SIT4 protein phosphatase is required for the normal accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs during late G_1

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In *Saccharomyces cerevisiae*, the RNA levels of the G_1 cyclins *CLN1*, *CLN2*, and *HCS26* increase dramatically during the late G_1 phase of the cell cycle. The *SIT4* gene, which encodes a serine/threonine protein phosphatase, is required for the normal accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs during late G_1. This requirement for *SIT4* in normal G_1 cyclin RNA accumulation is at least partly via *SWI4*. Strains containing mutations in *SIT4* are sensitive to the loss of either *CLN2* or *CLN3* function. At the nonpermissive temperature, temperature-sensitive *sit4* strains are blocked for both bud emergence and DNA synthesis. Heterologous expression of *CLN2* in the absence of *SIT4* function results in DNA synthesis, but most of the cells are still blocked for bud emergence. Therefore, *SIT4* is required for at least two late G_1 or G_1/S functions: the normal accumulation of G_1 cyclin RNAs (which is required for DNA synthesis) and some additional function that is required for bud emergence or cell cycle progression through late G_1 or G_1/S.

[*Key Words*: Protein phosphatase; SIT4; G_1 cyclins; SWI4; G_1 regulation]

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The *Saccharomyces cerevisiae SIT4* gene was originally identified by *sit4* mutations that give increased transcription of the *HIS4* gene in the absence of BAS1, BAS2, and GCN4 (Arndt et al. 1989). BAS1, BAS2, and GCN4 (which bind directly to the *HIS4* promoter) are normally required for transcription of *HIS4*. The *SIT4* gene predicts a protein that is 55% identical to the mammalian type 2A and 43% identical to the mammalian type 1 protein phosphatase catalytic subunits. The SIT4 protein phosphatase is a unique phosphatase that is distinct from the *S. cerevisiae* type 1 and type 2A phosphatases (Sutton et al. 1991a,b).

Whether or not the *SIT4* gene is essential depends on a second gene, termed *SSD1/SRK1* (Sutton et al. 1991a; Wilson et al. 1991). The *SSD1* (suppressor of sit4 deletion) locus is polymorphic in that some laboratory strains contain a *ssd1-d* allele of *SSD1* and other laboratory strains contain a *SSD1-v* allele of *SSD1* (Sutton et al. 1991a). Deletion of *SIT4* is lethal in strains containing a *ssd1-d* (d = dead in combination with Δ*sit4*) allele or a deletion allele of *SSD1*. In contrast, strains containing a deletion of *SIT4* are viable, but with a slow growth rate, if they have a *SSD1-v* (v = viable in combination with Δ*sit4*) allele of *SSD1*. Deletion of *SSD1* by itself causes only subtle phenotypic alterations (Sutton et al. 1991a). That a Δ*sit4 SSD1-v* strain is viable allows us to determine the effect due to the complete absence of *SIT4*. An additional effect of the *SSD1* gene is that our standard temperature-sensitive *SIT4* allele, *sit4-102*, results in a temperature-sensitive phenotype only in *ssd1-d* backgrounds.

At the nonpermissive temperature, temperature-sensitive *sit4* strains arrest in late G_1 as growing cells with no bud, a 1n or near 1n DNA content, a single nucleus, a single microtubule organizing center, and an unduplicated spindle pole body (Sutton et al. 1991a). The point in the cell cycle where SIT4 executes its functions is in late G_1 (Sutton et al. 1991a). Parallel experiments show that the execution point of the CDC28 protein kinase is very close to the time in G_1 during which SIT4 is required (Sutton et al. 1991a). The requirement for SIT4 in late G_1, combined with the genetic interactions between mutations in *SIT4* with mutations in the *CDC28* protein kinase gene (Sutton et al. 1991a), led us to examine the role of *SIT4* for expression of the G_1 cyclins.

The original set of *S. cerevisiae* G_1 cyclins included *CLN1*, *CLN2*, and *CLN3* (Cross 1988; Nash et al. 1988; Hadwiger et al. 1989). A strain containing mutations in any two of these *CLN* genes is viable. However, a strain is inviable in the absence of *CLN1*, *CLN2*, and *CLN3* function (Richardson et al. 1989). In addition, *cln1 cln2*

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cells that are limited for CLN3 have a cell cycle arrest as growing G_1 cells (Richardson et al. 1989; Cross 1990). The G_1 cyclins interact with the CDC28 kinase (Wittenberg et al. 1990; Tyers et al. 1992) and are required for the late G_1 activation of the CDC28 kinase for progression through Start (the commitment step for entry into the cell cycle) (Cross 1990; Wittenberg et al. 1990). The *HCS26* gene, which encodes a possible additional G_1 cyclin, was recently identified as a high copy number suppressor that allows growth of a *swi4* mutant strain under nonpermissive conditions (Ogas et al. 1991). The *SWI4* gene product may function as a direct DNA-binding factor for the activation of *CLN1*, *CLN2*, and *HCS26* transcription (Nasmyth and Dirick 1991; Ogas et al. 1991).

The RNA levels of *CLN3* undergo relatively modest variations during the cell cycle (this paper, Nash et al. 1988). In contrast, the RNA levels of *CLN1* and *CLN2* undergo large variations during the cell cycle (Wittenberg et al. 1990). When cells first enter G_1, they have very low levels of *CLN1* and *CLN2* RNAs. However, during late G_1, the *CLN1* and *CLN2* RNA levels increase very rapidly and then decrease as the cells progress through S phase. The rate of accumulation of *CLN1* and *CLN2* RNAs determines, at least in part, the timing of Start (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). The rapid increase in the levels of *CLN1* and *CLN2* RNAs in G_1 may occur by a positive feedback loop that requires both the *CDC28* kinase and the G_1 cyclins themselves (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). Therefore, the mechanism controlling the rate of *CLN1* and *CLN2* RNA accumulation is of great importance for control of entry into the cell cycle. In this report we show that the *SIT4* protein phosphatase is also required for the normal accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs during late G_1. In contrast, *SIT4* is not required for transcription of *CLN3*. We also show that the *SIT4* phosphatase has a function in addition to the late G_1 accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs. This additional function of SIT4 is essential in *ssd1-d* backgrounds.

Results

A sit4-102 *strain arrests with low levels of* CLN1, CLN2, *and* HCS26 *RNAs*

S. cerevisiae cells in the G_1 phase of the cell cycle have much higher levels of *CLN1*, *CLN2*, and *HCS26* RNAs as compared with the overall levels of these RNAs in an asynchronous population of cells (Fig. 1). In contrast, the levels of *CLN3* RNA are similar in G_1 cells as compared with asynchronous cells (Fig. 1). These results agree with previous findings that the levels of *CLN1* and *CLN2* RNAs vary greatly during the cell cycle, reaching maximal level in late G_1 (Wittenberg et al. 1990) but that the levels of *CLN3* RNA vary modestly during the cell cycle (Nash et al. 1988).

