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G1 Cyclins Block the Ime1 Pathway to Make Mitosis and Meiosis Incompatible in Budding Yeast

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Running Title: G1 Cyclins Make Mitosis and Meiosis Incompatible

Summary

Diploid yeast cells switch from mitosis to meiosis when starved for essential nutrients. While G1 cyclins play a key role in initiating the mitotic cell cycle, entry into meiosis depends on Ime1, a transcriptional activator regulated by both nutritional and cell-type signals. We show here that G1 cyclins block the Ime1 pathway and inhibit entry into meiosis. G1 cyclins repress transcription of the *IME1* gene and prevent the accumulation of Ime1 within the nucleus, which results in repression of meiotic gene expression. As G1-cyclin deficient cells do not require nutrient starvation for entry into meiosis, G1 cyclins exert its role by transmitting essential nutritional signals to Ime1 function. The existence of a negative cross-talk mechanism between mitosis and meiosis may help explain why these two developmental options are incompatible in budding yeast.

Introduction

Nutrients are among the most important trophic factors for yeast and, like most other eukaryotes, Saccharomyces cerevisiae takes different developmental options depending on environmental conditions during the G1 phase of the cell cycle. Depending on the nutrient limitation conditons, haploid cells either arrest in G1 or initiate invasive growth. Diploid cells also arrest in G1 or produce pseudo-hyphae, but they have an additional option: entry into meiosis.

Ime1 is a transcriptional activator that routes both nutritional and cell-type signals to the expression of meiotic genes, and has a central role in triggering meiosis (see Kupiec et al., 1997 for a review). Only diploid cells are able to enter meiosis as they possess both components of the Mata1-Matα2 complex, which allows expression of the *IME1* gene by two separate pathways. In addition, nutrient starvation signals regulate *IME1* at both transcriptional and post-transcriptional levels. The *IME1* promoter is repressed by glucose and nitrogen whereas is induced in the presence of acetate (Kassir et al., 1988; Sagee et al., 1998). On the other hand, Ime1 function as a transcriptional activator depends on its ability to interact with Ume6, a protein that binds at the promoters of early meiotic genes, and plays a dual role by inhibiting or activating gene expression depending on the interacting proteins (Strich et al., 1994; Bowdish et al., 1995; Kadosh and Struhl, 1997). Interaction between Ime1 and Ume6, which is elicited by the Rim11 and Rim15 kinases (Rubin-Bejerano et al., 1996; Vidan and Mitchell, 1997), has been shown to be a key target for glucose-mediated inhibition of Ime1 activity (Malathi et al., 1997).

S-phase entry during meiosis is completely dependent on Ime1 (Kassir et al., 1988), partly through the Ime2 protein kinase (Foiani et al., 1996). However, the mechanisms by which this transcriptional activator is able to trigger initiation of DNA replication have not been characterized. It has been proposed that Cdc28, the central cyclin-dependent kinase that regulates the mitotic cell cycle, may not have a role since *cdc28* thermosensitive cells arrest meiosis after DNA replication (Shuster and Byers, 1989), but no additional evidences are available yet that confirm this idea. Although Cln1, Cln2 and Cln3, the three yeast G1 cyclins, show a clear functional redundancy, they perform different roles during the G1-S transition in the mitotic cell cycle. Cln3 is the most potent activator regarding SBF- and MBF-dependent transcription of a set of genes including *CLN1* and *CLN2* (Tyers et al., 1993; Dirick et al., 1995; Stuart and Wittenberg, 1995; Levine et al., 1996). On the other hand, Cln1 and Cln2 have more specialized roles in budding initiation (Benton et al., 1993; Cvrcková and Nasmyth, 1993) and DNA synthesis initiation through degradation of the Clb-Cdc28 inhibitor Sic1 (Schwob et al., 1994; Schneider et al., 1996; Feldman et al., 1997; Skowyra et

al., 1997). The possible role of G1 cyclins in regulating entry into premeiotic S phase has not been characterized.

This work deals with the relationships between key molecules involved in initiating either mitosis or meiosis. Here we show that, although mitosis and meiosis share some important similarities during S-phase entry, G1 cyclins are not required at all to trigger premeiotic DNA replication, which depends on Ime1. In fact we have found that G1 cyclins block the Ime1 pathway to inhibit meiosis by two different mechanisms: (1) repressing *IME1* transcription and (2) preventing Ime1 accumulation within the nucleus. Our results indicate that yeast cells have established a negative cross-talk mechanism between mitosis and meiosis to make these cell cycle choices incompatible.

Results

G1 Cyclins Are Lost Early during Entry into Meiosis

In accordance with their essential role in the G1-S transition, we have shown previously that G1-cyclin levels are down-regulated very rapidly in haploid yeast cells deprived of an essential nutrient such as the nitrogen source (Gallego et al., 1997). Contrary to haploid cells, which arrest in G1, diploid yeast cells switch from the mitotic to the meiotic cell cycle under nitrogen starvation conditions. To understand the basis of these different cell fates we first focused on the key molecules of the mitotic G1-S transition during entry into meiosis.

Figure 1 shows the experimental model used for entry into meiosis (see Experimental Procedures). Diploid wild-type 1788 cells exponentially growing in acetate-based rich medium are allowed to reach a high cell density and accumulate in G1 as their growth becomes limited by the carbon source. Upon transfer to fresh medium with carbon source added but lacking the nitrogen source, these G1 cells readily initiate a premeiotic S phase in about 4h (Figure 2A), and proceed into the meiotic nuclear divisions to produce spores in 24h. After an initial burst of transcription due to carbon-source re-feeding, G1-cyclin expression was repressed rapidly under nitrogen deprivation conditions (Figure 2B). Accordingly to its role in *CLN1* and *CLN2* gene expression, Cln3 protein levels were also down-regulated. However, expression of the S-phase cyclin gene *CLB5*, which also depends on Cln3 during mitosis, was strongly induced. While Clb5 levels increased, Sic1 levels decreased steadily as cells entered the premeiotic S phase, suggesting that firing of replication origins during meiosis also depends on Clb-Cdc28 activity.

Although the absence of Cln proteins may explain the lack of budding, some questions arise from the early G1-cyclin loss during entry into meiosis. How is *CLB5*, and perhaps other MBF-regulated genes as *TMP1*, expression induced during meiosis? What triggers Sic1 loss in the absence of Cln-Cdc28 activity? As Ime1 is a key transcriptional factor essential for premeiotic S-phase entry (Kassir et al., 1988; Foiani et al., 1996), we asked whether it could also play an essential role in *CLB5* induction and Sic1 down-regulation. Figure 3 shows that Ime1-deficient diploid cells failed to induce *CLB5* and *TMP1* expression up to the wild-type levels while, more importantly, Sic1 protein levels were not down-regulated at a post-transcriptional level. In agreement to the essential role of Rim11 in Ime1 function, *CLB5* induction was also prevented in a *rim11* null mutant (data not shown). Thus, besides activating early-gene expression during meiosis, Ime1 has a key role in increasing Clb-Cdc28 activity during entry into premeiotic S phase.

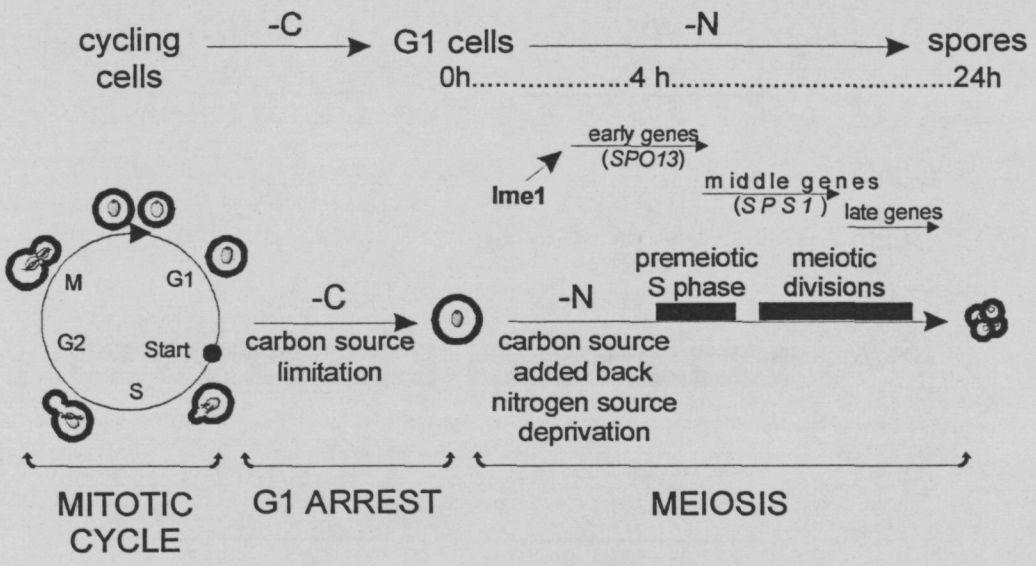

Figure 1. Experimental Model Used for Entry into Meiosis

Diploid wild-type 1788 cells (CYC) growing in YPA (acetate-based rich medium) are allowed to accumulate in G1 as the carbon source becomes limiting (-C) and transferred to sporulation medium (-N). Premeiotic S phase is initiated in ~4h and cells sporulate with an efficiency of 70-80% after 24h. Approximate time intervals for Ime1-dependent expression of early (e.g. *SPO13*), middle (*e.g. SPS1*) and late meiotic gens are indicated.

