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Roles and regulation of Cln–Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*

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In budding yeast G_1 cells increase in cell mass until they reach a critical cell size, at which point (called Start) they enter S phase, bud and duplicate their spindle pole bodies. Activation of the Cdc28 protein kinase by G_1-specific cyclins Cln1, Cln2 or Cln3 is necessary for all three Start events. Transcriptional activation of *CLN1* and *CLN2* by SBF and MBF transcription factors also requires an active Cln–Cdc28 kinase and it has therefore been proposed that the sudden accumulation of *CLN1* and *CLN2* transcripts during late G_1 occurs via a positive feedback loop. We report that whereas Cln1 and Cln2 are required for the punctual execution of most, if not all, other Start-related events, they are not required for the punctual activation of SBF- or MBF-driven transcription. Cln3, on the other hand, is essential. By turning off cyclin B proteolysis and turning on proteolysis of the cyclin B–Cdc28 inhibitor $p40^{SIC1}$, Cln1 and Cln2 kinases activate cyclin B–Cdc28 kinases and thereby trigger S phase. Thus the accumulation of Cln1 and Cln2 kinases which starts the yeast cell cycle is set in motion by prior activation of SBF- and MBF-mediated transcription by Cln3–Cdc28 kinase. This dissection of regulatory events during late G_1 demands a rethinking of Start as a single process that causes cells to be committed to the mitotic cell cycle.

Keywords: CLN cyclins/MBF/SBF/Start/transcriptional regulation

Introduction

To sustain proliferation cells need to increase in mass (growth), as well as duplicate chromosomes (S phase) and equally segregate sister chromatids to daughter cells (M phase). In between S and M phases are two intervening periods or gaps, G_1 prior to S phase and G_2 prior to M phase (Howard and Pelc, 1951). In contrast to the discrete phases of the chromosome cycle, growth is a rather continous process and occurs at all stages of the cell cycle (Mitchison, 1970). To maintain a constant size during cell proliferation cells need to grow and double in mass during each round of the chromosome cycle. How are growth and the chromosome cycle coupled? Preventing the initiation of S phase until cells grow to a critical cell size is one solution to this problem (Killander and Zetterberg, 1965).

After reaching a critical size in the presence of sufficient nutrients yeast cells initiate DNA replication, direct growth to buds and duplicate their spindle pole body in preparation for mitosis and cytokinesis. Once they have passed this point in the cell cycle haploid cells no longer arrest in G_1 in the presence of mating pheromone (Hereford and Hartwell, 1974). This point is therefore known as Start (Hartwell *et al.*, 1974).

What is the molecular basis for Start? Genetic analysis pinpointed a single gene, *CDC28*, which encodes a cyclin-dependent kinase (Cdk). Cdc28 is essential not only for S phase, but also for mitosis (Nasmyth, 1993). Inactivation of Cdc28 in G_1 cells prevents the occurrence of all Start events, without affecting growth, and cells arrest as large unbudded cells that retain the ability to mate (Reid and Hartwell, 1977). Nine different cyclins associate with Cdc28 and thereby confer stage-specific functions (Nasmyth, 1993). Six different B-type cyclins (*CLB1–6*) are involved in different aspects of S phase and mitosis, while three G_1-specific cyclins (*CLN1–3*) are necessary for Start. None of the three *CLN* genes are essential, but mutants lacking all three *CLN* genes arrest in G_1 with a phenotype resembling *cdc28* mutants (Richardson *et al.*, 1989; Cross, 1990). The three G_1 cyclins are thought therefore to have overlapping or even equivalent functions. Recent work suggests, however, that *CLN1* and *CLN2* have some different properties to *CLN3* (Tyers *et al.*, 1993; Oehlen and Cross, 1994).

The timing of Start is determined by the activity of Cln cyclins. Unlike Cdc28, all three Cln cyclins are unstable proteins and mutant forms of Cln2 and Cln3 with increased stability allow cells to start the cell cycle at a smaller than normal cell size (Sudbery *et al.*, 1980; Nash *et al.*, 1988; Hadwiger *et al.*, 1989). Control of *CLN1* and *CLN2* transcription plays an important part in triggering Start. *CLN1* and *CLN2* mRNAs are absent in early G_1 cells, but appear suddenly in late G_1. They then decline during G_2, as do the Cln1 and Cln2 protein levels and their Cdc28 associated kinase (Wittenberg *et al.*, 1990; Tyers *et al.*, 1993). Thus the onset of *CLN1* and *CLN2* transcription could determine when cells undergo Start. *CLN1* and *CLN2* transcription is down-regulated at high growth rates, thereby delaying Start until cells have increased their mass, an effect that is mediated by cAMP-dependent protein kinases, which are also required for cell growth (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994). The third G_1 cyclin *CLN3* is only distantly related to *CLN1* and *CLN2* and is strikingly different in its regulation. Unlike other Cdc28-associated cyclins, *CLN3* mRNA and protein levels do not greatly fluctuate during the cell cycle (Tyers *et al.*, 1993).

The late G_1-specific transcription of *CLN1* and *CLN2* is due to the SBF and MBF transcription factors (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). SBF (SCB binding

factor) and the closely related MBF (MluI binding factor) are regulators of a large family of genes transcribed specifically in late G_1. Both factors are composed of a common regulatory subunit, Swi6, complexed with a different DNA binding protein: Swi4 in SBF (Andrews and Herskowitz, 1989; Primig *et al.*, 1992) and Mbp1 in MBF (Koch *et al.*, 1993). SBF target genes include the *HO* endonuclease gene, *CLN1* and *CLN2*, as well as two cyclin-like genes *HCS26* (*PCL1*) and *ORFD* (*PCL2*) involved in phosphate metabolism, while MBF activates numerous genes, most of which are involved in DNA metabolism and encode stable proteins, like thymidylate synthase (*TMP1*) and DNA polymerase α (*POL1*) (Koch and Nasmyth, 1994). Among the genes whose transcription is regulated by MBF are two that encode unstable B-type cyclins, *CLB5* and *CLB6*, which are activators of Cdc28 and thereby promote S phase (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). Mutants lacking both SBF and MBF are inviable, but can be rescued by ectopic expression of *CLN2* (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Koch *et al.*, 1993). The late G_1-specific transcription programme is therefore required to start the yeast cell cycle.

Transcription of genes regulated by SBF and MBF does not occur in mutant cells lacking either Cdc28 or any one of the three Cln cyclins. Reactivation of any one Cln in a triple *cln* mutant immediately restores the transcriptional programme and causes cells to start the cell cycle. The ability of Cln1–Cdc28 and Cln2–Cdc28 kinases to activate SBF/MBF-regulated genes, including their own genes, suggested that the sudden activation of *CLN1* and *CLN2* transcription during late G_1 might involve a positive feedback loop mechanism (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). According to this model, Cln1 and Cln2 stimulate their own transcription, as well as that of all SBF/MBF-regulated genes, in late G_1 by activating the Cdc28 kinase, which in turn activates SBF, leading to more *CLN1* and *CLN2* transcription, and so on (Figure 1B). Because it is a potent activator of SBF/MBF-regulated transcription and is already present in early G_1, it has been proposed that Cln3 could act to trigger this loop when cells reach a critical cell size (Tyers *et al.*, 1993).

This hypothesis predicts that full activation of SBF/MBF-regulated genes should depend on the activities of Cln1 and Cln2 (Figure 1B). We now show that this is not the case. Target genes for both the SBF and the MBF transcription programmes are activated on schedule in *cln1 cln2 CLN3*$^+$ mutant cells at a size virtually identical to the wild-type cell size for Start. Despite this, cells lacking functional *CLN1* and *CLN2* genes do not undergo other Start-related events until they reach a much larger size. Budding, S phase, shutting off cyclin B proteolysis and acquisition of pheromone resistance are all greatly delayed. The delayed DNA replication in *cln1 cln2* cells can be abolished by deletion of the Clb5–Cdc28 and Clb6–Cdc28 kinase inhibitor p40^{SIC1}, suggesting that targeting p40^{SIC1} for destruction could be one of the functions of the Cln1–Cdc28 and Cln2–Cdc28 kinases. We show that G_1 cells of a *cln3* mutant are unable to activate SBF/MBF target genes until they reach a very large size. Our data, therefore, show that, contrary to previous conclusions based simply on synthetic lethality, the three Cln cyclins have very different functions. Cln3 is needed for activation of the SBF and MBF transcription factors, whereas Cln1 and Cln2 are needed to regulate the other Start-regulated events (Figure 7).