When a *cdc28-13* strain or a *sit4-102 ssd1-d2* strain is arrested for 4 hr at 37.5°C, the levels of *CLN1*, *CLN2*, and *HCS26* RNAs are much lower than in G_1 cells (Fig. 1).

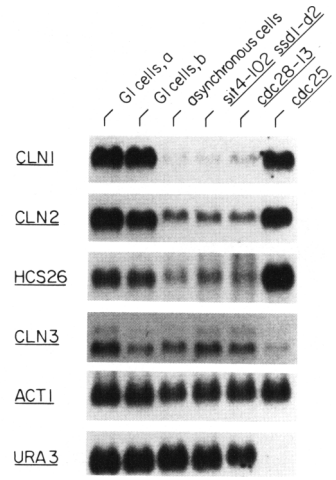

Figure 1. The levels of G_1 cyclin RNA in *sit4-102-*, *cdc28-13-*, and *cdc25*-arrested cells. Strains AY926 (lane 3; wild-type *SIT4* isogenic to CY146), CY146 (lane 4; *sit4-102 ssd1-d2*), CY12 (lane 5; *cdc28-13*), and CY11 (lane 6; *cdc25-1*) were grown at 24°C to an OD_{600} of ~0.2. The cultures were then shifted to 37.5°C and grown for 4 hr before collection of the cells. Lanes *1* and *2* use RNA prepared from two different populations of G_1 phase-enriched cells. For lane *1*, RNA was prepared from strain CY1518 (*cdc15-2*) grown for 3 hr at 37°C (which arrests the cells in late mitosis) and then shifted to a 24°C water bath and grown for 150 min (at this time, the cells are in the second cycle G_1 after release). For lane *2*, strain AY925 (isogenic to AY926 but *MAT***a**) was arrested in G_1 by treatment with α-factor for 3.5 hr. The cells were filtered, washed, resuspended in fresh medium, and grown for 100 min before collection of the cells (which are in the second cycle G_1 after α-factor release). YEPD medium was used for all cultures. For all samples, total RNA was prepared and 8 μg was loaded into each lane. The *URA3* signal is absent for strain CY11 (*cdc25-1*) owing to a deletion allele of *URA3* present in this strain.

The levels of these RNAs in the *cdc28-13-* or the *sit4-102*-arrested G_1 cells are similar to the low overall levels of these RNAs in an asynchronous population of cells (Fig. 1). In contrast, the levels of *CLN3* RNA in the *cdc28-13-* or the *sit4-102*-arrested cells are similar to the levels in the G_1 population of cells (Fig. 1). These results with the *cdc28-13* strain agree with those of Cross and Tinkelenberg (1991) and with those of Dirick and Nasmyth (1991) in that the CDC28 kinase is required for transcription of *CLN1* and *CLN2* but not *CLN3*. In addition, these results suggest that the *SIT4* protein phosphatase is also required for the accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs but not for the accumulation of *CLN3* RNA. At the *cdc28-13* arrest point or at the *sit4-102* arrest point, transcription of *CLN3* probably continues because the *CLN3* RNA is very unstable (half-life of <2 min; Tyers et al. 1991, 1992). Alternatively, but less likely, *CLN3* RNA could be specifically stabilized at the *sit4* and *cdc28* arrest points. Moreover, the *cdc28-13* strain and the *sit4-102 ssd1-d2* strain have normal levels of *ACT1* and *URA3* RNAs under these arrest conditions.

As a control for the levels of *CLN1*, *CLN2*, and *HCS26* RNAs in cells arrested in G_1 for 4 hr at 37.5°C, we used

a temperature-sensitive *cdc25* strain. Strains containing temperature-sensitive mutations in *CDC25* (which is required to activate the cAMP-dependent protein kinases) arrest at the nonpermissive temperature in G_1 (or a G_1-like state), where the overall level of protein synthesis is very low (Pringle and Hartwell 1981). When *cdc25-1* cells are arrested for 4 hr at 37.5°C, the levels of *CLN1*, *CLN2*, and especially *HCS26* RNAs are high (Fig. 1). Therefore, the cell cycle block in *cdc25-1* mutants at the nonpermissive temperature is not the result of failure to transcribe *CLN1*, *CLN2*, and *HCS26* (and high levels of G_1 cyclin RNAs are not sufficient to execute Start). The high levels of *CLN1*, *CLN2*, and *HCS26* RNAs for the *cdc25-1* strain show that the arrest conditions (4 hr at 37.5°C) used for the *sit4-102* and *cdc28-13* strains should allow *CLN1*, *CLN2*, and *HCS26* transcripts to accumulate to high levels if these genes were transcribed. Interestingly, *CLN3* RNA levels may be partially dependent on *CDC25* (Fig. 1).

SIT4 *is required for the normal late* G_1 *accumulation of* CLN1, CLN2, *and* HCS26 *RNAs*

When MATa cells are treated with α-factor, the levels of *CLN1* and *CLN2* RNAs are repressed while the levels of *CLN3* RNA are slightly induced (Nash et al. 1988; Wittenberg et al. 1990). After release from α-factor arrest, the levels of *CLN1* and *CLN2* RNAs increase dramatically (Wittenberg et al. 1990). We investigated the requirement of *SIT4* for this increase in *CLN1* and *CLN2* RNA levels after α-factor release using two different *sit4* mutant strains: a *sit4-102 ssd1-d1* strain arrested with α-factor at 30°C and then shifted to 37°C after α-factor release or a Δ*sit4 SSD1-v1* strain arrested with α-factor at 30°C and maintained at 30°C after α-factor release. Each of these strains was compared with an isogenic wild-type *SIT4* strain.

When wild-type cells (*SIT4 ssd1-d1*) are released from α-factor arrest and shifted to 37°C (time 0, Fig. 2A), the levels of *CLN1*, *CLN2*, and *HCS26* RNAs are initially very low. At 30 min after release, the levels of *CLN1*, *CLN2*, and *HCS26* RNAs increase dramatically to very high levels and the cells have not yet formed a visible bud (Fig. 2A). During later times after α-factor release, the levels of *CLN1*, *CLN2*, and *HCS26* RNAs decrease and the cells form a visible bud (Fig. 2A). Therefore, similar to the results of Wittenberg et al. (1990), maximal levels of *CLN1*, *CLN2*, and *HCS26* RNAs are achieved in late G_1 before visible bud formation.

When *sit4-102 ssd1-d1* cells are released from α-factor arrest and shifted to 37°C, the maximal levels of *CLN1*, *CLN2*, and *HCS26* RNAs are not achieved until 45 min after α-factor release (Fig. 2B). These maximal levels of *CLN1*, *CLN2*, and *HCS26* RNAs are much lower than the maximal levels of the same RNAs for the *SIT4 ssd1-d1* strain. Even 4 hr after α-factor release, <10% of the *sit4-102 ssd1-d1* cells form a visible bud. Therefore, in a *ssd1-d* background, *SIT4* is required for the normal accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs during late G_1. In contrast, *SIT4* function is not required for *CLN3* transcription (Fig. 2). The levels of *ACT1* RNA are used as controls.

