, the whole Clb5 population is stabilized in the presence of HU. Cells under replication stress show fully stabilized Clb5 when protein synthesis is blocked with CHX (Fig. 10C). Therefore, both S phase cyclins are regulated in response to replication stress, albeit by different mechanisms; whereas Clb6 remains highly unstable, and its continued presence results from reactivated CLB6 expression, the unstable Clb5 pool is stabilized as a result of decreased turnover, and no de novo protein synthesis is required.

Regulation of the Unstable Clb5 Pool Does Not Require MBF Activity—We analyzed the behavior of the CLB5 transcript in an unperturbed S phase and in S phase under replication stress. The quantitative reverse transcription-PCR analysis in Fig. 11 shows that the CLB5 transcript peaks 20 min after release from the α-factor pre-Start arrest. CLB5 mRNA then decreases in S phase, but in contrast to the decay of CLB6 mRNA (Fig. 6, left panel), it remains stably present (Fig. 11, left panel). The presence of CLB5 transcript would be a necessary condition to support continued Clb5 translation during the S phase (Fig. 10).

Interestingly, CLB5 mRNA levels are not increased in response to replication stress with respect to an unperturbed S phase. The simplest explanation for this observation would be that the checkpoint-activated, MBF-driven transcription affects not all but only a subset of the genes whose transcription is under MBF in the G1 phase. In that case, a prediction would be that inhibition of MBF during replication stress should have no effect on the overall levels of Clb5. This happens to be the case, as shown in Fig. 7.

DISCUSSION

We have shown here that the S phase cyclins Clb5 and Clb6 are regulated in response to genotoxic stress. However, the mechanisms mediating such regulations are altogether different.

Clb6, which is eliminated early in an unperturbed S phase, is stabilized in response to genotoxic stress. Such stabilization requires the activity of the S phase checkpoint central kinase Rad53. However, Clb6 remains a highly unstable protein, and its continued presence requires de novo protein synthesis and MBF activity in the S phase.

The checkpoint effector kinase Dun1 is dispensable for the transcriptional up-regulation of Clb6 in response to replication stress. Dun1 operates downstream of Rad53 and is involved in the transcriptional response to genotoxic stress (15, 63). Signif-
significantly, the alteration in the DNA damage transcriptional response of dun1 mutants is largely the same as in mec1 mutants, suggesting that most of the checkpoint transcriptional response is mediated by Dun1 (16). However, our observation indicates that Dun1 is not universally required for the transcriptional response to replication stress. Dun1 induces ribonucleotide reductases by removal of the transcriptional repressor Rfx1/Crt1 (64), which may be involved in the control of a subset of, but not all, genes inducible in response to genotoxic stress. The CLB6 promoter lacks any canonical match to the consensus X-box to which Rfx1/Crt1 binds. It will be of interest to explore whether overall, the MBF-mediated response to genotoxic stress in the S phase is Dun1-independent.

We also showed that Swi6, the transactivatory subunit of MBF, remains nuclear in cells under replication stress, despite the presence of Clb6. Clb6-CDK activity, but not Clb5-CDK, mediates the nuclear exclusion of Swi6 in a normal S phase, as part of the down-regulation control of MBF activity (58). Our observation indicates that the S phase checkpoint overrides such control. In this respect Rad53 has been shown to phosphorylate Swi6 (65). In addition, we originally identified Clb6 as an in vitro Rad53 substrate. Although we have not confirmed whether Rad53 phosphorylates Clb6 also in vivo, a plausible possibility is that Rad53 phosphorylation of Swi6, Clb6, or both abrogates their affinity for each other, making possible the nuclear localization of Swi6.