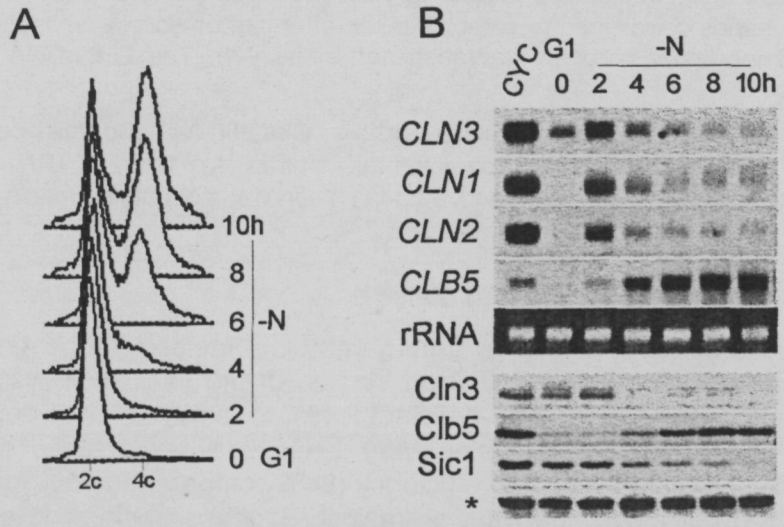

Figure 2. G1 Cyclins are Lost Early during Entry into Meiosis

(A) DNA content distributions of wild-type 1788 cells subject to the experimental model shown in Figure 1 for entry into meiosis. Samples were obtained during mitotic growth in YPA medium (CYC), after carbon-source limitation (G1, 0h), and at different times under nitrogen starvation conditions (-N).

(B) mRNA levels for G1-cyclins and the *CLB5* S-phase cyclin from samples taken as in (A). Protein levels for the Cln3 and Clb5 cyclins, and the Clb-Cdc28 inhibitor Sic1 are also shown. The 25S rRNA and a 12CA5 cross-reactive band (*) serve as loading controls.

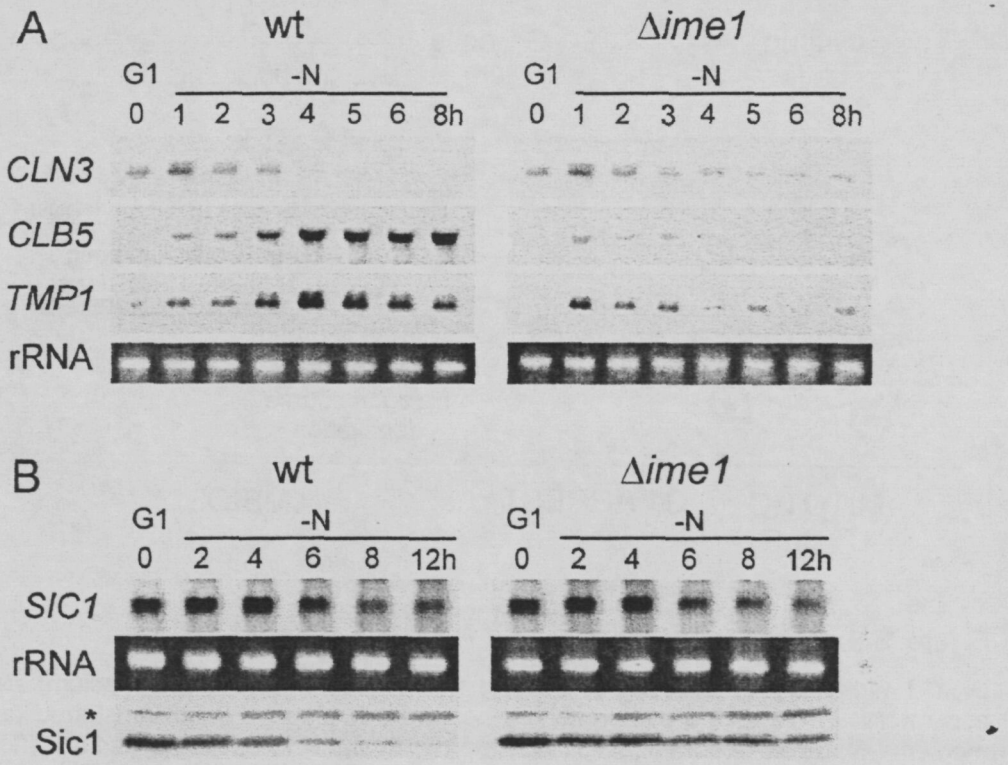

Figure 3. *CLB5* Cyclin Induction and Sic1 Loss Depend on Ime1 during Premeiotic S-Phase Entry

(A) *CLN3*, *CLB5* and *TMP1* mRNA levels in wild-type 1788 (wt) and Ime1-deficient CML268 (Δ*ime1*) cells. Lanes correspond to samples taken after carbon-source limitation (G1, 0h), and at different times under nitrogen starvation conditions (-N). The 25S rRNA is shown as a loading control.

(B) *SIC1* mRNA and Sic1 protein levels in wild-type CML260 (wt) and Ime1-deficient CML365 (Δ*ime1*) cells were determined from samples taken as in (A). The 25S rRNA is shown as a loading control and a 12CA5 cross-reactive band (*) serve as controls for loading.

Cln3 Is Not Required for Entry into Meiosis

The loss of G1 cyclins early during entry into meiosis suggests that they may not be required at all in this specialized version of the yeast cell cycle. To test this possibility further we used Cln3-deficient cells to analyze Ime1-dependent gene expression and premeiotic S-phase entry kinetics. Unexpectedly, not only Cln3-deficient cells sporulated with high efficiency (84% compared to 75% for the wild type, see Figure 5A) but they underwent premeiotic S phase earlier and more efficiently (Figure 4A), while induction of *CLB5, SPO13 (*an early gene), and *SPS1* (a middle gene) also occurred much earlier (Figure 4B). The *TMP1* expression patterns paralleled those observed for *CLB5* (data not shown), supporting the notion that Ime1 replaces Cln3-dependent mechanisms to induce mitotic MBF-dependent genes during entry into meiosis. Expression of *IME4*, which transmits both diploid- and nutrient-specific signals to the *IME1* promoter, as well as *IME1* mRNA levels, did not differ significantly in Cln3-deficient cells compared to the wild type (Figure 4B). As Ime1 protein levels did not differ either when comparing Cln3-deficient and wild-type cells (data not shown), these results suggest a negative role for Cln3 on *IME1* function at a post-translational level. Although wild-type cells produced an initial Cln3-dependent burst of transcription of both *CLN1* and *CLN2* due to carbon-source re-feeding (see

Figure 2), Cln1 and Cln2 protein levels remained at very low levels (about 50-fold lower compared to cycling cells in acetate-based rich medium, data not shown) under the nitrogen starvation conditions used for entry into meiosis. Thus, the observed Cln3 negative effects on Ime1 function are most likely independent of downstream effects mediated by Cln1 and/or Cln2.

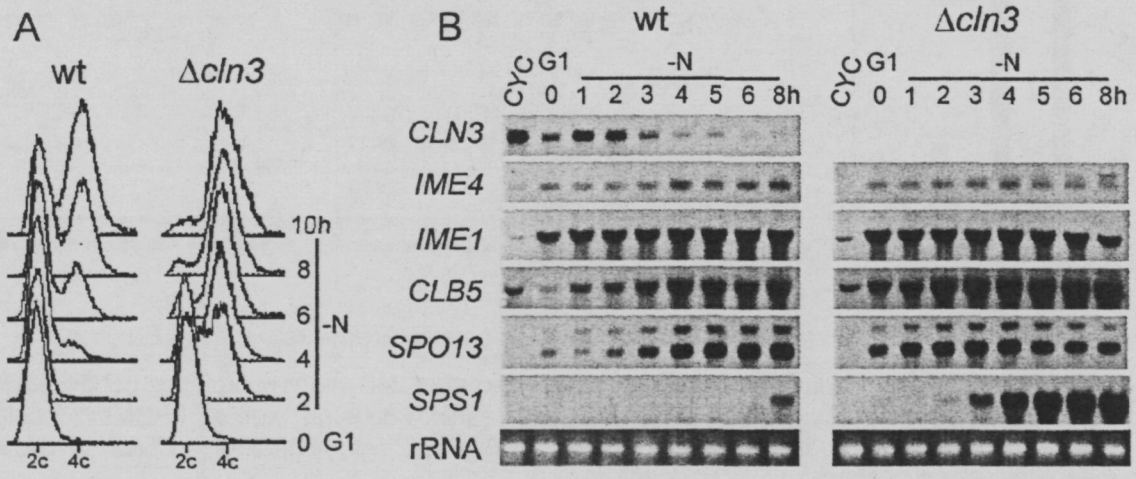

Figure 4. Cln3-Deficient Cells Enter Meiosis More Efficiently

(A) DNA content distributions of wild-type 1788 (wt) and Cln3-deficient CML254 (Δ*cln3*) cells during entry into meiosis. Samples were obtained during mitotic growth in YPA medium (CYC), after carbon-source limitation (G1, 0h), and at different times under nitrogen starvation conditions (-N).

(B) mRNA levels for cyclins *CLN3* and *CLB5*, the meiotic genes *IME4* and *IME1*, as well as *SPO13* (an Ime1-dependent early gene) and *SPS1* (a middle gene) were determined by northern blot analysis from samples taken as in (A). The 25S rRNA serves as a loading control.