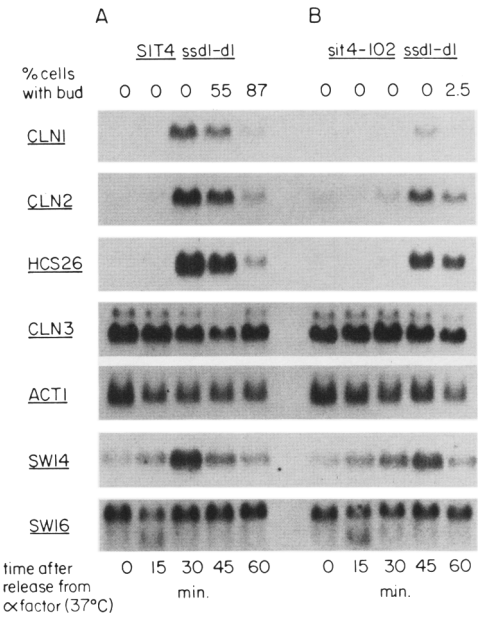

Figure 2. The effect of *sit4-102* on G_1 cyclin and *SWI4* RNA accumulation. Strains CY1176 (*A*, *SIT4 ssd1-d1*) and CY1178 (*B*, *sit4-102 ssd1-d1*) are isogenic except for *SIT4*. These strains were grown exponentially at 24°C in SC minus leucine medium. At an OD_{600} of ~0.20, α-factor was added to 0.015 mM and the cells were grown at 30°C. After growing for 3.5 hr, the cultures were filtered in parallel. The cultures were then washed with SC minus leucine medium, resuspended in fresh SC minus leucine medium, and shifted to 37°C. The filtration, washing, and resuspension were completed in <3 min. Samples were collected at the indicated times after the shift to 37°C. Total RNA was prepared, and 8 μg was loaded into each lane. The probes used for Northern analysis are listed in Materials and methods.

To further examine the role of *SIT4* for accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs, we determined the levels of these RNAs for a Δ*sit4 SSD1-v1* strain after release from α-factor arrest. Yeast cells can survive (but with a slow growth rate) in the absence of *SIT4* if they have a *SSD1-v* allele of *SSD1* (Sutton et al. 1991a). When a *SIT4 SSD1-v1* strain is released from α-factor arrest (at 30°C), the levels of *CLN1*, *CLN2*, and *HCS26* increase dramatically and achieve their maximal levels ~10 min after release (Fig. 3A). By 30 min, the levels of *CLN1*, *CLN2*, and *HCS26* RNAs have decreased and 88% of the cells have budded. In contrast, when the Δ*sit4 SSD1-v1* strain is released from α- factor arrest (at 30°C), the *CLN1*, *CLN2*, and *HCS26* RNAs do not reach their maximal levels until 20 min after α-factor release (Fig. 3B). In addition, the maximal levels of these RNAs are lower for the Δ*sit4 SSD1-v1* strain than for the *SIT4 SSD1-v1* strain (Fig. 3). Therefore, the absence of *SIT4* results in both a delay in the accumulation of and lower maximal levels of *CLN1*, *CLN2*, and *HCS26* RNAs. In contrast,

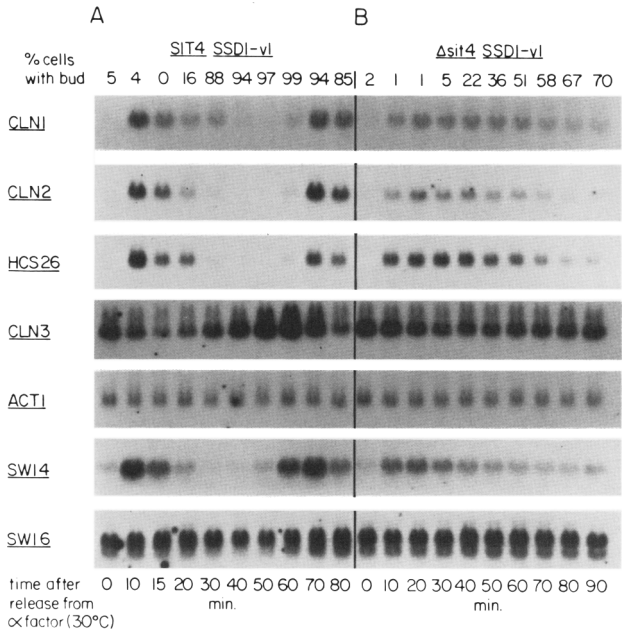

Figure 3. The effect of the deletion of *SIT4* on G_1 cyclin and *SWI4* RNA accumulation. Strains CY1394 (*A*, *SIT4 SSD1-v1*) and CY1393 (*B*, Δ*sit4*::*HIS3 SSD1-v1*) are isogenic except for *SIT4*. Strains were grown exponentially at 30°C in SC minus leucine medium. At an OD_{600} of ~0.20, α-factor was added to 0.0045 mM. After growing for 3.5 hr, the cultures were filtered in parallel. The cells were then washed with SC minus leucine medium, resuspended in fresh SC minus leucine medium, and grown at 30°C. Samples were collected at the indicated times after resuspension of the cells in fresh medium. Total RNA was prepared, and 5 μg was loaded into each lane. The probes used for Northern analysis are listed in Materials and methods.

SIT4 is not required for normal *CLN3* RNA levels (Fig. 3). Note, however, that *CLN3* RNA levels for the *SIT4* strains are somewhat cell cycle regulated and reach their maximum before *CLN1*, *CLN2*, and *HCS26* RNA levels reach their maximum (Figs. 2 and 3).

A Δ sit4 SSD1-v1 *strain requires* CLN2 *and* CLN3, *which are normally dispensable*

As shown above, a Δ*sit4 SSD1-v1* strain (compared with an isogenic *SIT4 SSD1-v1* strain) takes longer to accumulate *CLN1*, *CLN2*, and *HCS26* RNAs, and the maximal levels to which these RNAs accumulate are also lower. Therefore, Δ*sit4 SSD1-v1* cells might be sensitive to partial loss of CLN activity. To test this hypothesis, we crossed a Δ*sit4 SSD1-v1* strain to three different strains, each containing a disruption or deletion of either *CLN1*, *CLN2*, or *CLN3*. Normally, wild-type *SIT4* cells that contain a single mutation in either *CLN1*, *CLN2*, or *CLN3* have a doubling time that is the same as, or very close to, that of wild-type cells (Nash et al. 1988; Hadwiger et al. 1989).

When a Δ*sit4 SSD1-v1* strain is crossed to a strain containing a disruption of *CLN1*, the Δ*sit4 SSD1-v1* *cln1::TRP1* progeny grow at the same rate as the Δ*sit4 SSD1-v1* progeny (Fig. 4A). However, when a Δ*sit4 SSD1-v1* strain is crossed to a strain containing a disruption of *CLN2*, the Δ*sit4 SSD1-v1* *cln2::TRP1* progeny grow much slower than the Δ*sit4 SSD1-v1* progeny (Fig. 4B). In addition, when a Δ*sit4 SSD1-v1* strain is crossed to a strain containing a deletion of *CLN3*, the Δ*sit4 SSD1-v1* Δ*cln3* progeny grow extremely slowly compared with the Δ*sit4 SSD1-v1* progeny (Fig. 4C). The Δ*sit4 SSD1-v1* Δ*cln3* colonies are barely visible in Figure 4C.