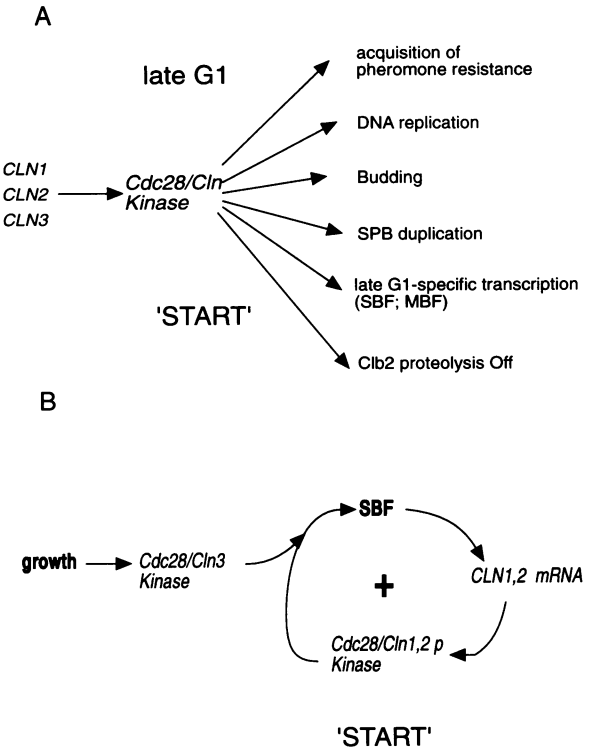

Fig. 1. 'Start' of the budding yeast cell cycle. (**A**) Genetic redundancy. Start seen as activation of the Cdc28 protein kinase by the G_1-specific cyclins, Cln1, Cln2 and Cln3. All the events listed on the right occur around Start and are dependent on activation of the Cdc28 kinase. *CLN*s are thought to encode redundant genes, since only the triple *cln* deletion strain fails to pass Start and arrest in late G_1. (**B**) The positive feedback loop model. *CLN1* and *CLN2* mRNAs appear transiently around the timing of Start. This transcription is both Cdc28- and Cln-dependent. Moreover, increased expression of *CLN* genes leads to increased *CLN1* and *CLN2* transcription. Therefore, it is proposed that Cln1 and Cln2 are required for *CLN* gene expression. The third G_1 cyclin *CLN3* is not transcriptionally regulated during the cell cycle and is thought to be a trigger of *CLN1* and *CLN2* autoactivation in response to increased cell size in late G_1.

Results

Kinetics of activation of the late G_1-specific transcripts

SBF- and MBF-driven transcription in late G_1 requires an active Cln-associated Cdc28 kinase. To address whether *CLN1* and *CLN2* are required for their own transcription, as predicted by the positive feedback loop model (Figure 1B), we compared the kinetics of SBF- and MBF-regulated transcription in wild-type and *cln1 cln2* double mutant strains as small daughter cells approached the critical size for Start.

cln1 cln2 double mutants divide at twice to three times the size of congenic wild-type cells and hence their daughters are born larger. To compare wild-type and *cln* mutant daughter cells of similar sizes we integrated a *CLN2* gene under control of the methionine repressible *MET3* promoter (Amon *et al.*, 1994) in wild-type (K3928) and *cln1 cln2* (K3652) strains. When grown in medium lacking methionine both strains resemble a *CLN*$^+$ strain.

Addition of methionine to the medium quickly represses *MET3–CLN2* and, because Cln2 is unstable, the strains then behave in the next cycle according to their respective genotypes. G_1 daughter cells were isolated by centrifugal elutriation 100 min after addition of methionine and incubated in fresh medium containing methionine. The starting G_1 populations were composed of unbudded cells of similar size (9 fl; Figure 2A). Transcripts from *PCL1*, an SBF-regulated gene, are undetectable in both starting populations, but accumulate (to 50% of the wild-type maximum level) as cells attain a volume of 19 fl (Figure 2A). It is striking that the rate of accumulation of *PCL1* mRNA is identical in wild-type and *cln1 cln2* double mutant cells. The subsequent decline was greatly delayed, however, in the double mutant. Transcripts from the mutant *cln2* allele in the *cln1 cln2* double mutant (see Material and methods) accumulated at a slightly larger size (+2–5 fl) when compared with the wild-type (Figure 2A). We were not able to ascertain whether this was due to the *cln2* mutation. *TMP1* transcript appeared at identical sizes in wild-type and mutant cells (Figure 2A), suggesting that the onset of MBF-regulated transcription is also unaffected by loss of *CLN1* and *CLN2* function. We conclude that *CLN1* and *CLN2* are barely, if at all, required for transcriptional activation of genes regulated by SBF and MBF as cells reach a critical cell size.

The lethality of triple, but not double, *cln* mutations suggested that the three *CLN* genes might have equivalent functions. Might this include gene activation? If so, *CLN1* and *CLN2* might be unnecessary for punctual activation, because *CLN3* fulfils the same function. According to this notion, *cln3* mutants should also activate gene transcription normally.

To test this we performed a similar experiment with a *cln3 MET3–CLN2* strain (K4690). This time the accumulation of SBF- and MBF-regulated transcripts was greatly delayed; *PCL1*, *CLN2* and *TMP1* mRNAs only appeared after cells had grown to 40–50 fl (Figure 2A). *CLN3* is therefore essential for the appearance of late G_1-specific transcripts at a normal cell size. The three *CLN* genes do not, therefore, have equivalent functions. The above data do not exclude the possibility that other genes activated as part of the late G_1-specific programme might substitute for *CLN*s.

Pcl1 and Pcl2, which associate with the Pho85 Cdk, are thought to play a role in the G_1–S transition (Espinoza *et al.*, 1994; Measday *et al.*, 1994). Both are regulated by SBF and could therefore be part of a feedback loop if they were able to activate late G_1-specific transcription. However, *TMP1* transcripts accumulated at similar sizes in *cln1 cln2* (K2008) double and *cln1 cln2 pcl1 pcl2* quadruple (K2884) mutants (data not shown; see Material and methods). Moreover, deletion of *PHO85* does not affect activation of *PCL1* transcription (C.Koch, personal communication). We conclude that neither *CLN1/CLN2* nor *PCL1/PCL2* nor any combination of these genes is needed for punctual late G_1-specific transcription.

Another set of genes which could contribute to a transcriptional positive feedback are those encoding the B-type cyclins, *CLB*s. Activation of *CLB5*, for example, has been shown to be capable of activating *PCL1* mRNA in cells arrested in G_1 due to inactivation of all three *CLN* genes (Schwob and Nasmyth, 1993). As will become apparent below, it is likely that activation of Clb5–Cdc28 and Clb6–Cdc28 kinase is greatly delayed in *cln1 cln2* double mutants. It is therefore not possible that Clbs could be responsible for punctual gene activation in these cells.

Budding and DNA replication require functional CLN1 and CLN2

Unlike transcription, both budding and DNA replication are greatly delayed in *cln3* single mutant and *cln1 cln2* double mutant cells (Figure 2B and C). To confirm these results with cells lacking *MET3–CLN2* we measured DNA content and budding index in wild-type, *cln1 cln2* double mutant and *cln3* single mutant cell populations after separation of log phase cells into fractions of increasing size by centrifugal elutriation. In rich medium (YEPRaff) wild-type cells start to accumulate with 2*n* DNA content around 23 fl (Figure 3). In contrast, no *cln1 cln2* below 70 fl and very few *cln3* cells below 50 fl had undergone S phase. The large (50–70 fl) unbudded *cln1 cln2*, but not the *cln3* G_1 cells, nevertheless had high levels of the MBF-regulated *CLB5* mRNA (data not shown).