G1-Cyclin Overexpression Inhibits Meiosis and Down-Regulates Ime1 Function

If Cln3 exerts a negative effect on Ime1 function, entry into meiosis should be inhibited by overexpression of G1 cyclins from a constitutive promoter. We used the tetracycline-regulatable promoter $tetO_2$ to drive expression of *CLN1*, *CLN2* or *CLN3* from a centromeric vector in cells growing exponentially in acetate-based rich medium without tetracycline to induce the $tetO_2$ promoter. Shown in Figure 5A are the percentages of asci and budded cells 24 hours after nitrogen deprivation in cells overexpressing *CLN1*, *CLN2* or *CLN3*. As expected from a negative role of Cln3 during entry into meiosis, G1-cyclin overexpression inhibited sporulation and forced cells to enter mitosis as deduced from the final budding indexes. Similar results were obtained in W303 diploid cells (data not shown). Constitutive overexpression of *CLN3* from the $tetO_2$ promoter did not prevent the G1 arrest produced by carbon-source limitation in acetate-based rich media as deduced from the DNA content distributions (data not shown). Wild-type cells arrested in G1 by carbon-source limitation did not increase their number significantly during 24 hours upon transfer to sporulation conditions. On the contrary, *CLN3*-overexpressing cells doubled their number and arrested with a high percentage of cells with a 4c DNA content (data not shown), which is in agreement with the high final budding index attained by these cells. Thus, G1-cyclin overexpression not only inhibits sporulation but also drives cells into mitosis under conditions where wild-type cells enter meiosis very efficiently.

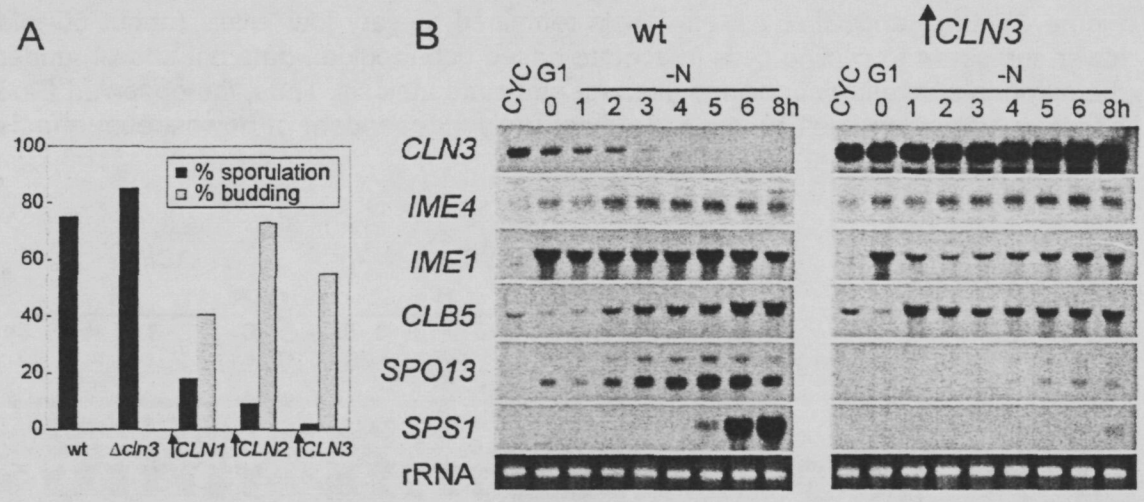

Figure 5. G1-Cyclin Overexpression Inhibits Meiosis by Repressing Ime1 Function

(A) Sporulation and budding indexes were determined after 24h under sporulation conditions in wild-type CML256 (wt) and Cln3-deficient CML254 (Δ*cln3*) cells, as well as in CML256 cells overexpressing either *CLN1* from pCM165 (↑*CLN1*), *CLN2* from pCM250 (↑*CLN2*) or *CLN3* from pCM166 (↑*CLN3*).

(B) Wild-type CML256 cells transformed with the empty vector (wt) or pCM166 to overexpress *CLN3* (↑*CLN3*) were used to determine mRNA levels for cyclins *CLN3* and *CLB5*, the meiotic genes *IME4* and *IME1*, as well as *SPO13* (an Ime1-dependent early gene) and *SPS1* (a middle gene). Lanes correspond to samples obtained during mitotic growth in YPA medium (CYC), after carbon-source limitation (G1, 0h), and at different times in sporulation medium (-N). The 25S rRNA serves as a loading control.

The proposal that Cln3 inhibits Ime1 function at a post-translational level also predicts that constitutive overexpression of *CLN3* from the $tetO_2$ promoter should block Ime1-dependent expression. Figure 5B shows that expression of both *SPO13*, which depends directly on Ime1 as an early gene, and *SPS1*, which is induced further downstream in the Ime1 pathway as a middle gene, is strongly repressed under transfer to sporulation conditions. As expected, *CLB5* (Figure 5B) and *TMP1* (data not shown) expression was readily induced by *CLN3* overexpression, most likely by Cln3-dependent mechanisms as deduced from the behavior of double *cln3 ime1* null mutants (data not shown). Although *CLN3* overexpression did not affect the *IME4* expression pattern, *IME1* mRNA levels were clearly down-regulated after transfer to sporulation conditions. It has been proposed that Ime1 may retro-activate its own transcription during the earliest steps of meiosis (Shefer-Vaida et al., 1995). In order to avoid indirect effects on *IME1* transcription due to Ime1 inhibition at a post-translational level, Ime1-deficient cells were subject to *CLN3* overexpression during entry into meiosis. *IME1* expression was evaluated (see Figure 6) from a plasmid construct that lacks a functional *IME1* ORF (*ime1-2*), and from the homozygous *kanMX4*-disrupted chromosomal copies (*ime1-1*). In both constructs *CLN3* overexpression was able to repress the *IME1* promoter. Accordingly, neither *IME1* transcription nor *CLN3*-overexpression repression effects on *IME1* transcription changed significantly in *rim11* null mutants (data not shown), where Ime1-dependent transcription is completely repressed (Bowdish et al., 1994). Thus, in addition to the post-translational mechanism aforementioned, our results indicate that high Cln-cyclin levels similar to those found in cycling cells have a role in repressing *IME1* transcription.

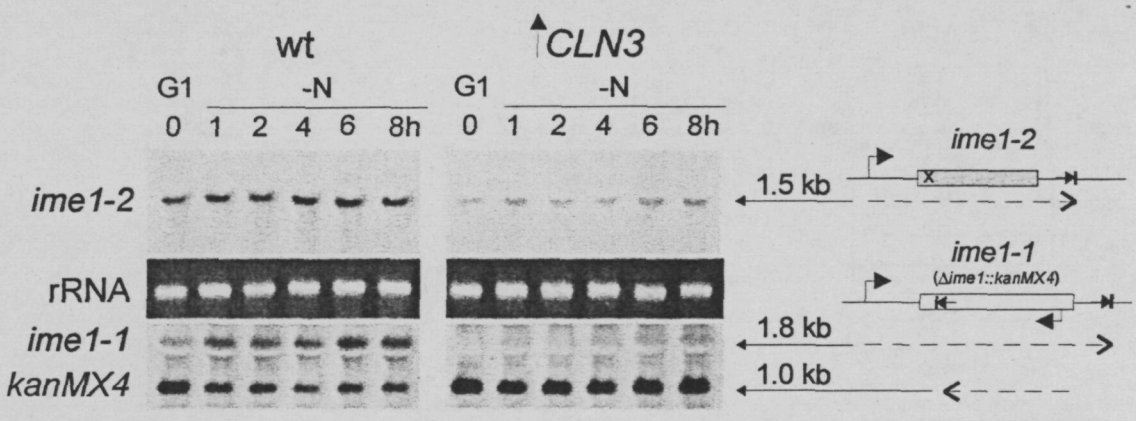

Figure 6. *CLN3* Overexpression Inhibits *IME1* Transcription

Ime1-deficient CML342 cells carrying a homozygous *ime1-1* deletion (*Δime1::kanMX4)* and a truncated *ime-2* allele on pCM268, were transformed with empty vector (wt) or pCM166 to overexpress *CLN3* (↑*CLN3*). mRNA levels produced by the *IME1* promoter from the chromosomal *ime1-1* allele were determined with a *kanMX4* probe, which also detects the *kanMX4* transcript. The plasmid-borne *ime1-2* mRNA was detected with a *IME1* probe that does not cover any of the *IME1* sequences left in *ime1-1*. Lanes correspond to samples taken after carbon-source limitation (G1, 0h), and at different times under sporulation conditions (-N). The 25S rRNA is shown as a loading control. The origin of the transcripts detected is outlined on the right.

Cln3-Deficient Cells Initiate Meiosis under Conditions where Wild-Type Cells Remain Arrested in G1

Nitrogen starvation has been the most efficient environmental condition used to induce meiosis in diploid yeast cells (Freese et al., 1982). We have shown that (1) Cln3 is lost very early during entry into meiosis, (2) Cln3-deficient cells undergo meiosis more efficiently than wild-type cells, and (3) *CLN3* overexpression inhibits meiosis and forces cells into mitosis even under nitrogen starvation conditions. Thus, nitrogen starvation could exert its essential role in inducing meiosis through down-regulation of G1-cyclin levels. As Cln3 is the only detectable G1 cyclin in carbon-source-limited G1 cells prior to induction of meiosis by nitrogen starvation in the experimental model used (see above and Figure 7D), we asked whether Cln3-deficient cells are able to enter meiosis in the presence of the nitrogen source. Figure 7A shows that this is indeed the case. Wild-type and Cln3-deficient cells growing exponentially in acetate-based rich medium were allowed to reach a high cell density and arrest in G1 by carbon-source limitation. Instead of starving cells for nitrogen they were left under carbon-source limitation conditions during the rest of the experiment. While wild-type cells remained arrested in G1, Cln3-deficient cells slowly initiated DNA replication with no signs of budding. That this S phase was in fact premeiotic was concluded from the observation that double *ime1 cln3* null mutant cells remained arrested in G1, as deduced from their DNA content distributions under the same experimental conditions (data not shown). Although more than 50% of Cln3-deficient cells entered premeiotic S phase, the final sporulation percentages were 15-20% (those attained by wild-type cells were always lower than 5%). As Cln3 levels remained invariable in wild-type cells subject to carbon-source limitation (Figure 7B), our results indicate that the only essential role of nitrogen starvation for entry into premeiotic S phase is the down-regulation of Cln3.