The genetic interaction between mutations in *SIT4* and *CLN3* provides further support for a model where *SIT4* is required for normal transcription of *CLN1* and *CLN2* (for an explanation of the genetic interactions between *SIT4* and *CLN2*, see Discussion). Wild-type cells require at least one of the three *CLN* genes for viability (Richardson et al. 1989). In the absence of *SIT4* (a Δ*sit4 SSD1-v1* strain), the levels of *CLN1* and *CLN2* (and *HCS26*) RNAs are lower than normal. Therefore, our model predicts that Δ*sit4 SSD1-v1* cells should be dependent on the function of *CLN3*, whose transcription does not depend on *SIT4*. That Δ*sit4 SSD1-v1* Δ*cln3* cells are almost inviable supports the model. A further prediction of the model is that if *CLN2* RNA is expressed from a promoter that does not depend on *SIT4*, then *CLN3* should be dispensable in a Δ*sit4 SSD1-v1* strain. To test this prediction, the diploid from the cross of the Δ*sit4 SSD1-v1* strain to the Δ*CLN3* strain was transformed with a low copy number *LEU2*/centromere (cen) plasmid containing the *CLN2*-coding sequences expressed from the *Schizosaccharomyces pombe ADH* promoter (Nasmyth and Dirick 1991). In *S. cerevisiae*, this promoter gives low-level expression of *CLN2* that is not *SIT4* dependent. The haploid progeny from the transformed diploid show that Δ*sit4 SSD1-v1* Δ*cln3* [*S. pombe ADH:CLN2* on *LEU2*/cen] strains are fully viable and grow at about the same rate as Δ*sit4 SSD1-v1* strains (Fig. 4D). Furthermore, Leu$^-$ segregants of Δ*sit4 SSD1-v1* Δ*cln3* [*S. pombe ADH:CLN2* on *LEU2*/cen] strains grow at extremely reduced rates (equivalent to the Δ*sit4 SSD1-v1* Δ*cln3* strain in Fig. 4C). Therefore, the requirement of a Δ*sit4 SSD1-v1* strain for *CLN3* is completely eliminated if *CLN2* is expressed from a promoter that is not *SIT4* dependent.

SIT4 *is required for the normal late* G_1 *accumulation of* SWI4 *RNA*

The *SWI4* and *SWI6* genes encode proteins that function as transcription factors for *CLN1*, *CLN2*, and *HCS26* expression (Nasmyth and Dirick 1991; Ogas et al. 1991). Although the levels of *SWI6* RNA vary only slightly during the cell cycle, the levels of *SWI4* RNA are cell cycle regulated (Breeden and Mikesell 1991) and begin to accumulate just before *CLN1*, *CLN2*, and *HCS26* RNAs (see Figs. 2 and 3). Therefore, it is possible that the requirement of *SIT4* for the normal accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs could be the result of the requirement of *SIT4* for the accumulation of *SWI4* and/or *SWI6* RNAs.