Acquisition of pheromone resistance in wild-type and cln mutant strains

Wild-type haploid cells become refractory to G_1 arrest by mating pheromone around the time they enter S phase and bud. To compare the state of pheromone sensitivity in wild-type, *cln1 cln2* double and *cln3* single mutants of different cell sizes cells from asynchronous populations grown in rich medium were elutriated (see Figure 3) and fractions of increasing size were plated at low density on α-factor plates. Unbudded cells from all three strains failed to bud and formed schmoos, indicating that they were still sensitive to mating pheromone. Wild-type cells acquired the ability to divide at the time of budding, at a volume between 20 and 30 fl. In contrast, *cln1 cln2* (K3651) cells up to 70 fl and *cln3* cells up to 50 fl were incapable of budding in the presence of pheromone (see legend to Figure 3). Acquisition of pheromone resistance therefore requires *CLN1* and *CLN2* in addition to *CLN3*. This is consistent with results obtained by Oehlen and Cross (1994).

The S phase delay in cln1 cln2 mutants is dependent upon p40^{SIC1}

B-Type cyclin–Cdc28 kinases are necessary for S phase in budding yeast. The delayed DNA replication of *cln1 cln2* double mutant cells is not due to delayed transcription of *CLB5* and *CLB6* (data not shown), but it might nevertheless be due to a delay in the activation of Clb–Cdc28 complexes. G_1 cells contain an inhibitor of B-type cylin–Cdc28 kinases called p40^{SIC1}, which must be destroyed via the Cdc34 ubiquitin conjugating enzyme before cells can start DNA replication (Schwob *et al.*, 1994). To address whether *CLN1* and *CLN2* might have a role in triggering inactivation of p40^{Sic1} we tested whether deletion of the *SIC1* gene could eliminate the S phase delay of *cln1 cln2* double mutants.

Small G_1 cells (10 fl) were isolated by centrifugal elutriation from logarithmically growing populations of *cln1 cln2 MET3–CLN2* double (K3652) and *cln1 cln2 sic1 MET3–CLN2* triple mutants (K4900) and were allowed to grow under the same conditions as described in Figure 2.

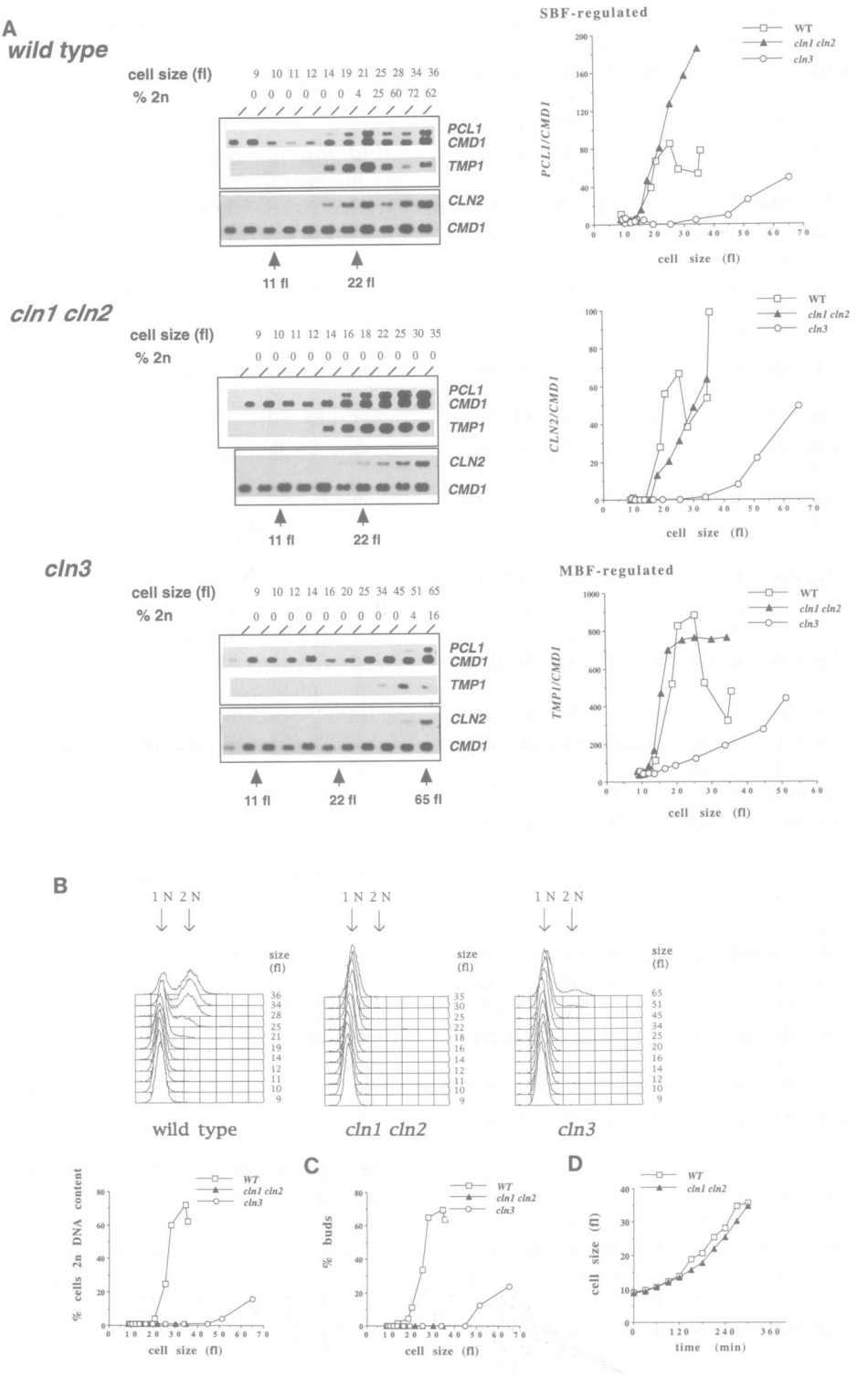

Fig. 2. Cln3, but not Cln1 and Cln2, is required for SBF- and MBF-regulated gene expression. Wild-type (K3852), *cln1 cln2* (K3652) and *cln3* (K4690) cells, all harbouring a *MET3–CLN2* construct integrated at *TRP1*, were grown to logarithmic phase at 30°C in 10 l methionine-free/raffinose medium (OD_{600} 1–1.5); *MET3–CLN2* was switched off 100 min prior to elutriation by addition of 2 mM methionine; the cells were maintained in the presence of methionine at all subsequent steps. Daughter G_1 cells were isolated by centrifugal elutriation and inoculated in fresh methionine/raffinose medium at 30°C; samples were taken every 30 min. (**A**) RNA levels of *PCL1* and *CLN2* (SBF-regulated), *TMP1* (MBF-regulated) and *CMD1* (internal control) determined by Northern blot analysis. (Left) The mean cell size (in femtoliters) and the percentage of cells with replicated DNA (%2*n*) in those samples. The arrows (cell sizes of 11 and 22 fl) should facilitate comparison between the three strains. (Right) Normalized quantification of the the RNAs in all three strains, relative to cell size. The abscence of *CMD1* signal at 9 fl in the *cln1 cln2* panel is due to the the very low level of total RNAs in this particular lane. (**B**) DNA distribution in wild-type, *cln3* and *cln1 cln2* strains described above, as determined by FACS analysis. (Below) The percentage of cells with 2*n* DNA content (S/G_2 phases) versus cell size (fl). (**C**) Budding index (% budded cells) in the same cultures, relative to cell size (fl). (**D**) Growth of wild-type and *cln1 cln2* cells during the time course of the experiment (fl/min).