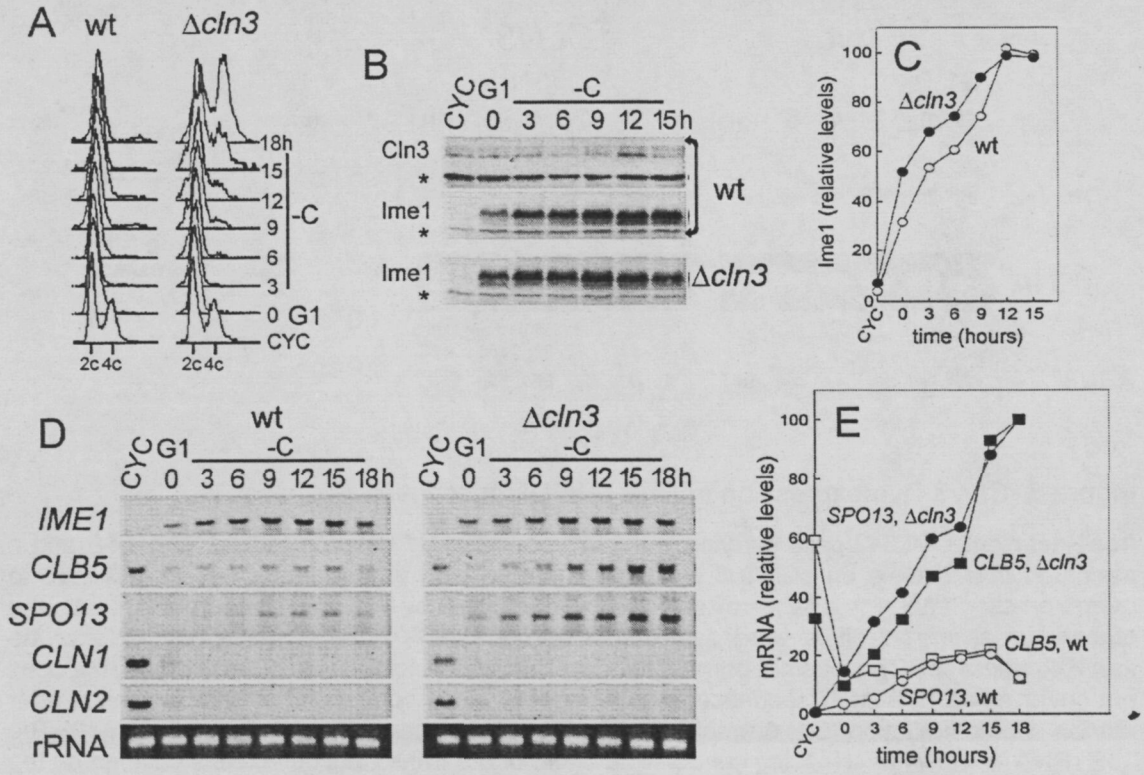

Figure 7. Cln3-Deficient Cells Undergo Meiosis under Incomplete Starvation Conditions for Sporulation, Where Wild-type Cells Remain Arrested in G1

(A) DNA content distributions of wild-type CML337 (wt) and Cln3-deficient CML319 (Δ*cln3*) cells during entry into meiosis. Samples were obtained during mitotic growth in YPA medium (CYC), and at different times under carbon-source limitation (-C), but in the presence of the nitrogen source.

(B) Ime1 levels in wild-type CML337 (wt) and Cln3-deficient CML319 (Δ*cln3*) cells containing the 3HA-tagged *IME1* gene were determined from samples taken as in (A). Cln3 levels were determined from CML257, a wild-type strain that contains a 3HA-tagged version of *CLN3*. A 12CA5 cross-reactive band (*) serves as a control for loading.

(C) Quantification of Ime1 protein levels shown in (B) for wild-type (wt, open circles) and Cln3-deficient (Δ*cln3*, closed circles) cells.

(D) mRNA levels for cyclins *CLN1, CLN2* and *CLB5*, the meiotic gene *IME1*, and *SPO13* (an Ime1-dependent early gene) were determined by northern blot analysis from samples taken as in (A). The 25S rRNA serves as a loading control.

(E) Quantification of *CLB5* (squares) and *SPO13* (circles) mRNA levels shown in (D) for wild-type (wt, open symbols) and Cln3-deficient (Δ*cln3*, closed symbols) cells.

We have shown that *CLN3* overexpresion is able to repress *IME1* transcription (see above). As expected from the low Cln3 levels normally attained in wild-type cells, neither *IME1* mRNA nor Ime1 protein levels were up-regulated in Cln3-deficient cells compared to wild-type cells under carbon-source limitation (Figure 7). However, Ime1-dependent transcription was clearly induced only in Cln3-deficient cells. Expression levels of both *CLB5* and *SPO13* attained by the *cln3* mutant strain were 5-fold higher than the corresponding levels in wild-type cells. Moreover, induction of *CLB5* and *SPO13* in Cln3-deficient cells strictly depended on Ime1, as it was completely abolished in a double *cln3 ime1* null mutant strain (data not shown). These results confirm the notion that even low levels of Cln3 block Ime1 function at a post-

confirm the notion that even low levels of Cln3 block Ime1 function at a post-translational level, which agrees with the fact that *CLN3* overexpression represses *SPO13* transcription much more severely than *IME1* transcription.

G1-Cyclin Deficient Cells Switch from Mitosis to Meiosis in Rich Media

Cln3-deficient cells are able to enter premeiotic S phase only after a temporary G1 arrest where *CLN1* and *CLN2* expression becomes strongly repressed by carbon-source limitation (Figure 7D). This observation suggests that all three G1 cyclins must be down-regulated to allow entry into premeiotic S phase. In agreement with this idea we have shown that overexpression of any of the three G1 cyclins inhibits meiosis and forces cells into mitosis under otherwise normal sporulation conditions (see above).

To test whether G1-cyclin down-regulation is not only a necessary but also a sufficient condition to allow entry into premeiotic S phase independently of the nutritional status of the cell, we used a homozygous *Δcln1 Δcln2 GAL1p-CLN3* strain that depends on the presence of galactose to execute the mitotic G1-S transition. In order to determine first the essential role of G1 cyclins on Ime1 function inhibition at a post-translational level, *IME1* was consitutively expressed in a centromeric vector from the Schiz. pombe *adh* promoter, which attains expression levels similar to those produced by the natural *IME1* promoter under sporulation conditions (data not shown). Figure 8A shows that when these cells were transferred to acetate-based rich medium to repress *CLN3* expression, they arrested temporarily in G1, proceeded into an S phase with no signs of budding, and finally sporulated with high efficiency, indicating that G1-cyclin down-regulation is sufficient for entry into meiosis in rich media as long as *IME1* constitutive expression is provided. S-phase entry and sporulation efficiencies were as high as those obtained when cells were transferred to sporulation medium and starved for nitrogen once cells had arrested in G1 after 3 h in acetate-based rich medium. Thus, and in agreement with our results with Cln3-deficient cells under carbon-source limitation conditions, nitrogen starvation exerts its essential role in meiosis induction by down-regulating G1 cyclins. As expected, when *CLN3* expression was provided from its natural promoter sequences in a centromeric vector, those cells also arrested temporarily in G1 but resumed cell proliferation by mitosis as deduced from budding indexes (Figure 8A) and cell number increase (data not shown).

Although the *IME1* promoter senses a variety of nutritional signals to become fully active (Sagee et al., 1998), when we performed the same cell cycle analysis with a *Δcln1 Δcln2 GAL1p-CLN3* strain with *IME1* under its own promoter, cells did indeed underwent S-phase entry with no budding (data not shown) and sporulated, albeit at lower frequencies (15% in acetate-based rich medium compared to 40% in sporulation medium). These results suggest that *IME1* expression from its own promoter in acetate-based rich medium is sufficient for entry into meiosis as long as G1 cyclins are not present in the cell, which agrees with the fact that *CLN3* overexpression represses *IME1* transcription (Figure 6, and see above). Figure 8B shows that, after an initial increase in *IME1* transcription due to the transfer from galactose to acetate-based rich media, Cln-deficient cells induced *IME1* transcription at levels much higher than those attained by cells containing Cln3. In addition, Ime1-dependent induction of *SPO13* expression did only take place in Cln-deficient cells. This transcriptional activation was not merely due to higher Ime1 levels as *IME1* transcription increased. Cln3-containing cells that expressed constitutive levels of *IME1* mRNA were not able at all to activate *SPO13* transcription (Figure 8B). The same inhibitory effects on Ime1 were observed with a strain where progression through the mitotic cycle depended on Cln1 (data not shown), which agrees with the fact that overexpression of *CLN1* and

CLN2 inhibits meiosis under optimal nutritional conditions for sporulation (see above). These results indicate that G1 cyclins are able to block Ime1 function at both transcriptional and post-translational levels.