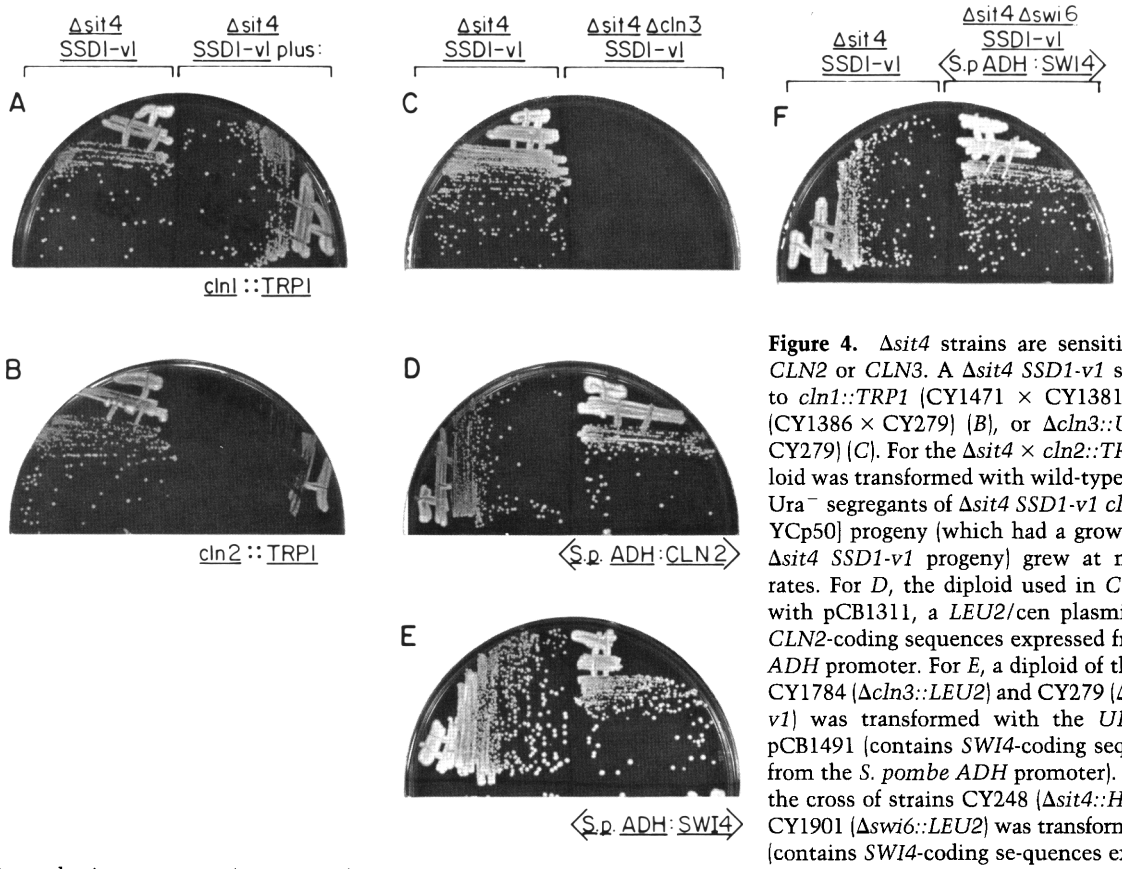

Figure 4. Δ*sit4* strains are sensitive to the loss of *CLN2* or *CLN3*. A Δ*sit4 SSD1-v1* strain was crossed to *cln1::TRP1* (CY1471 × CY1381) (*A*), *cln2::TRP1* (CY1386 × CY279) (*B*), or Δ*cln3::URA3* (CY1507 × CY279) (*C*). For the Δ*sit4* × *cln2::TRP1* cross, the diploid was transformed with wild-type *CLN2* on YCp50. Ura$^-$ segregants of Δ*sit4 SSD1-v1 cln2::TRP1* [*CLN2*/YCp50] progeny (which had a growth rate similar to Δ*sit4 SSD1-v1* progeny) grew at markedly reduced rates. For *D*, the diploid used in *C* was transformed with pCB1311, a *LEU2*/cen plasmid containing the *CLN2*-coding sequences expressed from the *S. pombe ADH* promoter. For *E*, a diploid of the cross of strains CY1784 (Δ*cln3::LEU2*) and CY279 (Δ*sit4::HIS3 SSD1-v1*) was transformed with the *URA3*/cen plasmid pCB1491 (contains *SWI4*-coding sequences expressed from the *S. pombe ADH* promoter). For *F*, a diploid of the cross of strains CY248 (Δ*sit4::HIS3 SSD1-v1*) and CY1901 (Δ*swi6::LEU2*) was transformed with pCB1491 (contains *SWI4*-coding se-quences expressed from the *S. pombe ADH* promoter). For *A–F*, the diploids were sporulated. Tetrads (at least 40 for each cross) were dissected, and the spores were germinated for at least 7 days at 30°C. Shown are colonies of representative haploid progeny on YEPD plates grown at 30°C for 4 days (*A,B,C,D,F*) or for 4.5 days (*E*).

The levels of *SWI6* RNA are not altered in either a *sit4-102 ssd1-d1* strain at 37°C (compared with an isogenic *SIT4* strain; Fig. 2) or a Δ*sit4 SSD1-v1* strain at 30°C (compared with an isogenic *SIT4* strain; Fig. 3). In contrast, the levels of *SWI4* RNA are reduced in the absence of functional SIT4. After release from α-factor arrest, both the *sit4-102 ssd1-d1* strain (at 37°C, compared with an isogenic *SIT4 ssd1-d1* strain; Fig. 2) and the Δ*sit4 SSD1-v1* strain (at 30°C, compared with an isogenic *SIT4 SSD1-v1* strain; Fig. 3) take longer to achieve the maximal levels of *SWI4* RNA and these maximal levels are lower.

The requirement of SIT4 *for accumulation of* CLN1, CLN2, *and* HCS26 *RNAs is at least partly via* SWI4

As shown above, *SIT4* is required for maximal accumulation of *SWI4* RNA levels during late G_1. To demonstrate that *SIT4* is required for *SWI4* function in vivo, we looked at two genetic interactions involving mutations in *SWI4*: the near lethality of mutations in *SWI4* combined with mutations in *CLN3* and the lethality of mutations in *SWI4* combined with mutations in *SWI6*. As shown by Nasmyth and Dirick (1991), deletion of *SWI4* combined with deletion of *CLN3* causes a very slow growth rate. We have found similar results in our strain backgrounds (data not shown). The very slow growth rate of Δ*swi4* Δ*cln3* strains presumably results from lower levels of *CLN1*, *CLN2*, and *HCS26* RNAs (owing to deletion of *SWI4*) combined with the absence of CLN3 (owing to deletion of *CLN3*). Indeed, transcription of *CLN2*-coding sequences from the *S. pombe ADH* promoter causes a Δ*swi4* Δ*cln3* strain to grow at almost wild-type rates (data not shown). As shown above, deletion of *SIT4* (which is normally viable in a *SSD1-v1* background) results in near inviability in combination with deletion of *CLN3* (Fig. 4C). This extremely slow growth rate is also probably the result of lower levels of *CLN1*, *CLN2*, and *HCS26* RNAs (owing to deletion of *SIT4*) combined with the absence of CLN3 (owing to deletion of *CLN3*) because transcription of *CLN2* from the *S. pombe ADH* promoter restores the growth rate of a Δ*sit4 SSD1-v1* Δ*cln* strain to that of a Δ*sit4 SSD1-v1* strain (Fig. 4D). Therefore, mutations in *SIT4* are similar to mutations in *SWI4* with respect to their interactions with mutations in *CLN3*. To determine whether the near inviability caused by deletion of *SIT4* in a Δ*cln3* strain is the result of a defect in *SWI4* transcription, we

transformed a Δ*sit4*/+ Δ*cln3*/+ diploid with a *URA3* plasmid containing the *SWI4*-coding sequences transcribed from the *S. pombe ADH* promoter. The progeny from this diploid show that transcription of *SWI4*-coding sequences from a promoter that is not *SIT4* dependent restores the growth rate of a Δ*sit4 SSD1-v1* Δ*cln* strain to that of a Δ*sit4 SSD1-v1* strain (Fig. 4E). Furthermore, Ura$^-$ segregants of the Δ*sit4 SSD1-v1* Δ*cln3* [*S. pombe ADH:SWI4* on *URA3*/cen] strains grow at extremely reduced rates (equivalent to the Δ*sit4 SSD1-v1* Δ*CLN3* strain in Fig. 4C). The transcription of *SWI4* (or *CLN2*) from a *SIT4*-independent promoter does not restore the growth rate of a Δ*sit4 SSD1-v1* Δ*cln3* strain or a Δ*sit4 SSD1-v1* strain to wild-type levels because *SIT4* is required for a function or functions in addition to the normal accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs (see below).

For a second test of the requirement of *SIT4* for *SWI4* function, we looked at the interaction of mutations in *SWI4* or *SIT4* with mutations in *SWI6*. Mutations in *SWI4* are lethal in combination with mutations in *SWI6* (Breeden and Nasmyth 1987). If *SIT4* is required for *SWI4* function, then a Δ*sit4* Δ*swi6* strain should also be inviable. To test this possibility, we crossed a Δ*sit4::HIS3 SSD1-v1* strain to a Δ*swi6::LEU2* strain. In 80 tetrads, no His$^+$ Leu$^+$ (Δ*sit4* Δ*swi6*) progeny were obtained. However, when the Δ*sit4*/+ Δ*swi6*/+ diploid was transformed with a *URA3* plasmid containing the *SWI4*-coding sequences transcribed from the *S. pombe ADH* promoter, viable His$^+$ Leu$^+$ (Δ*sit4* Δ*swi6 SSD1-v1*) progeny were obtained (Fig. 4F). All of the His$^+$ Leu$^+$ (Δ*sit4* Δ*swi6 SSD1-v1*) progeny contained the *S. pombe ADH:SWI4 URA3*/cen plasmid (they were Ura$^+$). In addition, these progeny were not able to grow in the absence of the *S. pombe ADH:SWI4 URA3*/cen plasmid. That the lethality of a Δ*sit4* Δ*swi6* strain is cured by transcribing *SWI4* from a *SIT4*-independent promoter demonstrates further that *SWI4* function requires *SIT4*.