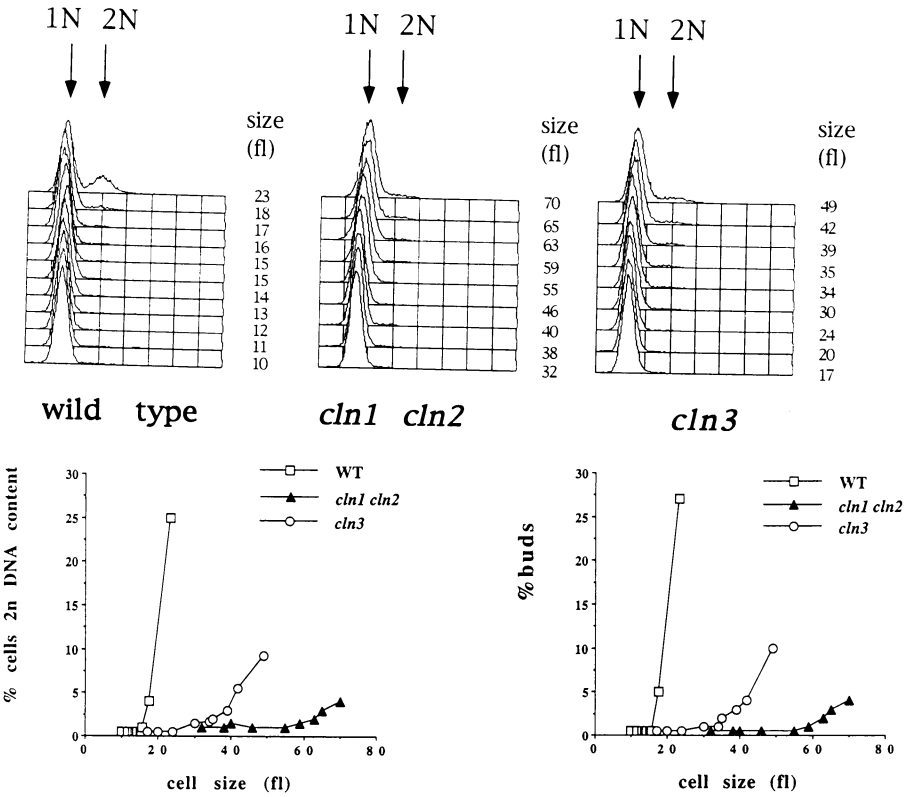

Fig. 3. DNA replication, budding and mating pheromone sensitivity of wild-type and *cln* mutant strains. MATa G_1 cells from wild-type (K3928), *cln1 cln2* (K3652) and *cln3* (K4953) strains were isolated by centrifugal elutriation starting from 4 l log phase populations in YEP raffinose medium (OD_{600} 1.5–2.0). Samples of increasing size were collected from the elutriator and analysed directly. Because they do not harbour the *MET3–CLN2* construct, the smallest daughters of the *cln3* and *cln1 cln2* strains are born larger than wild-type daughters (17 and 32 fl respectively). DNA distribution was analysed by FACS. (Below) Plots of the budding index. (Side) Per cent of cells with 2*n* DNA content in all the fractions versus cell size (fl). Cells of all fractions (500 cells/strain) were used to determine the state of pheromone sensitivity by plating at low density on YEP raffinose plates containing 2 μg/ml α-factor and scored for schmoo formation by microscopical observation.

Deletion of *SIC1* in *cln cln2* mutants allowed those cells to start replicating at a similar size to the wild-type strain (25 fl) and by the time *cln1 cln2* cells had started replicating (40 fl) the majority of the wild-type and *cln1 cln2 sic1* cells had already completed DNA replication (Figure 4A). This suggests that the S phase delay of *cln1 cln2* cells is due to a failure to inactivate/destroy the Clb–Cdc28 inhibitor Sic1. The budding delay of *cln1 cln2* cells compared with the wild-type (+20 fl) is only partially reversed by deletion of *SIC1* (+10 fl) (Figure 4B). These results are consistent with the observation that Clb5–Cdc28 and Clb6–Cdc28 kinases are mainly involved in triggering DNA replication, but are also able to promote budding in the absence of *CLN1* and *CLN2* only in larger cells (Schwob and Nasmyth, 1994).

Proper stabilization of Clb2 protein requires functional Cln1–Cdc28 and Cln2–Cdc28 kinases

Another function of Cln cyclins at Start is to turn off the proteolytic machinery that degrades mitotic cyclins such as Clb2 (Amon *et al.*, 1994). To determine whether *CLN1/CLN2* or *CLN3* perform this function we analysed the pattern of accumulation of Clb2 protein synthesized from the *GAL* promoter in small daughter cells from wild-type and *cln1 cln2* double mutants. We integrated one copy of the *CLB2* gene under the galactose-inducible promoter *GAL1-10* in wild-type and *cln1 cln2* double mutants carrying the *MET3–CLN2* construct (see legend to Figure 2). Asynchronous cultures of the wild-type and the mutant grown in medium lacking methionine (*MET3–CLN2* on), with raffinose plus galactose (*CLB2* mRNA induced) accumulated similar levels of Clb2 (data not shown). No Clb2 is detectable in small unbudded daughter cells isolated by elutriation in the presence of galactose. The half-life of Clb2 is less than 1 min in these cells and this prevents any accumulation (Amon *et al.*, 1994). Clb2 appeared in wild-type cells when they reached 16–20 fl, which is when they enter S phase and start budding, but it remained undetectable in the *cln1 cln2* double mutant until they reached 35 fl (Figure 5A). These data suggest that the activity of Cln1 and Cln2 in late G_1 is required to switch off the Clb2 proteolysis machinery at a normal cell size; Cln3 is not sufficient.

Cln2 is a rate limiting factor for S phase entry

While Cln1 and Cln2 are unnecessary for activation of late G_1-specific transcription, they are essential for budding, DNA replication, acquisition of pheromone resistance and shutting off of Clb proteolysis in a normal sized cell. Since Cln1 and Cln2 are capable of activating the late G_1-specific transcription programme, it is possible that activation by *CLN3* of *CLN1* and *CLN2* transcription when cells reach a critical cell size is the rate determining step for all Start events. To test this notion we tested the effect of moderate ectopic expression of *CLN2* in early G_1 using the *MET3–CLN2* fusion construct (*MET3–CLN2*