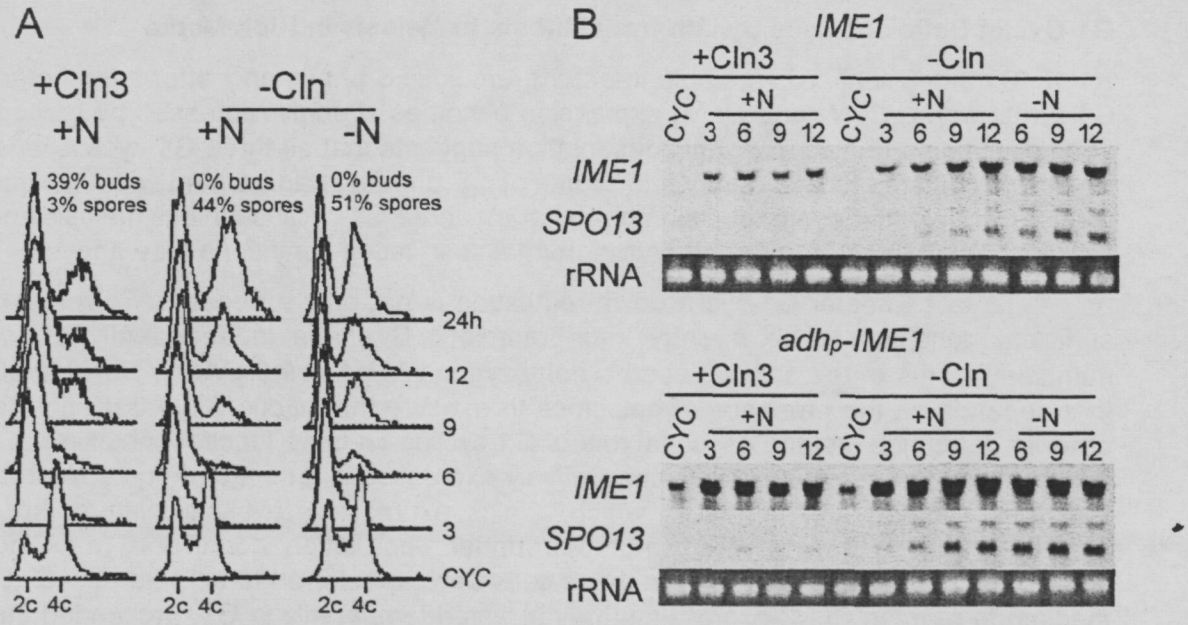

Figure 8. G1-Cyclin Deficient Cells Switch from Mitosis to Meiosis in Rich Media

(A) DNA content distributions of CML353, a homozygous Δ*cln1* Δ*cln2* *GAL1p-CLN3* strain that expresses *IME1* consitutively from pCM284, transformed either with an empty vector (-Cln) or pCM194 (+Cln3), which contains the *CLN3* gene under its own promoter sequences. Samples were obtained during mitotic growth in YPGal medium (CYC) and at different times after transfer to YPA medium (+N) to repress *CLN3* expression from the *GAL1* promoter. After 3h in YPA, a portion of G1-cyclin deficient cells (-Cln) were transferred to sporulation medium (-N) and samples were taken thereafter. Budding and sporulation indexes attained at 24h are indicated.

(B) CML353 cells constitutively expressing (*adhp-IME1*) or not (*IME1*) from plasmid pCM284, and transformed with either an empty vector (-Cln) or pCM194 (+Cln3), were used to determine mRNA levels of *IME1* and *SPO13* by northern blot analysis from samples taken as in (A). The 25S rRNA is shown as a loading control.

Figure 8B shows that both *IME1* and *SPO13* expression levels were very similar in Cln-deficient cells independently of the presence of the nitrogen source, supporting the idea that the essential effect of nitrogen starvation to induce meiosis is the down-regulation of G1 cyclins, which will in turn fully activate Ime1 function.

Not all nutritional requirements could be mimicked by G1-cyclin deprivation. Upon transfer of Δ*cln1* Δ*cln2* *GAL1p-CLN3* cells from galactose to glucose-based rich medium, which also represses *CLN3* expression, they rapidly arrested in G1 but did not undergo meiosis as deduced from DNA content distributions, lack of *SPO13* expression and absence of asci (data not shown). Transcription of *IME1* was not induced under these conditions (data not shown), which is in agreement with the fact that the *IME1* promoter is repressed by fermentable carbon sources such as glucose (Sagee et al., 1998) and galactose (see Figure 8B). In addition, and possibly through Rim15 (Vidan and Mitchell, 1997), glucose inhibits the physical interaction between Ume6 and Ime1 proteins (Malathi et al., 1997), which is essential to activate early-

gene promoters during meiosis. Accordingly, although constitutive expression of *IME1* resulted also in increased Ime1 protein levels in Cln-deprived cells by glucose, it did not allow for any detectable *SPO13* induction (data not shown).

G1-cyclin deficient cells are able to complete meiosis in the presence of nitrogen, which agrees with the fact that glucose but not the presence of a nitrogen source inhibits late steps during meiosis (Lee and Honigberg, 1996). On the other hand, triggering G1-cyclin overexpression late during meiosis did not inhibit sporulation (data not shown), which indicates that G1 cyclins only block the earliest steps of meiosis.

G1 Cyclins Prevent Ime1 Accumulation in the Nucleus

To activate transcription Ime1 must interact with Ume6, a DNA-binding protein that plays a dual role in regulating meiotic early-gene expression. While Ume6 represses early-gene promoters in mitotically active cells, its interaction with Ime1 converts the complex into a transcriptional activator under sporulation conditions (Rubin-Bejerano et al., 1996). This interaction depends on two protein kinases, Rim11 and Rim15 (Rubin-Bejerano et al., 1996; Vidan and Mitchell, 1997). Although Rim15 may transmit some nutritional signals to Ime1 function (absence of fermentable carbon sources such as glucose), no physiological role has yet been established for Rim11. Since we have shown that G1 cyclins inhibit Ime1 function at a post-translational level, we decided to test whether this effect was exerted on the interaction between Ume6 and Ime1. To test this possibility we used the tetracycline-regulatable expression system that we had adapted to yeast (Garí *et al.* 1997) and built a two-hybrid analysis model by fusing the *tetO*-binding domain of the *E. coli* Tet repressor (TetR) to the interaction domain of Ime1 (Ime1id) and, on the other hand, the VP16 transactivator to the interaction domain of Ume6 (Ume6id). Two-hybrid interaction was monitored from a construct where the *E. coli lacZ* gene is under the control of a $tetO_7$-driven promoter (Garí et al., 1997). Similarly to previous work where the Ime1-Ume6 interaction was first shown by two-hybrid analysis (Rubin-bejerano et al., 1996), the presence of both TetR-Ime1id and Ume6id-VP16 constructs in diploid cells gave rise to high β-galactosidase levels (comparable to those obtained with TetR-VP16), but only under sporulation conditions, while these high expression levels were completely dependent on Rim11 (Figure 9A).

We then used wild-type and homozygous *cln3* null mutant strains to determine β-galactosidase activity in cycling and G1-arrested cells in glucose-based or acetate-based media, and under the nitrogen starvation conditions used to induce entry into meiosis. Figure 9B shows that the main nutritional requirement for the Ume6-Ime1 interaction is the presence of a non-fermentable carbon source such as acetate, independently of cell cycle position (cycling *vs.* G1-arrested cells) or the presence or absence of the nitrogen source. Cln3-deficient cells showed a very similar behavior, which indicates that the Ume6-Ime1 interaction is not modulated by the presence of Cln3. Similar negative results were obtained when using *Δcln1 Δcln2 GAL1p-CLN3* cells to analyze the Ume6-Ime1 interaction by two-hybrid analysis in the experimental approach shown in Figure 8 (data not shown). Thus we concluded that G1 cyclins block Ime1 function by mechanisms that seem not to involve its interaction with Ume6.

Ime1 is a nuclear protein under sporulation conditions (Smith et al., 1993) and its localization does not depend on Rim11 (Rubin-Bejerano et al., 1996). By using a constitutively expressed HA-tagged *IME1* gene that fully complements homozygous *ime1* null mutants (data not shown), we determined its cellular localization by immunofluorescence in the *Δcln1 Δcln2 GAL1p-CLN3* strain after transfer to acetate-

rich media. We have shown that, under these conditions, Cln-deficient cells induce Ime1-dependent transcription and enter into premeiotic S phase, while mitotically cycling cells do not. Figure 10 shows that the overall Ime1 protein levels were similar as measured in immunoblots. However, the Ime1 protein did only accumulate in the nuclei of Cln-deficient cells. Mitotically cycling cells could prevent Ime1 accumulation in the nucleus either by G1-cyclin activity or more indirectly by the action of other molecules only present in mitotically active cells. To test these two possibilities we determined the cellular localization of Ime1 in *cdc28-13* cells arrested in G1 with very low Cln-Cdc28 kinase activity (Wittenberg and Reed, 1988; Wittenberg et al., 1990), and *cdc34-2* cells arrested at the G1-S transition with high Cln-Cdc28 levels (Deshaies et al., 1995; Yaglom et al., 1995). As seen in Figure 10, Ime1 was clearly detected in the nuclei of *cdc28-13* cells, while *cdc34-2* cells showed a non-localized signal. Similar results were obtained in the W303 background strain. *SPO13* induction took only place in *cdc28-13* cells (data not shown), indicating that Ime1 accumulation in the nucleus may be a key target for G1-cyclin inhibition of entry into meiosis.