SIT4 *and* CLN3 *provide additive pathways for the accumulation of* CLN1, CLN2, *and* HCS26 *RNAs*

Efficient transcription of *CLN1* and *CLN2* was shown previously to depend on at least one functional *CLN* gene: either *CLN1*, *CLN2*, or *CLN3* (Cross and Tinkelenberg 1991). Therefore, *CLN3* can stimulate the accumulation of *CLN1* and *CLN2* RNAs. As shown above, the *SIT4* gene is also providing an activation pathway for the accumulation of *CLN1* and *CLN2* RNAs. As shown in Figure 1, *sit4-102 ssd1-d2 CLN3* cells arrested in G_1 after 4 hr at the nonpermissive temperature have low levels of *CLN1*, *CLN2*, and *HCS26* RNAs. To determine whether this *SIT4*-independent low level of *CLN2* and *HCS26* RNAs is *CLN3* dependent, we compared the levels of these RNAs for a *sit4-102 ssd1-d1 CLN3* strain and isogenic *sit4-102 ssd1-d1* Δ*cln3* strains. At the permissive temperature (24°C), these *CLN3* and Δ*CLN3* strains have very similar levels of *CLN2* and *HCS26* RNA (data not shown). Therefore, the contribution of *CLN3* to the transcription of *CLN2* and *HCS26* is not observable when *SIT4* is functional (24°C), which agrees with the results of Cross and Tinkelenberg (1991). However, at the nonpermissive temperature (37°C), the low *SIT4*-independent levels of *CLN2* and *HCS26* RNAs seen for the *sit4-102 ssd1-d1 CLN3* strain are reduced further if the cells also have a deletion of *CLN3* (Fig. 5A). These data suggest that *SIT4* and *CLN3* provide additive pathways for the accumulation of *CLN2* and *HCS26* RNAs.

Further support for additive activation pathways by SIT4 and CLN3 comes from the effects of the *CLN3-1* mutation on the cell size of a strain containing a deletion of *SIT4*. Compared with an isogenic *SIT4 SSD1-v1* strain, deletion of *SIT4* results in a biphasic cell size distribution: Some of the cells are larger than normal and other cells are smaller than normal (Fig. 5B). Microscopic analysis of pedigrees of dividing cells shows that the smaller cells are the newly released daughter cells and the larger cells are the mother cells. Therefore, in the absence of *SIT4*, the daughter cells are released from the mother cell at a smaller than normal daughter cell size. However, these daughter cells do not initiate budding (and probably Start) until they reach a larger than normal cell size. Expression of *CLN2* from the *S. cerevisiae ADH* promoter in Δ*sit4 SSD1-v1* cells results in a monophasic cell size distribution and a reduced modal cell size of 26 fl (cf. Fig. 5B) (data not shown). Therefore, the initiation of bud formation at a larger than normal cell size by Δ*sit4 SSD1-v1* daughter cells is a result of the defect in *CLN1*, *CLN2*, and *HCS26* RNA accumulation owing to the absence of *SIT4*.

The *CLN3-1* or *CLN3-2* mutation causes a smaller than normal cell size (Cross 1988; Nash et al. 1988). The smaller than normal cell size for *CLN3-1* or *CLN3-2* strains is probably the result of a more rapid increase in the levels of *CLN1* and *CLN2* RNA during G_1, which would advance Start. When a Δ*sit4 SSD1-v1* strain is transformed with a high copy number plasmid containing the *CLN3-1* gene, the cell size distribution becomes monophasic and the modal cell size becomes very small (~20 fl) (Fig. 5C). This reduced cell size is very similar to that of an isogenic *SIT4 SSD1-v1* strain containing the same *CLN3-1* plasmid (~18 fl; data not shown). Therefore, a hyperactivated allele of *CLN3* does not require *SIT4* for the small cell size phenotype, again suggesting that *SIT4* and *CLN3* stimulate the accumulation of *CLN1* and *CLN2* RNAs by additive pathways.

SIT4 *provides an essential function in addition to the accumulation of* CLN1, CLN2, *and* HCS26 *RNAs*

Several lines of evidence indicate that *SIT4* is required for a function in addition to the late G_1 accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs. First, the *CLN2*-coding sequences transcribed from the *S. pombe ADH* promoter (gives low-level *SIT4*-independent expression) (Fig. 6A) or from the *S. cerevisiae ADH* promoter (gives high-level *SIT4*-independent expression) (data not shown) do not allow a *pGAL:SIT4* Δ*ssd1* strain to grow on medium containing glucose (which represses the expression of *SIT4*

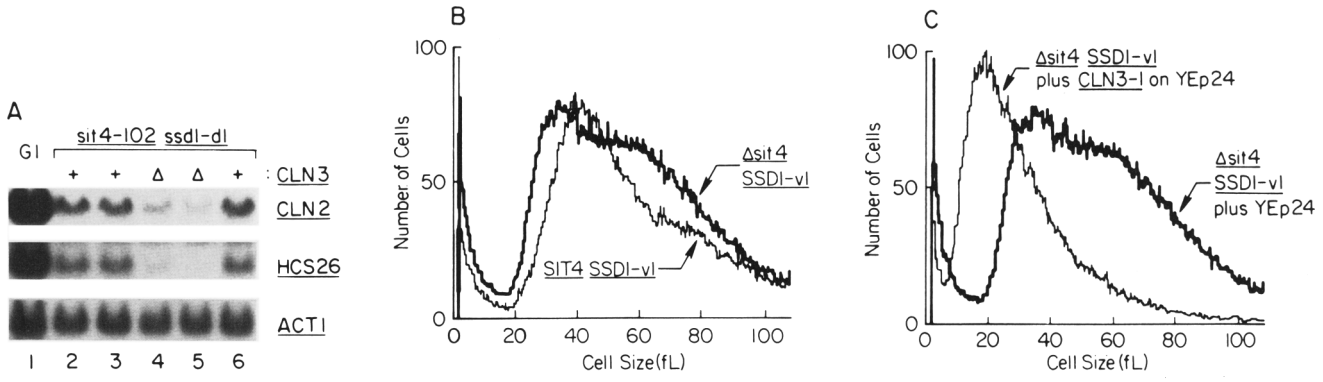

Figure 5. *SIT4* and *CLN3* function in additive pathways. (*A*) Northern analysis showing that most of the low *SIT4*-independent levels of *CLN2* and *HCS26* RNAs are *CLN3* dependent. (Lane *1*) RNA prepared from a G_1-enriched population of cells obtained by α-factor arrest/release (see Fig. 1). For lanes *2* and *6*, the RNA was from the same preparation of RNA isolated from a culture of strain CY1680 [CY1680 is CY738 (=*sit4-102 ssd1-d1*) containing *LEU2*/cen plasmid pAB484]. (Lane *3*) RNA prepared from a separate culture of strain CY1680. (Lanes *4,5*) RNA prepared from cultures of strains CY1681 and CY1682, respectively, which are independent Δ*cln3::LEU2* transformants of strain CY738. For lanes *2–6*, the cultures were grown exponentially in SC minus leucine medium, shifted (at an OD_{600} of ~0.20) to 37°C, and grown for 4 hr before collection of the cells. For all samples, total RNA was prepared and 8 μg was loaded into each lane. The probes used are listed in Materials and methods. (*B*) Coulter Channelyzer plots of cell volume (in fl) distributions for a Δ*sit4 SSD1-v1* strain (CY248) transformed with either a *LEU2*/cen control plasmid or pCB182, which contains the wild-type *SIT4* gene on the same *LEU2*/cen plasmid. The cultures were grown exponentially at 30°C in SC minus leucine medium and analyzed as described in Nash et al. (1988). (*C*) Coulter Channelyzer plots of cell volume distributions for a Δ*sit4 SSD1-v1* strain (CY248) containing either a control 2μ plasmid (YEp24) or pCB1451 (*CLN3-1* on YEp352). The cultures were grown exponentially at 30°C in SC minus uracil medium.