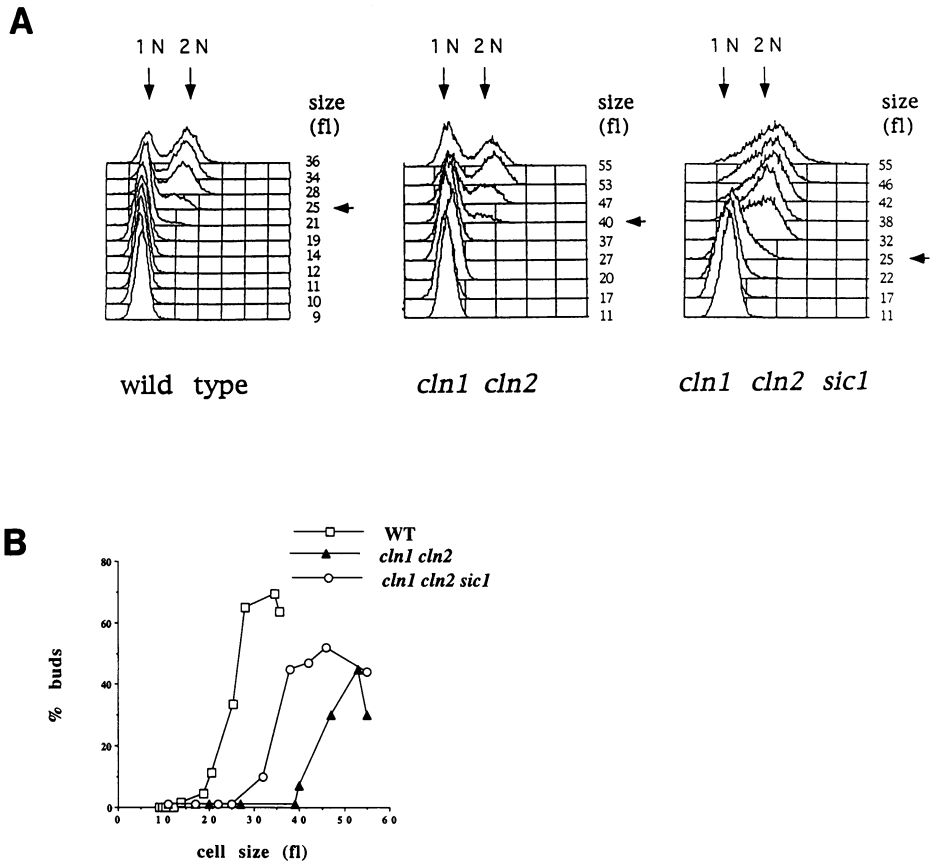

Fig. 4. Deletion of $p40^{SIC1}$ suppresses the S phase entry delay of *cln1 cln2* cells. Kinetics of DNA replication in *cln1 cln2* (K3652) and *cln1 cln2 sic1* (K4900) cells, as determined by FACS analysis. The wild-type control (K3852) is identical to that presented in Figure 2B. All three strains carry an integrated copy of the *MET3–CLN2* construct. Cells were grown, elutriated and re-inoculated in methionine/raffinose medium at 30°C, as described in Figure 2. Samples were taken for (**A**) size determination and FACS analysis and (**B**) budding index. The arrows on the side of the FACS data point to the size at which cells start replicating DNA.

produces similar amounts of *CLN2* mRNA as the endogenous gene in asynchronous cultures; data not shown). Daughter cells were isolated by elutriation from a culture pre-grown in the presence of methionine and were then incubated in medium containing or lacking methionine. Premature expression of *CLN2* from *MET3–CLN2* had no effect on cell growth (Figure 6B), but it advanced both S phase entry and budding by 5–10 fl (Figure 6A and C). These data indicate that the onset of *CLN2* transcription could be a rate determining step for Start in wild-type cells.

Discussion

Starting the cell cycle in yeast

Cell division in *Saccharomyces cerevisiae* (in most media) gives rise to progeny of different sizes, a larger mother cell and a smaller daughter derived from the bud. Mother cells start preparations for another division almost immediately. Budding (an early step of cytokinesis), DNA replication and spindle pole body duplication (the first step torwards building a mitotic spindle) commence almost simultaneously soon after cytokinesis. Daughter cells, on the other hand, must first grow to a critical size before these three events, known collectively as Start, can occur. The different behaviour of mother and daughter cells in this respect is entirely due to their different cell sizes, because larger than normal daughter cells, which can be created by treatments that delay late events of the chromosome cycle, resemble mother cells in not having to grow extensively before starting the next cell cycle. A natural version of this process occurs during pseudomycelial growth, where a control that prevents nuclear division until buds have grown as large as their mothers produces large daughter cells, which like their mothers enter S phase and form buds soon after cytokinesis (Kron *et al.*, 1994). Growth to a critical cell size before starting chromosome duplication is a mechanism common to many eukaryotic cells for maintaining a constant nuclear to cytoplasm ratio during proliferation. How they achieve this feat remains poorly understood.

How cell growth drives entry into the chromosome cycle is also relevant to the relationship between proliferation and differentiation, because most eukaryotic cells undergo differentiation from G_1. In *S.cerevisiae* pheromones prevent passage through Start of haploid cells that express the appropriate receptors and induce them instead to differentiate into gametes capable of conjugation. Once cells have passed Start, however, their chromosome cycles become refractory to arrest by pheromones, until the next round of cell divsion creates new G_1 cells.

What then do cells produce that trigger Start events when they reach a critical size and how is its production linked to cell size? It has become clear in recent years that a large group of mRNAs whose proteins are concerned with early events of the chromosome cycle accumulate suddenly shortly before or at Start in *S.cerevisiae* (Koch

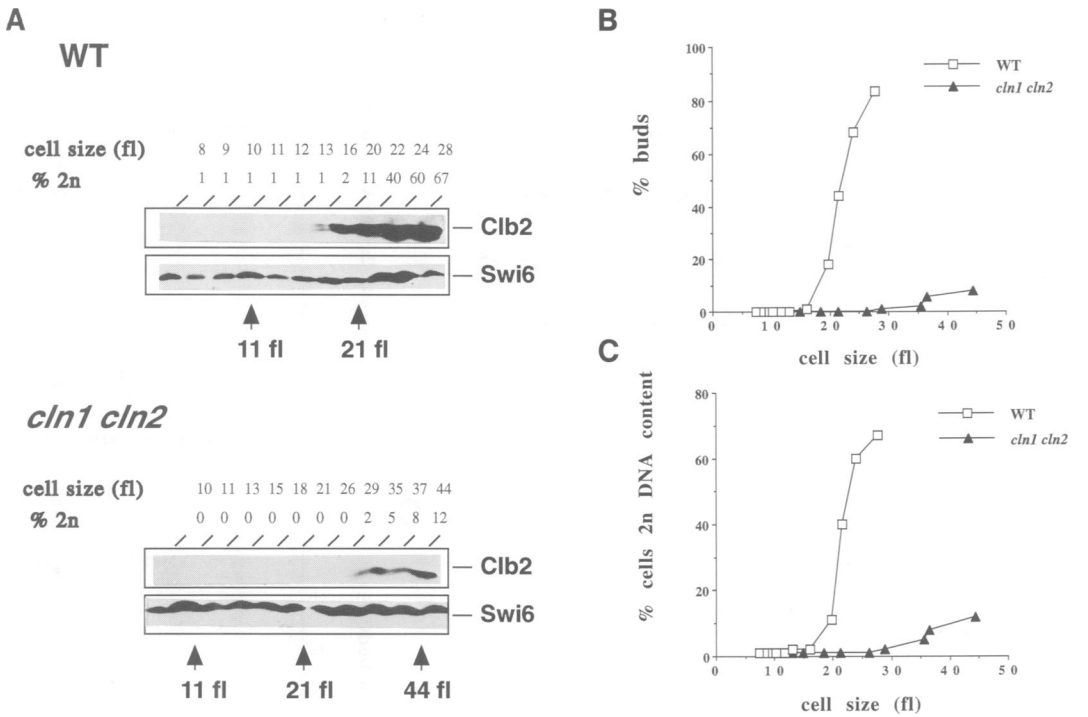

Fig. 5. Proper stabilization of the Clb2 protein requires functional *CLN1* and *CLN2*. Wild-type (K4255) and *cln1 cln2* (K4684) strains carrying an integrated copy of the wild-type *CLB2* gene under control of the *GAL1-10* promoter (pGAL-CLB2 [URA3]) and the *MET3–CLN2* fusion construct (pMET3-CLN2 [TRP1]) were grown to mid log phase (OD_{600} 1.5–2.0) in minimal medium/raffinose lacking methionine. Methionine (2 mM final) was added to the medium 100 min prior to elutriation. Cells (6 l) were elutriated in minimal medium containing 2 mM methionine, 2% raffinose and 2% galactose (*MET3–CLN2* off, *GAL–CLB2* on) and the isolated G_1 daugther cells incubated in the same medium at 30°C. Samples for size, budding index, FACS and Western blot analysis were taken every 30 min. (**A**) Western blot analysis using anti-Clb2 specific antibodies and anti-Swi6 specific antibodies as an internal control for equal loading. (**B**) Budding index. (**C**) Per cent cells with 2*n* DNA content/cell size (fl).

and Nasmyth, 1994). Most of these transcripts encode stable proteins, like DNA polymerase α, whose *de novo* synthesis is not needed for a new cell cycle, but others encode unstable regulatory subunits of the Cdc28 protein kinase, called cyclins. Activation of Cdc28 by one of three G_1 cyclins encoded by *CLN1*, *CLN2* and *CLN3* is necessary for Start in *S.cerevisiae*. Mutations that cause Cln2 and Cln3 proteins to become more stable cause cells to undergo Start at a smaller than normal cell size, which suggests that changes in the activity of Cln cyclins could be instrumental in triggering Start. *CLN3* mRNA and protein are present throughout G_1, but those from *CLN1* and *CLN2* (which are closely related to each other in sequence and are very different to *CLN3*) are absent in small G_1 cells and only appear as they reach the size critical for Start (reviewed in Nasmyth, 1993). Furthermore, as we have shown here, premature activation of *CLN2* transcription due to moderate expression from the constitutive *MET3* promoter is sufficient to advance all Start events (Figure 6). It is therefore plausible that accumulation of *CLN1* and *CLN2* transcripts is one of the key events that determines the onset of Start. This is the earliest change, other than cell growth itself, that can be detected in cells about to undergo Start and it seems to be rate limiting.