A

β-galactosidase activity (Miller units)

Protein fusions	+C (glucose)	8h -N (acetate)	
	wt	wt	Δ*rim11*
TetR-VP16	221	301	273
TetR-Ime1id	3	5	4
TetR-Ime1id + Ume6id-VP16	9	233	7

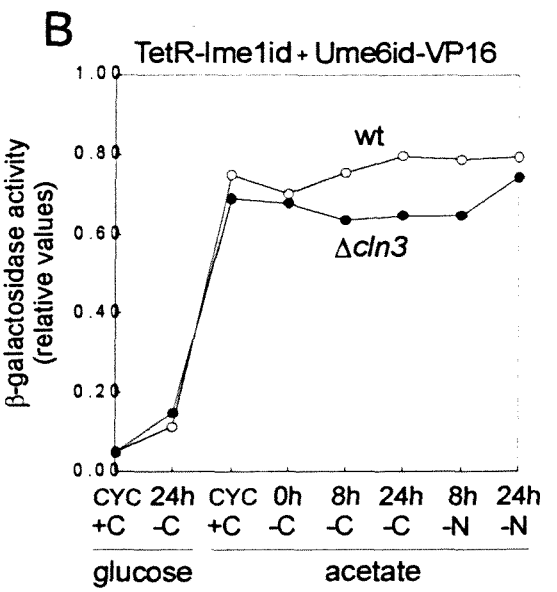

Figure 9. G1 Cyclins Do Not Affect the Ume6-Ime1 Interaction

(A) β-galactosidase activities were determined in wild-type 1788 (wt) and Rim11-deficient CML359 (Δ*rim11*) cells expressing different hybrid proteins from pCM293 (TetR-VP16), pCM295 (TetR-Ime1id) and pCM298 (TetR-Ime1id + Ume6id-VP16). TetR-driven expression of the *lacZ* gene was monitored with plasmid pCM286. Samples were taken from cells growing exponentially in glucose-based minimal media (+C), or 8h after transfer to sporulation conditions (8h -N) following the experimental model described in Figure 1.

(B) β-galactosidase activities were determined in wild-type 1788 (wt, open circles) and Cln3-deficient CML254 (Δ*cln3*, closed circles) cells containing either pCM293 (TetR-VP16) or pCM298 (TetR-Ime1id + Ume6id-VP16). TetR-driven expression of the *lacZ* gene was monitored with plasmid pCM286. Samples were taken from cells either growing exponentially (+C, CYC), or at different times under carbon-source limitation (-C) or nitrogen starvation conditions (-N) in the presence of a fermentable (glucose) or a nonfermentable (acetate) carbon source. β-galactosidase activities determined from pCM298 (TetR-Ime1id + Ume6id-VP16) were made relative to those from pCM293 (TetR-VP16) for each condition.

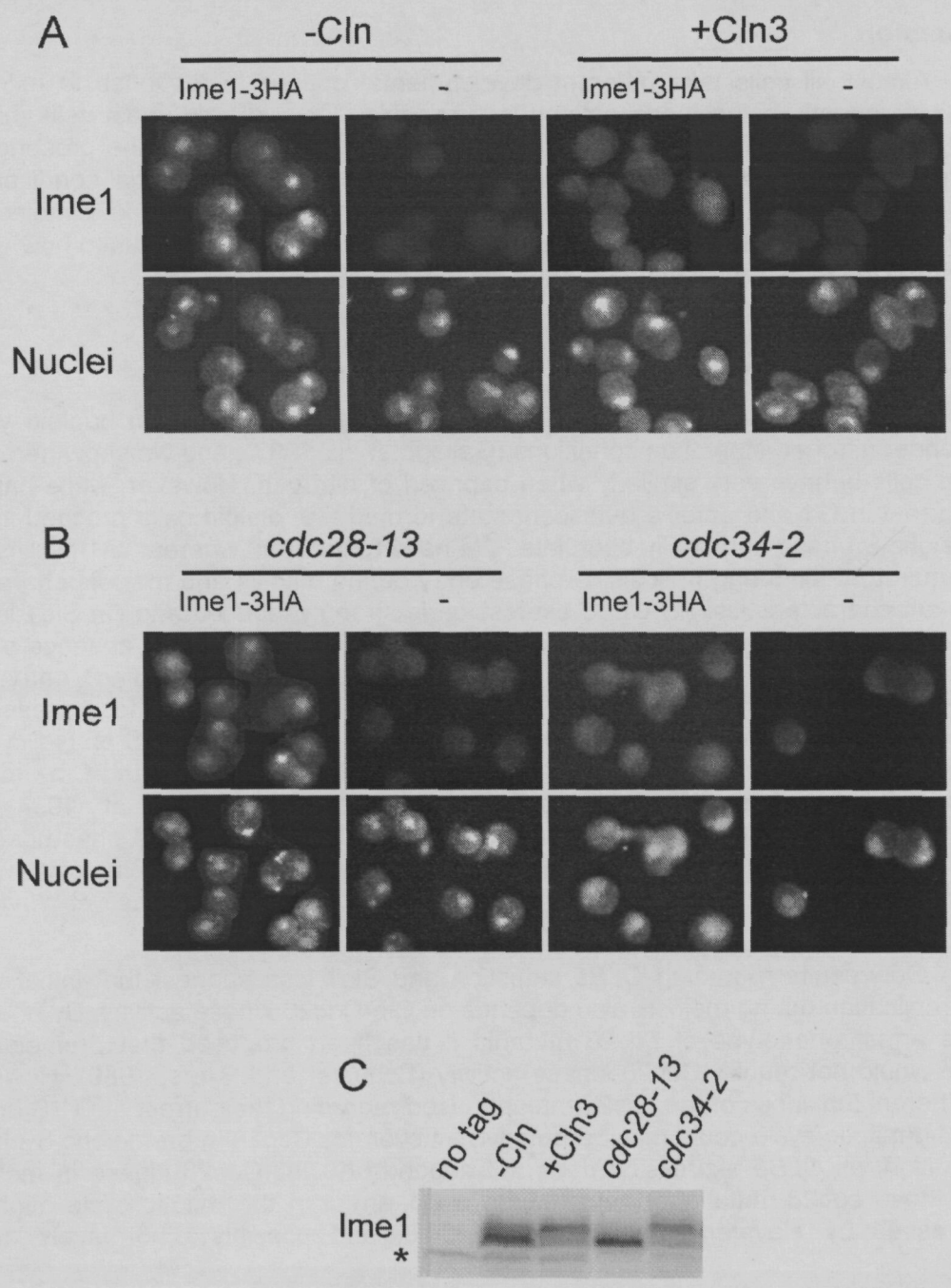

Figure 10. G1 Cyclins Prevent Ime1 Accumulation within the Nucleus

(A) CML353 cells constitutively expressing the 3HA-tagged *IME1* ORF from plasmid pCM284, were transformed with either an empty vector (-Cln) or pCM194 (+Cln3). CML353 cells without pCM284 were used as control (no tag). Samples were taken 9h after transfer to YPA medium as described under Figure 8. The Ime1-3HA protein was visualized by immunofluorescence (Ime1) and nuclei were counterstained with propidium iodide.

(B) Cdc28-thermosensitive CML200 (*cdc28-13*) and Cdc34- thermosensitive CML344 (*cdc34-2*) cells were transformed with an empty vector or plasmid pCM279 to constitutively express the 3HA-tagged *IME1* ORF (Ime1-3HA). Cells were grown in YPA at 25ºC and samples were taken 4h after transfer to 37ºC. The Ime1-3HA protein was localized by immunofluorescence (Ime1) and nuclei were counterstained with propidium iodide.

(C). Ime1 levels were determined by western blot in samples taken as described in (A) and (B). A 12CA5 cross-reactive band (*) serves as a control for loading.

Discussion

Almost all cells take different developmental options in response to external signals, frequently being these options incompatible. Thus, diploid yeast cells initiate either mitosis or meiosis depending on environmental signals. While the presence of nutrients exerts an inducing role for mitosis, their absence is an essential condition for entry into meiosis. This work concerns the relationships between key molecules involved in initiating either mitosis or meiosis, and provides some clues as to how yeast cells make these cell cycle choices incompatible.

Ime1 Replaces G1-Cyclin Function for Entry into Premeiotic S Phase

We have shown that G1 cyclins are down-regulated rapidly in haploid yeast cells under nitrogen starvation conditions (Gallego et al., 1997), and we show here that diploid cells behave very similarly when deprived of nitrogen. However, while haploid cells arrest in G1 and enter a quiescent state termed G0, diploid cells proceed into a premeiotic S phase and finally sporulate. We have found that, whereas G1 cyclins are not required at all during meiosis, S-phase entry during mitosis and meiosis share two important characteristics: (1) *CLB5* expression is strongly induced and (2) Sic1 levels are down-regulated by post-transcriptional mechanisms. Although the absence of G1 cyclins may explain why the meiotic cycle proceeds without budding, entry into premeiotic S phase must be exerted by mechanisms different than those present in mitosis, where Cln3 has a key role in MBF-dependent expression of S-phase cyclins *CLB5* and *CLB6* (Schwob et al., 1993) and, on the other hand, Cln1 and Cln2 trigger degradation of Sic1, the yeast Clb-Cdc28 kinase inhibitor (Schwob et al., 1994). We have found that both *CLB5* induction and Sic1 loss before premeiotic S phase depend on Ime1, a transcriptional activator involved in the earliest steps of meiosis, which would explain why *ime1* mutants arrest before premeiotic S phase (Kassir et al., 1988; Foiani et al., 1996).