from the *GAL* promoter). The *S. pombe ADH:CLN2* and the *S. cerevisiae ADH:CLN2* plasmids express functional CLN2 because they give full viability to a *cln1::HIS3 cln2::TRP1* Δ*cln3* strain (data not shown). Therefore, expression of *CLN2* from a heterologous promoter does not cure the requirement for *SIT4* in a Δ*ssd1* genetic background.

Second, transformation of a temperature-sensitive *sit4-102 ssd1-d1* strain with either a *S. pombe ADH:CLN2* plasmid or a *S. cerevisiae ADH:CLN2* plasmid does not cure the temperature-sensitive phenotype (data not shown). If the only function of *SIT4* were for accumulation of *CLN* RNAs, these strains should be Ts^+. When asynchronous *sit4-102 ssd1-d1* cells are shifted to 38°C, they arrest in G_1 with a 1n or near 1n DNA content (Fig. 6B). In contrast, when asynchronous *sit4-102 ssd1-d1* cells that contain the *S. pombe ADH:CLN2* plasmid are shifted to 38°C, they arrest with a 2n (or very near 2n) DNA content (Fig. 6B). However, even with the *S. pombe ADH:CLN2* plasmid, most (76%) of the cells cannot form a visible bud (Fig. 6B; b.i. is percent cells with bud) and are blocked in some other late G_1 or G_1/S phase function. To show that the *sit4-102 ssd1-d1* cells containing the *S. pombe ADH:CLN2* plasmid arrest with a 2n (or very near 2n) DNA content only after progression through a 1n G_1 phase at the nonpermissive temperature, we performed a similar experiment using synchronized cells. Unbudded G_1 cells (which have a 1n DNA content; top of Fig. 6C) were obtained by centrifugal elutriation (Sutton el al. 1991a) and grown at 24°C. When most of the cells had budded (middle of Fig. 6C), the cultures were shifted to 38°C. The cells were monitored by flow cytometry and by budding index. The *sit4-102 ssd1-d1* cells containing the control plasmid go through mitosis and arrest primarily as unbudded cells with a 1n (or near 1n) DNA content (bottom of Fig. 6C). In contrast, the *sit4-102 ssd1-d1* cells containing the *S. pombe ADH:CLN2* plasmid go through mitosis, a 1n unbudded stage (data not shown), and arrest primarily as unbudded cells with a 2n (or very near 2n) DNA content (bottom of Fig. 6C). Therefore, the failure of temperature-sensitive *sit4-102 ssd1-d1* cells to replicate their DNA is the result of insufficient accumulation of *CLN2* RNA (and probably *CLN1* and *HCS26* RNAs). If *CLN2* is provided from a promoter that is not *SIT4* dependent, the cells can replicate all or almost all of their DNA in the absence of *SIT4* (and *SSD1*) function. However, these cells are still mostly blocked for bud formation, which indicates that *SIT4* is required for some additional cell cycle function during late G_1.

Discussion

A model for SIT4 *function*

A model for *SIT4* function, incorporated into the current model of *CLN* transcription, is shown in Figure 7. When yeast cells exit mitosis or during α-factor arrest, they have very low levels of *CLN1* and *CLN2* RNAs (Wittenberg et al. 1990). In contrast, the levels of *CLN3* RNA are more constant throughout the cell cycle and are present during early G_1 (Nash et al. 1988). At some time during late G_1, the levels of *CLN1* and *CLN2* RNAs increase dramatically (Wittenberg et al. 1990). This late G_1 in-