Do Clns 1 and 2 promote their own synthesis?

A key question then is what regulates *CLN1* and *CLN2* transcription? Their transcription during late G_1 is part of a wider programme of gene activation mediated by a pair of related transcription factors called SBF and MBF. Transcription of all genes regulated by these two factors depends on Cdc28 and on at least one of the three *CLN* cyclins. None of the *CLN* genes are necessary for Start, but at least one is necessary, because triple mutants lacking all three arrest in G_1 prior to Start. Furthermore, restoration of any one of the three Cln cyclin activities to such mutant cells triggers both Start and transcription of all SBF/MBF-regulated genes. Two conclusions have been drawn from these observations: first, that the three Cln cyclins, despite differences between the Cln1/Cln2 pair and Cln3 in sequence and regulation, have equivalent functions; second, one of these functions is to activate transcription mediated by SBF and MBF.

The fact that Cln1 and Cln2 are capable of activating their own transcription led to the proposal that the sudden activation of SBF- and MBF-regulated genes when cells reach a critical cell size involves a positive feedback loop, whereby Cdc28 kinase activities associated with Cln1 and Cln2 promote their own increase through stimulation of *CLN1* and *CLN2* transcription (Figure 1). A key question arose concerning the mechanism for starting such an autocatalytic process and it has been proposed that Cln3, which unlike Cln1 and Cln2 is present in early G_1 cells, would be ideally suited for catalysing the transition from a state where Cln1 and Cln2 are insufficiently abundant to activate themselves to one where their synthesis becomes autocatalytic (Tyers *et al.*, 1993). Here then was a detailed hypothesis for the dynamics of Cln1 and Cln2 activation during late G_1. Is it correct?

An important prediction of the positive feedback model is that SBF- and MBF-mediated gene activation should be greatly delayed in cells lacking Cln1 and Cln2. Cln3

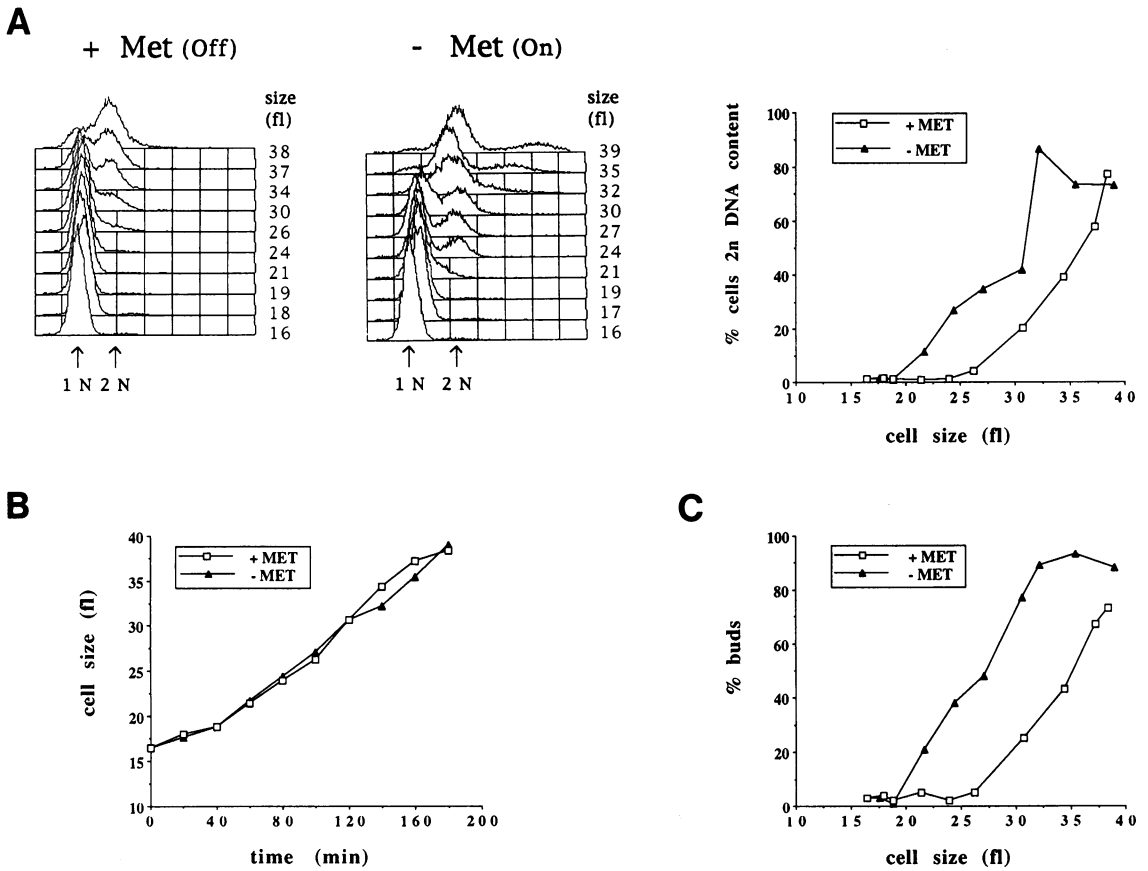

Fig. 6. Cln2 is a rate limiting factor for S phase entry. Wild-type cells carrying the *MET3–CLN2* gene fusion were grown to mid log phase (OD_{600} 1.3) in minimal medium/raffinose + 2 mM methionine. Small G_1 cells were isolated by centrifugal elutriation in the same medium. Half of the daughter cells were re-inoculated at 30°C at OD_{600} 1.0 in minimal medium/raffinose + 2 mM methionine (+MET) and the other half was washed with minimal medium/raffinose without methionine and re-inoculated in the same medium (–MET) at OD_{600} 1.0 at 30°C. Samples for FACS analysis, cell size determination and budding index were taken every 20 min. (**A**) FACS profile of the DNA content and per cent of 2*n* cells/cell size (fl). (**B**) Increase in cell size (fl) with time (min). (**C**) Budding index.

might start activation, but participation of Cln1 and Cln2 was envisaged to be necessary for full-blown activation. We have now tested this prediction using transient expression of *CLN2* from a *MET3–CLN2* promoter–gene fusion to generate daughter cells from a *cln1 cln2* double mutant which are of a similar size to wild-type daughter cells. Contrary to our expectations, we found that the rates of accumulation during growth of small daughter cells of most, if not all, transcripts under SBF/MBF regulation were very similar, if not identical, in wild-type and *cln1 cln2* double mutant cells (Figure 2A). We noticed a slight delay in accumulation of *CLN2* transcripts in *cln1 cln2* mutants, whose significance was unclear. Thus sudden activation of late G_1-specific transcription as cells reach a critical size does not require the Cln1/Cln2 cyclin pair.

Because all three Cln cyclins were thought, on the basis of their synthetic lethality, to have equivalent functions and because all three are capable of triggering late G_1-specific transcription, it seemed possible that either Cln1/Cln2 or Cln3 could be responsible for activating transcription. If this were the case we should find the same result with *cln3* mutants. However, in a similar experiment using a *cln3 MET3–CLN2* strain we found that the appearance of SBF- and MBF-regulated transcripts was greatly delayed in the absence of Cln3 function. We conclude that Cln1/Cln2 and Cln3 do not after all have equivalent functions: Cln3 is essential for the punctual activation of cell cycle-specific gene expression in late G_1, whereas neither Cln1 nor Cln2 is necessary. Our conclusion emphasises the difficulties of interpreting synthetic lethalities without accompaning such observations with extensive physiological studies. The different phenotypes of *cln3* and *cln1 cln2* mutants with regard to transcription is particularly striking because all other Start-related events are similarly delayed in both types of mutants.