Our results regarding *CLB5* induction and Sic1 loss suggest that initiation of DNA replication during meiosis also depends on Clb-Cdc28 kinase activity. By analysis of the arrest phenotype of *cdc28* mutants it has been proposed that premeiotic S phase would not require Cdc28-kinase activity (Schuster and Byers, 1989). However, the different leakiness of the *cdc28* mutants used regarding their arrest at G1-S or G2-M in the mitotic cycle could mask a putative function for Cdc28 in premeiotic S phase. In addition, as *CLB5* expression does not depend on Cln3-Cdc28 kinase in meiosis, even those *cdc28* mutations that produce a G1 arrest in the mitotic cycle might be suppressed by elevated Ime1-dependent Clb5, and possibly Clb6, levels during meiosis.

G1 Cyclins Transmit Essential Nutritional Signals to the Ime1 Pathway to Inhibit Meiosis

The essential nutritional requirements for entry into meiosis in budding yeast are: (1) nitrogen starvation conditions and (2) presence of only a nonfermentable carbon source. Ime1 function has been shown to be regulated by these nutritional signals through different transcriptional and post-transcriptional mechanisms.

Our results indicate that high G1-cyclin levels inhibit *IME1* expression under optimal nutritional conditions for sporulation. Moreover, G1-cyclin deficient cells enter meiosis in acetate-based rich media by increasing *IME1* expression levels regardless of the presence of the nitrogen source, while these cells show no alterations in the induction caused by acetate or in the repression exerted by glucose. In consequence, G1-cyclin deficiency mimicks most of the nitrogen starvation signals for *IME1*

G1-cyclin deficiency mimicks most of the nitrogen starvation signals for *IME1* expression. The *IME1* promoter is (1) activated in the presence of acetate, a nonfermentable carbon source, at the UASrm region, (2) repressed by glucose through Msn2,4 at the IREu sequence, and (3) repressed by nitrogen at the UCS1 region and, to a lesser extent, at IREu (Sagee et al., 1998). *IME1* expression is de-repressed 3 to 4-fold in *swi4* and *swi6* mutants, being this effect attributed to the IREu sequence, which contains an SCB-like motif (Sagee et al., 1998). Similar mechanisms could operate at the UCS1 region, since it also contains SCB-like sequences. Thus, in addition to its role as an activator during the mitotic G1-S transition, SBF could act as a repressor through G1-cyclin dependent mechanisms to inhibit *IME1* expression. Alternatively, other transcriptional factors involved in activating expression from the IREu and UCS1 sequences could be subject to Cln-Cdc28 mediated inhibition.

Overexpression of *IME1* overcomes *MAT*-mediated requirements for entry into meiosis, but only when growth becomes limited in acetate-based media (Kassir et al., 1988; Smith et al., 1990), which indicates that nutrients exert important repressor effects upon Ime1 activity. We show here that G1-cyclin loss activates Ime1 at a post-translational level to increase expression of *SPO13* in acetate-based media, independently of the presence of the nitrogen source, while this effect is not observed in the presence of glucose. As for transcriptional control of *IME1*, G1-cyclin deficiency also mimicks most of the nitrogen starvation signals required for post-translational activation of Ime1.

The Ime1 protein interacts with Ume6 to transactivate early-gene expression and this interaction is mediated by the Rim11 and Rim15 kinases. Many observations indicate that this interaction is a key target for regulation of Ime1 function by the carbon source present, but not by nitrogen starvation signals (Bowdish et al. 1994; Rubin-Bejerano et al., 1996; Malathi et al. 1997; Vidan and Mitchell, 1997). Ume6-Ime1 interaction by two-hybrid analysis is readily detected in cycling cells grown in acetate-based rich media, but not in glucose-based media. Both Ume6-Rim11 and Ime1-Rim11 interactions are detected by coimmunoprecipitation in cycling cells under the presence of the nitrogen source in acetate-based media, while the Ume6-Rim11 interaction is repressed by glucose as deduced from two-hybrid analysis. As *RIM15* expression is repressed by glucose, the Rim15 kinase has also been proposed to transmit carbon-source signals to the Ume6-Ime1 interaction. Using a different two-hybrid approach our results confirm that the Ume6-Ime1 interaction is mainly regulated by the carbon source regardless of nitrogen starvation conditions and cell cycle position, i.e. G1-arrested vs. cycling cells. In addition, we have found that G1-cyclin deficiency does not increase transcription levels driven by the Ume6-Ime1 interaction in acetate-based rich media, whereas glucose-mediated inhibition of Ime1-Ume6 interaction is not relieved by G1-cyclin deficiency. Thus, *IME1* post-translational regulation by G1 cyclins is not exerted at the level of Ime1 protein interaction with Ume6.

Ime1 is a nuclear protein during sporulation (Smith et al., 1993) and its localization does not depend on the Rim11 kinase (Rubin-Bejerano et al., 1996). We have found that Ime1 accumulates in the nucleus of G1-cyclin deficient cells but not in cycling cells growing in acetate-based rich media. As *SPO13* expression is only induced in G1-cyclin deficient cells, Ime1 accumulation in the nucleus may be an important mechanism for entry into meiosis by regulating Ime1 function at a post-translational level. That Cln cyclins have a role in regulating Ime1 localization to the nucleus is supported by the fact that *cdc28*-arrested cells, which contain very low Cln-Cdc28 kinase activity, accumulate Ime1 in their nuclei and express *SPO13*, while *cdc34*-arrested cells, which contain high Cln-Cdc28 levels, do not accumulate Ime1 in their nuclei and show no *SPO13* mRNA detectable levels. Whether Cln-Cdc28 activity

prevents Ime1 accumulation in the nucleus by direct mechanisms remains to be elucidated. However, some data suggest that this may be the case since (1) Ime1 is phosphorylated in vitro by both Cln2 and Cln3 immunoprecipitates with equal efficiency, and (2) similar hyperphosphorylated forms of Ime1 can be detected by IEF in extracts obtained from cycling and *cdc34*-arrested cells but not in extracts from G1-cyclin deficient or *cdc28*-arrested cells (our unpublished results). Phosphorylation is a widespread mechanism used to link environmental and internal signals to the activity of transcriptional factors, often regulating their import to the nucleus. Thus, nuclear import of the Swi5 transcriptional activator is restricted to the G1 phase of the cell cycle in budding yeast (Nasmyth et al., 1990), and the Cdc28 kinase phosphorylates Swi5 at its NLS to prevent its import to the nucleus (Moll et al., 1991). Further work will be required to determine whether Ime1 import to the nucleus is regulated by similar Cdc28-dependent mechanisms.

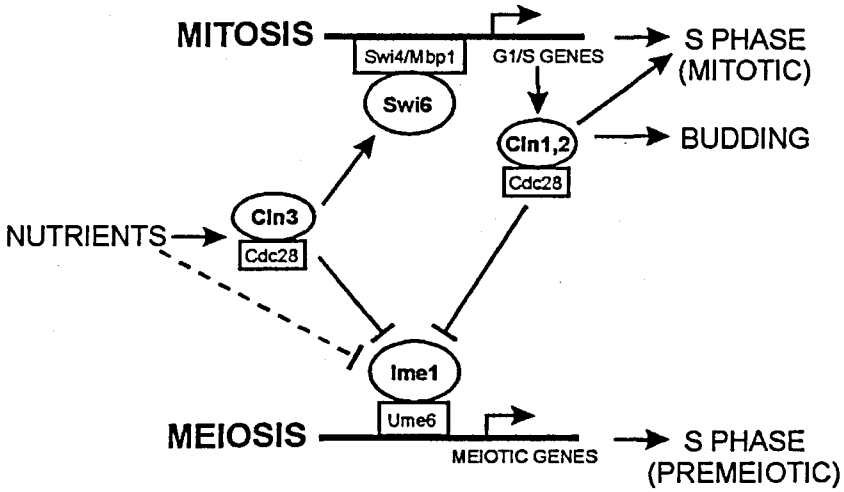

Figure 11. G1 Cyclins, the Key Activators of the Mitotic G1-S Transition, Block the Ime1 Pathway to Inhibit Meiosis.

G1 cyclins transmit essential nutritional signals to the Ime1 pathway. In the presence of nutrients, G1-cyclin levels are high and cells are driven to the mitotic G1-S transition. G1 cyclins have a key role in activating SBF(Swi4/Swi6)- and MBF(Mbp1/Swi6)-dependent gene expression and in the degradation of Sic1, the Clb-Cdc28 kinase inibitor. In addition, high G1-cyclin levels repress *IME1* transcription and prevent the accumulation of Ime1 in the nucleus, thus inhibiting meiotic gene expression. As G1-cyclin levels fall down rapidly under nitrogen starvation conditons, cells arrest mitotic proliferation in G1 and, if in the presence of a nonfermentable carbon source, derepression of Ime1 at both transcriptional and post-translational levels takes place, thus allowing cells to enter premeiotic S phase. The broken line indicates G1-cyclin independent mechanisms that down-regulate Ime1 function also at both transcriptional and post-transcriptional levels by the carbon source.