Why stabilize Clb6 in response to replication stress or DNA damage in the S phase? Of the two S phase cyclins in budding yeast, Clb6 apparently plays a dispensable role in DNA replication, because clb6 mutants display a normal onset time and duration of the S phase (17), whereas S phase duration is severely

![Figure 10](image-url) **FIGURE 10.** An unstable pool of Clb5 is stabilized in response to replication stress. A log phase culture (strain YGP23) was synchronized with α-factor and released under the conditions described below. The samples were taken at the indicated time points. Whole cell extracts were analyzed by immunoblot with anti-Clb5 antibodies (Clb5) or anti-Rad53 (Rad53) antibodies where indicated. A Ponceau S-stained region of the same membranes used for immunoblot is shown as a loading control. Budding indexes (BI) are shown on top as measures of synchronicity and cell cycle progression and to confirm that cells progress into the cell cycle despite the absence of DNA replication or protein synthesis. A, stable Clb5 levels in an unperturbed and in a compromised S phase. The cells were released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase either in the absence (YPD) or in the presence of HU. B, two pools of Clb5 can be distinguished according to turnover in an unperturbed S phase. The cells were released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase either in the absence (YPD) or in the presence of HU. C, the whole Clb5 population is stabilized in response to replication stress.

![Figure 11](image-url) **FIGURE 11.** CLB5 mRNA persists in an unperturbed S phase and is not increased in the presence of replication stress. The samples in Fig. 6 were quantified for CLB5 mRNA levels (see details in Fig. 6).
extended in clb5 mutants (17, 26, 66). Interestingly, despite the apparent dispensability of Clb6, clb6 rad53 mutants present a synthetic growth defect (67). However, to the best of our ability, we could not identify increased sensitivity to genotoxic drugs, altered replication rate in the presence of DNA damage, nor a defect in stalled fork resumption after a replication block in clb6Δ mutants (data not shown). Neither could we detect premature mitotic CDK activity in HU (68) when monitored in vivo by phosphorylation of the B subunit of DNA polymerase α-primase (not shown). Perhaps understanding why cells need to get rid of Clb6 early in the S phase (38) will help elucidate the role of Clb6 stabilization by the S phase checkpoint.

With respect to Clb5, previous works had reported that over-expressed Clb5 is kept an unstable protein in the S phase by a still unidentified ubiquitin ligase (37). However, we have shown now that when the endogenous levels of Clb5 are examined in S phase, two subpopulations of the cyclin can be identified according to their stability. Therefore, the steady levels of Clb5 in an unperturbed S phase result in a balance between continued protein synthesis and protein degradation of the unstable Clb5 pool. In this regard and contrary to the decay of CLB6 mRNA in the S phase, CLB5 mRNA remains present, providing the necessary template for continued protein synthesis.

Interestingly, the unstable Clb5 pool is directly stabilized in response to genotoxic stress. Under these conditions, again in contrast with Clb6, Clb5 levels remain stable when protein synthesis is blocked with cycloheximide or when the hyperstable MBF repressor Nrm1ΔN is overexpressed. We are currently working to characterize the nature and function of the two Clb5 pools and to explore the role that stabilization of the unstable Clb5 pool plays in the response to replication stress.

In addition, contrary to CLB6 mRNA, CLB5 mRNA levels are not increased in response to replication stress (compare CLB5 in Fig. 11 with CLB6 in Fig. 6). The simplest explanation for this observation is that reactivation of MBF in response to genotoxic stress (56, 69) involves only a subset of the genes that are expressed under the control of MBF at Start in S. cerevisiae. It will be of interest to identify whether a specific MBF repressor binds to the promoter of CLB5 and perhaps other genes also under MBF at Start to exclude them from the MBF-mediated response to genotoxic stress in the S phase.

Finally, it is worth mentioning that although CLB5 mRNA remains present during the replication stress crisis, no Clb5 build-up is detected (Fig. 10A), despite the fact that the cyclin appears to be fully stabilized under these conditions (Fig. 10B). These observations suggest that Clb5 translation may be repressed in response to genotoxic insults in the S phase.

In summary, we report here that the two S phase cyclins in budding yeast, Clb5 and Clb6, are regulated in response to genotoxic stress. This finding adds to the previously reported checkpoint regulation of Dbf4 (39), the activating subunit of Dbf4-dependent kinase, also essential for the initiation and timely progression of the S phase. Future experiments are required to address the role that targeting the S phase cyclins plays in the preservation of genomic integrity. In any case, checkpoint control of S phase-promoting cyclins is likely to be a conserved pathway of the cellular response to genotoxic insults, because human cyclin E and two negative regulators of cyclin E are phosphorylated at an ATR/ATM consensus sequence in response to DNA damage (70).

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