Our observations are not inconsistent with previous results showing that Cln1 and Cln2 are capable of activating late G_1-specific transcription. Our current experiments were designed to address the mechanism of gene activation in normal cells, which is a different issue to the mechanisms made possible by artifical conditions. This distinction between what does occur and what can occur is rarely appreciated in many current studies of cell cycle regulation, the majority of which, for methodological reasons, address potential rather than actual mechanisms. It is quite possible, if not probable, that in the absence of Cln3, Cln1 and 2 do promote their own accumulation, but this process only occurs when cells have grown to twice the size at which wild-type daughter cells undergo Start.

Cln3–Cdc28 is concerned with activation of transcription

Cln3 is necessary for punctual activation of gene expression. Might this be the only function of the Cln3–Cdc28

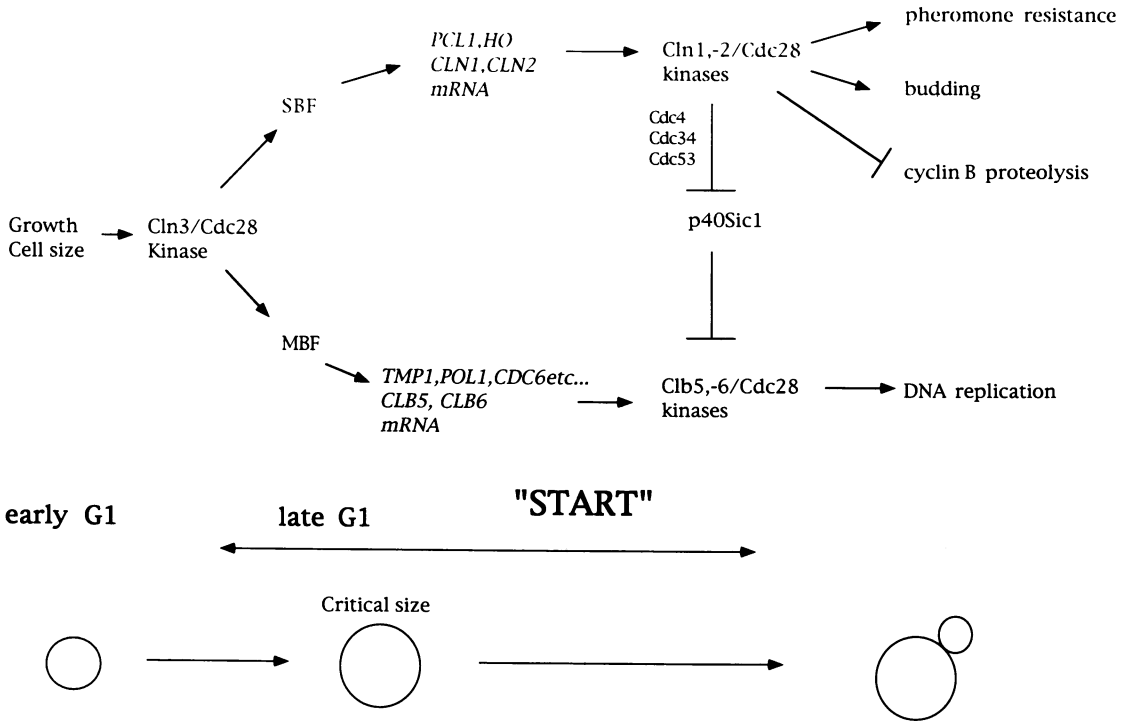

Fig. 7. Order of the late G_1-specific events. Starting from a small G_1 cells (left), the Cln3–Cdc28 kinase is activated when cells grow to a critical cell size. The mechanism of this activation remains unknown. As the main consequence Cln3–Cdc28 transiently induces expression of two families of genes, regulated by the SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) transcription factors. Whether or not this activation involves a direct phosphorylation of those factors by the Cln3–Cdc28 kinase is also not known, but it does not require the activity of the Cln1- and Cln2-associated kinases, not as proposed by the positive feedback loop model (Figure 1B). At this stage cells are still pheromone sensitive and neither bud nor replicate their DNA. As a consequence of SBF/MBF activation, four cyclin genes are now transcribed in late G_1: the SBF-regulated *CLN1* and *CLN2* genes and the MBF-regulated B-type cyclin genes *CLB5* and *CLB6*. The Cln1–Cdc28 and Cln2–Cdc28 kinases increase in parallel with the mRNA, while Clb5–Cdc28 and Clb6–Cd28 kinases remain inactive due to the presence of the $p40^{SIC1}$ inhibitor. Cln1–Cdc28 and Cln2–Cdc28 kinases are required for budding and to switch off the proteolytic activity acting on B-type cyclins. The cells are now pheromone resistant, a bud has emerged and spindle pole bodies are duplicated, but DNA replication is still not initiated. This situation can be artificially created in *cdc34* mutant cells, which possess high Cln1–Cdc28 and Cln2–Cdc28 activity, bud and duplicate spindle pole bodies, but possess no Clb–Cdc28 kinase. These cells are not commited to the mitotic cell cycle because they can still enter meiosis from this stage. Finally, the Cdc34-mediated proteolysis of $p40^{SIC1}$ (possibly targeted for destruction by the Cln1–Cdc28 and Cln2–Cdc28 kinases) releases active Clb5–Cdc28 and Clb6–Cdc28 kinases which trigger initiation of DNA replication. 'Start' can be seen as the narrow window of the cell cycle when all those events occur.

kinase? Two pieces of evidence are consistent with such a notion. First, premature activation during early G_1 of *CLN2* transcription (using the *MET3* promoter) is sufficient to advance all Start events. Thus Cln3 could, in principle, advance Start merely by activating *CLN1* and *CLN2* transcription. More persuasive, however, is the observation (M.Neuberg, personal communication) that over-production of Swi4 (one the components of SBF) in cells lacking MBF causes activation of all late G_1-specific genes independent of Cln3, Cdc28 and cell size. This causes cells to undergo Start at a third of their normal size and this reduction depends on Cln1 and Cln2, but is independent of Cln3. That hyperactivation of SBF by-passes the need for Cln3 (but not Cln1 and Cln2) suggests that its sole function may be to activate transcription.

How the Cln3–Cdc28 kinase activates SBF and MBF only when cells reach a certain size is still a mystery. The specific concentration of Cln3 protein does not vary much during the growth of small daughter cells (Tyers *et al.*, 1993; Siegmund, personal communication). There will, therefore, be a gradual increase in the amount of Cln3 per cell, but this would not lead to any change in the Cln3 concentration during G_1 unless Cln3 were localized to a compartment of the cell that does not increase in volume during this phase of the cell cycle. The location of Cln3 protein has never been established, but it is likely to act on chromatin, given its function as an activator of SBF and MBF transcription factors. Thus increases in the concentration of Cln3 in the vicinity of SBF and MBF transcription factors, which are known to be bound to SCBs and MCBs in early G_1 cells, could occur during the growth of small G_1 cells. However, this would not in itself explain the explosive appearance of late G_1-specific mRNAs when cell size crosses a threshold. A doubling of the *CLN3* gene dosage reduces size at Start by 20%, suggesting that the rate of Cln3 synthesis is a critical, but not the only, variable (Nash *et al.*, 1988). A key experiment that is lacking is measurement of Cln3–Cdc28 kinase activity during the growth of small daughter cells. This kinase cannot readily be assayed using histone H1 as a substrate and enzyme levels in wild-type cells have not been reliably measured.

Cln1 and Cln2, executors of the Start programme?