Mitosis or Meiosis: A Role for G1 Cyclins

It has been known for long that diploid yeast cells switch from mitosis to meiosis under nutrient starvation conditions, and since *IME1* was first isolated (Kassir et al., 1988) a pathway has emerged where different transcriptional and post-transcriptional regulatory mechanisms have been identified that link its activity to nutrient starvation. On the other hand, work done with haploid cells, where nutrient limitation causes a G1 arrest, has evidenced that G1 cyclins are a key target for cell cycle regulation by nutrient availability (Huble et al., 1993; Gallego et al., 1997). Here

we show that (1) G1 cyclins are also down-regulated by nutrient starvation in diploid cells during entry into meiosis, (2) G1 cyclins are not required throughout meiosis, (3) Ime1 replaces G1-cyclin function for entry into premeiotic S phase, (4) Ime1 function is blocked by G1 cyclins by both transcriptional and post-transcriptional mechanisms, and (5) G1-cyclin loss transmits essential nutritional signals to the Ime1 pathway to trigger meiosis. These observations, which are outlined in Figure 11, indicate that yeast cells have developed a negative cross-talk mechanism between the key initiator molecules of mitosis and meiosis. If nutrient availability were to regulate these two processes independently, intermediate nutrient-limitation situations would exist where no option or, much more detrimental for cell survival, both options could be taken. The existence of a negative cross-talk between G1 cyclins and Ime1, although unidirectional, may help explain why mitosis and meiosis are two incompatible choices for yeast cells.

Experimental Procedures

Strains and Plasmids

Table 1 lists the strains used in this work. Our parental diploid 1788 and haploid CML128 (*MAT***a**, *leu2-3,112*, *ura3-52*, *trp1-1*, *his4*, *can1*^{r}) strains have been described (Gallego et al. 1997). Some strains used derive from W303-1A (*MAT***a**, *leu2-3,112*, *ura3-52*, *trp1-1*, *his3-11,75, ade2-1, can1-100*) (N. Lowndes).

Table 1. Yeast Strains

Strain	Genotype	Source
CML128 derivatives		
CML133	*MAT***a**, *LEU2::tTA*	A
CML200	*MAT***a**, *cdc28-13, LEU2::tTA*	A
CML344	*MAT***a**, *cdc34-2, LEU2::tTA*	B
CML257	*MAT***a**/α, *CLN3-3HA/CLN3*	B
CML259	*MAT***a**/α, *CLB5-3HA/CLB5*	B
CML260	*MAT***a**/α, *SIC1-3HA/SIC1*	B
CML262	*MAT***a**/α, *CLN1-3HA/CLN1*	B
CML362	*MAT***a**/α, *CLN2-3HA/CLN2*	B
CML256	*MAT***a**/α, *LEU2::tTA/leu2-3,112*	B
CML337	*MAT***a**/α, *IME1-3HA/IME1-3HA*	B
CML315	*MAT***a**/α, *IME1-3HA/IME1-3HA, LEU2::tTA/leu2-3,112*	B
CML268	*MAT***a**/α, *Δime1::kanMX4/Δime1::kanMX4*	B
CML342	*MAT***a**/α, *Δime1::kanMX4/Δime1::kanMX4, LEU2::tTA/leu2-3,112*	B
CML365	*MAT***a**/α, *Δime1::kanMX4/Δime1::kanMX4, SIC1-3HA/SIC1*	B
CML359	*MAT***a**/α, *Δrim11::kanMX4/Δrim11::kanMX4*	B
CML331	*MAT***a**/α, *Δrim11::kanMX4/Δrim11::kanMX4, LEU2::tTA/leu2-3,112*	B
CML254	*MAT***a**/α, *Δcln3::LEU2/Δcln3::LEU2*	B
CML319	*MAT***a**/α, *Δcln3::LEU2/Δcln3::LEU2, IME1-3HA/IME1-3HA*	B
CML346	*MAT***a**/α, *Δcln3::LEU2/Δcln3::LEU2, Δime1::kanMX4/Δime1::kanMX4*	B
CML353	*MAT***a**/α, *Δcln1::HIS3/Δcln1::HIS3, Δcln2::TRP1/Δcln2::TRP1 GAL1p-CLN3/GAL1p-CLN3*	B
W303 derivatives		
CMY1036	*MAT***a**, *cdc34-2*	C
CML363	*MAT***a**/α, *LEU2::tTA/leu2-3,112*	B

A, Gallego et al., 1997; B, this work; C, C. Mann.

The tetracycline-repressible transactivator tTA was introduced in yeast cells by integration of pCM87 (Garí et al., 1997) at the *LEU2* locus. Chromosomal gene disruptions and C-terminal fusions to the 3HA epitope were obtained by gene transplacement as described (Gallego et al., 1997).

Plasmids pCM165, pCM250 and pCM166 carry the 3HA-tagged *CLN1*, *CLN2* and *CLN3* ORFs, respectively, under the control of the $tetO_2$ promoter (Garí et al., 1997) in YCplac33 (*URA3*, Gietz and Sugino, 1988). The 3HA-tagged *IME1* ORF under the control of the Schiz. pombe *adh* promoter (A. Bueno) was inserted in YCplac22 (*TRP1*, Gietz and Sugino, 1988) and YCplac111 (*LEU2*, Gietz and Sugino, 1988) resulting plasmids pCM279 and pCM284, respectively. The 3HA-tagged *IME1* gene under its own promoter sequences was placed in YCplac22 (*TRP1*; Gietz and Sugino, 1988), resulting plasmid pCM267. The *ime1-2* allele in pCM268 codes for an stop codon at amino acid 13, and was obtained from pCM267 by site-directed mutagenesis (Weiner et al.,1994). Plasmid pCM194 contains the *CLN3* gene under its own promoter (Gallego et al., 1997). The TetR-VP16 (tTA) protein fusion used has already been described (Garí et al., 1997). The TetR-Ime1id hybrid was obtained by fusing the TetR domain from tTA to amino acids 270-360 of Ime1. On the other hand the Ume6id-VP16 hybrid was constructed by fusing amino acids 1-232 from Ume6 to the VP16 domain of tTA. Expression of genes coding for the TetR-VP16 and TetR-Ime1id protein fusions is driven by the CMV promoter (Garí et al., 1997) in plasmids pCM293 and pCM295, respectively, which are both YCplac22 derivatives. Plasmid pCM298 is a derivative of pCM295 that also contains the gene coding for Ume6id-VP16 under the control of the Schiz. pombe *adh* promoter. The *lacZ*-reporter plasmid used to monitor TetR-driven transcription, pCM286, is a YCplac33-based version of pCM159 (Garí et al., 1997). Measurement of β-galactosidase activity in permeabilized cells has been described previously (Garí et al., 1997). Details of strain and plasmid constructions are available upon request.

Growth and Sporulation Conditions

In addition to 2% peptone and 1% yeast extract, YPD, YPGal and YPA contained 2% glucose, 2% galactose and 1% potassium acetate, respectively. Sporulation medium was 0.3% potassium acetate, to which the required amino acids were added to the following final concentrations: 15 μg/ml leucine, 5 μg/ml histidine and 10 μg/ml tryptophan. To obtain acceptable levels of synchrony in premeiotic S-phase entry, the nonstandard pregrowth regimen described by Padmore et al. (1991) was used with only slight modifications. Briefly, cells grown in YPD (with 50 μg/ml uracil for *ura3* strains) for 36-48 hours to reach OD_{600} values of 25-30 were washed, resuspended in YPA at an OD_{600}=0.3, and incubated at 30°C for 20 hours with vigorous agitation to reach an OD_{600} value of 2.5-3. At this point cells were uniformly arrested in G1 by carbon-source limitation, as deduced from both budding indexes and DNA content distributions. To initiate meiosis, cells were then washed, resuspended in sporulation medium at an OD_{600}=1, and incubated at 30°C. Minimal media with 2% glucose or 2% galactose with the required amino acids (Gallego et al., 1997) was substituted for YPD when using strains containing centromeric plasmids. In this case, most cells (>95%) had retained the plasmid after growth to saturation in YPA as deduced from plating efficiencies under selective and non-selective conditions for the plasmid marker used. To obtain samples from cells growing exponentially in YPA medium, cells grown in YPD as above were washed, resuspended at an OD_{600}=0.025 in YPA and incubated 16-20 hours at 30°C, unless otherwise indicated. Tetracycline was added to 2 μg/ml when repression of the tTA transactivator was needed (Garí et al., 1997).

Northern and Western Blot Analysis

Total RNA samples were analyzed by Northern blot as described (Gallego et al. 1997) DNA fragments containing only ORF sequences, either obtained by PCR or restriction digestion, were used to synthesize probes by random-PCR with a digoxigenin-dUTP labeling mixture as directed by Boehringer Mannheim. Western blot analysis of whole cell extracts with the mouse anti-HA antibody (clone 12CA5) from Boehringer Mannheim were performed as described previously (Gallego et al. 1997).

Immunofluorescence

The intracellular localization of the 3HA-tagged Ime1 protein was determined by indirect immunofluorescence techniques essentially as described by Rose et al. (1990). The rat anti-HA antibody (clone 3F10, Boehringer Mannheim) was used at 1 µg/ml, and the FITC-conjugated goat anti-rat antibody (Southern Biotechnology Associates) was used at 10 µg/ml in the presence of 100 µg/ml RNase. Nuclei were stained with 50 µg/ml propidium iodide and fluorescent images were obtained in a Zeiss LSM410 confocal microscope equipped with a 63x/1.4 objective and the required band-pass filters (515-545 nm for FITC, and 575-640 nm for propidium iodide).

Flow Cytometry and Morphological Determinations

DNA content distributions were obtained by propidium iodide staining as described (Nash et al., 1988) with an Epics XL flow cytometer (Coulter). Budding and sporulation percentages were obtained under a phase-contrast microscope by inspecting a minimum of 200 cells that had been fixed in 1% formaldehyde, 1xSSC, and sonicated for 5 sec.

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