Despite normal activation of late G_1-specific transcription, which implies that the Cln3–Cdc28 kinase is active, *cln1 cln2* double mutants show no other signs of passage through Start, until, that is, they reach a very large cell size. Cln1 and Cln2 are necessary for promoting budding, DNA replication, acquisition of pheromone resistance and

turning off of Clb2 proteolysis. The delayed replication of *cln1 cln2* double mutants is largely eliminated by deletion of the *SIC1* gene (Figure 4). This indicates that Cln1 and Cln2 might promote S phase by triggering proteolysis of $p40^{SIC1}$, which is a potent and specific inhibitor of the Clb–Cdc28 kinases needed for S phase. Thus Cln1 and Cln2 promote activation of Clb–Cdc28 kinases needed for DNA replication and for the formation of mitotic spindles by two mechanisms: by shutting off cyclin B proteolysis initiated during anaphase and by promoting proteolysis of $p40^{SIC1}$.

Cln1 and Cln2 could promote acquisition of pheromone resistance by down-regulating signal transduction (Oehlen and Cross, 1994), by triggering proteolysis of Far1 (McKinney *et al.*, 1993), which is an inhibitor of Cln–Cdc28 kinases, and/or by promoting activation of Clb–Cdc28 kinases, which are refractory or at least less sensitive to pheromone inhibition. How Cln1 and Cln2 promote budding is not yet clear.

Given the multitude of functions exerted by Cln1 and Cln2 at Start, it is perhaps surprising that *cln1 cln2* double mutants are alive at all. Their survival can be attributed to the ability of the mutant cells, when they grow sufficiently large, to trigger proteolysis of $p40^{SIC1}$. This will enable activation of the Clb5–Cdc28 and Clb6–Cdc28 kinases, which, in addition to their normal function of promoting S phase, are also capable of promoting budding. This hypothesis would explain the more permanent G_1 arrest of quadruple *cln1 cln2 clb5 clb6* mutants (Schwob and Nasmyth, 1993).

What is Start?

One of the more influential ideas in cell cycle research in the last 20 years has been the concept that cells make a decision in late G_1 that commits them to mitotic cell division. This point in the yeast cell cycle is called Start. An analogous point during G_1 in mammalian cells is the so-called Restriction point, after which cells can complete division in the absence of growth factors or in the face of a reduction in the overall rate of protein synthesis. Start or the Restriction point can be safely regarded as points or intervals of the cell cycle. As such, they are merely descriptive terms that make no assertion as to how cells execute the events that occur at these stages of the cell cycle. This then would be the 'soft' version of Start. However, this is not the only sense in which Start has been extensively discussed and used as a term. It was initially proposed that Start was an 'event' in late G_1 that 'committed' cells to the mitotic cell cycle. Cdc28 was the only gene at the time thought to be needed for Start events. The subsequent discovery that it encodes a cyclin-dependent protein kinase (Cdk) indicated that Start might correspond to 'activation' of this protein kinase. This then would be a 'hard' version of Start.

It is arguable that we now know enough about how different forms of Cdc28 are activated during G_1 in *S.cerevisiae* to assess the notion that its activation in late G_1 commits cells to division. The first surprise and complication has been the discovery that Cdc28 is not just required for 'Starting' the cell cycle, but is necessary for practically all events during the chromosome cycle. The second is that there is not just one form of the kinase that is suddenly activated in late G_1. Instead, we have Cln3–Cdc28 whose activation triggers transcription, Cln1–Cdc28 and Cln2–Cdc28 whose activation leads to budding and activation of six different Clb–Cdc28 kinases, which promote S phase, various aspects of spindle formation and finally activation of anaphase. Can any of these events be considered to correpond to cell cycle commitment? One of the problems with answering this question lies with the word 'commitment', which has no meaning unless we also specify the conditions with regard to which cells can be regarded as being committed. We can, however, ask which Cdc28 kinase commits a cell to undergoing division in the face of particular environmental circumstances that are inimical to sustained proliferation. In this regard it is clear that activation of the Cln1–Cdc28 and Cln2–Cdc28 kinases plays an important role in acquisition of the ability to divide in the presence of pheromones and in loss of ability to conjugate in haploid cells. Activation of these kinases is not, however, sufficient for commitment of diploid cells to mitosis in the face of transfer to conditions that support meiosis. For example, cells arrested in G_1 by *cdc4* or *cdc34* mutations, which have all three Cln–Cdc28 kinases fully activated but none of the Clb–Cdc28 forms, enter meiosis without completing a mitotic division upon transfer to the appropriate starvation medium (Simchen and Hirschberg, 1976). Activation of Clns and Clb–Cdc28 or something else besides is required for mitosis to proceed in diploid cells once they have been transferred to meiotic conditions. It is, therefore, not tenable to cling to the belief that Start corresponds to a unique event in late G_1 that commits the yeast cell to a mitotic division. Start may be better used simply as a description of a narrow interval of the cell cycle during which many events needed for division occur; i.e. we might be better off going for the 'soft' option.

Material and methods

Strains, media and reagents

All strains are derivatives of W303 (K699) but are $SSD1^+$ (K3928; *HMLa, HMRa, ho, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3,* $SSD1^+$). The synthetic medium lacking methionine (–Met medium) was nitrogen-based medium supplemented with amino acids as described by Rose *et al.* (1990). YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) was supplemented with 2% raffinose. Secondary antibodies were purchased from Amersham and proteins detected by enhanced chemiluminescence system according to the manufacturer's recommendations.

Genetic techniques, plasmid and strain constructions

Standard techniques (Mortimer and Hawthorne, 1969) were used for genetic crosses and DNA manipulations (Maniatis *et al.*, 1982). The *MET3–CLN2* construct fusion has been described (Amon *et al.*, 1994). The *cln2* frame-shift mutation (*cln2 FS*) was created by cutting the unique *XhoI* site within the *CLN2* coding region, filling in with Klenow and re-ligation. This creates a frame-shift mutation at nucleotide position +255. The translational stop of this construct is at position +300. The change was checked by sequencing. The mutant allele was transplaced back into yeast by transformation of a *cln1::hisG cln2::URA3* strain and selection for ura^- colonies on 5-FoA and the transplacement verified by Southern blot analysis. The resulting strain is K3651 (MATa *cln1::hisG cln2 FS* $CLN3^+$). K3652 and K3852 were derived from K3651 and K3928 respectively by integration of *MET3–CLN2* (c2433) at the *TRP1* locus. K4900 (MATa *cln1::hisG cln2 FS sic1::URA3 MET3–CLN2 [TRP1]*) was made by transformation of K3652 with a *sic1::URA3* disruption plasmid. K4255 and K4684 were made by integration of *GAL1-10::CLB2* (c2433) at *URA3* in K3852 and K3652 respectively. To test the effect of *PCL1* and *PCL2* on late G_1-specific transcription a white colony of a MATa *cln1::hisG cln2D pcl1::LEU2 pcl2::TRP1 YCP*

URA3 ADE3 ADH::CLN2 strain (K2884) was used (the color indicates plasmid loss). In this background (K1107; Nasmyth and Dirick, 1991) the quadruple mutant is alive. The *cln1 cln2* control strain is K2008 (Nasmyth and Dirick, 1991).

Northern and Western blot analysis

RNA isolation and Northern blot analysis were performed as described by Cross and Tinkelenberg (1991) and Price *et al.* (1991). The amounts of RNA were quantified using a Molecular Dynamics PhosphorImager. The probe used to detect *CLN2* mRNA is a 280 bp PCR fragment directly upstream of the ATG.

Western blot analysis was performed according to Surana *et al.* (1993) and Amon *et al.* (1994). Proteins were detected using the enhanced chemiluminescence detection system (ECL; Amersham).

Other techniques

Centrifugal elutriation was performed as described in Schwob and Nasmyth (1993). Flow cytometric DNA analysis was done according to Epstein and Cross (1992), using a Becton-Dickinson FACScan. Cell volume was measured using a Casy counter (Scharfe System, GHBH) and is the peak value of 10 000–15 000 cells.

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