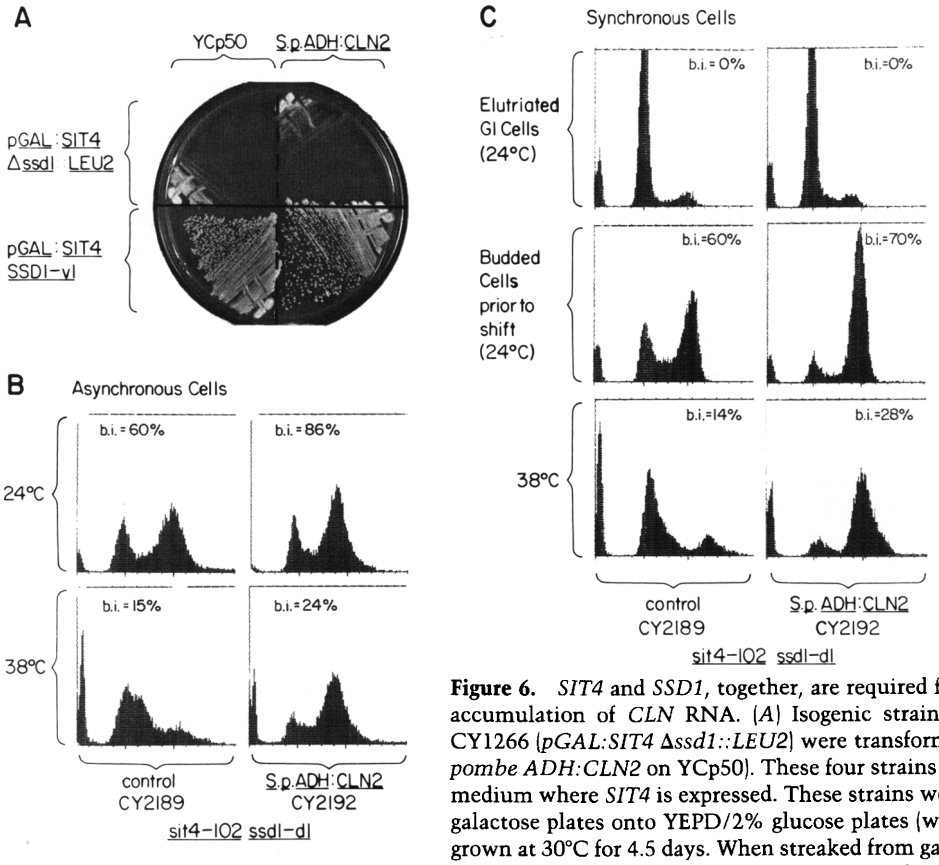

Figure 6. *SIT4* and *SSD1*, together, are required for an essential function in addition to accumulation of *CLN* RNA. (*A*) Isogenic strains CY1231 (*pGAL:SIT4 SSD1-v1*) and CY1266 (*pGAL:SIT4 Δssd1::LEU2*) were transformed with either YCp50 or pCB1342 (*S. pombe ADH:CLN2* on YCp50). These four strains grow at very similar rates on galactose medium where *SIT4* is expressed. These strains were streaked from SC minus uracil/2% galactose plates onto YEPD/2% glucose plates (which represses the *GAL* promoter) and grown at 30°C for 4.5 days. When streaked from galactose to glucose medium, the *pGAL:SIT4 Δssd1::LEU2* cells can give rise to tiny colonies because the levels of *SIT4* RNA and protein require dilution (by cell growth and division) before the cells arrest growth. The cells in these tiny colonies on the YEPD (glucose) plates do not divide if streaked onto a second YEPD plate. (*B,C*) FACS analysis of propidium iodide stained cells to determine DNA content. The vertical axis is the number of cells; the horizontal axis is fluorescence intensity. Cells were prepared for FACS as described by Nash et al. (1988) except that after RNase treatment, the cells were treated with 0.5 mg/ml of proteinase K (Boehringer Mannheim) for 30 min at 50°C. The budding index (b.i.) is the percent of cells with a visible bud (counting at least 100 cells). Both strains are *sit4-102 ssd1-d1*, but strain CY2189 contains pCB1126 (a control *TRP1*/cen plasmid) and strain CY2192 contains pCB1549 (*S. pombe ADH:CLN2* on pCB1126). SC minus tryptophan medium was used for all cultures. (*B*) Asynchronous cultures were grown exponentially at 24°C. Half of each culture was shifted to 38°C, and samples were collected after 4.5 hr. (*C*) Unbudded G_1 cells (*top* panels) were obtained by centrifugal elutriation of cultures grown at 24°C (Sutton et al. 1991a). Cultures of G_1 cells were grown at 24°C until most of the cells had formed a visible bud (*middle* panels). At this time, the cultures were shifted to 38°C and monitored for bud index and DNA content every 30 min for 3 hr (data not shown). The arrest state after 4 hr at 38°C is shown in the *bottom* panels.

crease in the levels of *CLN1* and *CLN2* RNAs requires at least one functional *CLN* gene (either *CLN1*, *CLN2*, or *CLN3*) and is stimulated by the hyperactive *CLN3-1* or *CLN3-2* mutation (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). In addition, the late G_1 increase in the levels of *CLN1* and *CLN2* RNAs requires the *CDC28* kinase (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). These results suggested that the *CLN* genes and *CDC28* jointly function by a positive feedback loop for stimulating the late G_1 accumulation of *CLN1* and *CLN2* RNAs (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). The targets of the CLN1/CDC28 and CLN2/CDC28 kinases in this potential feedback loop are not known. Also, the targets of the CLN3-associated kinase, which is CDC28 dependent (Tyers et al. 1991), are not known. For the model in Figure 7, the

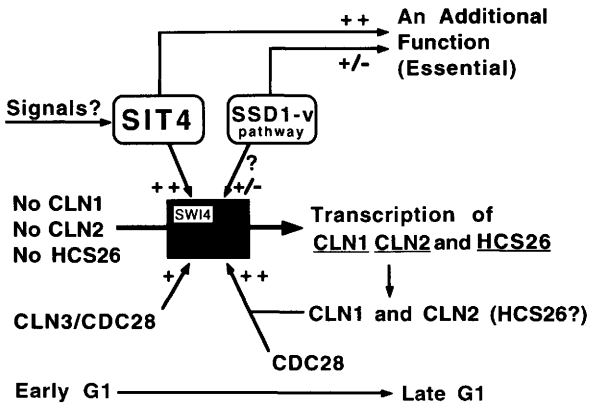

Figure 7. A model of the role of SIT4 in the cell cycle. This model is described in the Discussion.

substrates that require phosphorylation by the CLN/CDC28 kinases for the late G_1 accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs are included within the black box. These substrates could be the CLN/CDC28 complexes themselves, the SWI4 and SWI6 proteins [which function as transcription factors for *CLN1*, *CLN2*, and *HCS26* expression (Ogas et al. 1991; Nasmyth and Dirick 1991)], and/or some other currently unknown factor.

In this paper, we show that the SIT4 protein phosphatase is also required for the normal accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs during late G_1. As expected, the lower levels of *CLN2* RNA in *sit4* mutants result in lower levels of CLN2 protein (M.J. Fernandez-Sarabia and K.T. Arndt, unpubl.). In contrast, *SIT4* is not required for *CLN3* transcription. Also, *SIT4* is not required for *CDC28* transcription (A. Sutton and K.T. Arndt, unpubl.). Therefore, the requirement of *SIT4* for the accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs is not the result of *SIT4* being required for *CDC28* transcription (which would affect the positive feedback loop). In Figure 7, the arrow for SIT4 function points toward SWI4 within the black box because SIT4 functions for the accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs at least partly via accumulation of *SWI4* RNA. The *CLN3* pathway and the *CLN1*, *CLN2*, *HCS26* pathways may also function via *SWI4*.

Richardson et al. (1989) have shown that cells cannot survive in the absence of *CLN1*, *CLN2*, and *CLN3*. In our model of *SIT4* function, the near inviability of Δ*sit4 SSD1-v1* Δ*cln3* strains results from the lower levels of *CLN1*, *CLN2*, and *HCS26* RNAs (owing to the absence of *SIT4*) combined with the absence of *CLN3*. The near inviability of Δ*sit4 SSD1-v1* Δ*cln3* strains is cured by low-level transcription of either *CLN2*- or *SWI4*-coding sequences from a *SIT4*-independent promoter. The requirement of *SIT4* for normal accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs can also explain why cells lacking *SIT4* are sensitive to the loss of *CLN2*. The *CLN1*, *CLN2*, and *CLN3* cyclins are not a unique set of G_1 cyclins. A haploid *cln1 cln2 hcs26* mutant is very sick at 37°C and a diploid *cln1 cln2 hcs26* mutant is inviable (J. Ogas and I. Herskowitz, pers. comm.). Therefore, if cells lacking *SIT4* are surviving on the low *SIT4*-independent levels of *CLN1*, *CLN2*, and *HCS26* RNAs (and other possible *SWI4*-dependent G_1 cyclins), the loss of functional *CLN2* RNA by disruption of the *CLN2* gene would result in even lower G_1 cyclin function. That cells lacking *SIT4* are not sensitive to loss of *CLN1* function could result if *CLN1* provides less G_1 cyclin activity than *CLN2*. *CLN1 cln2 cln3* cells grow slower than *cln1 CLN2 cln3* cells (C. Di Como and K.T. Arndt, unpubl.). In addition, the in vitro kinase activity of CLN1 is weaker than that of CLN2 (Tyers et al. 1991).

SIT4 *and* SSD1 *together are essential*

The viability of a Δ*sit4* strain or the temperature-sensitive phenotype of a *sit4-102* strain depends on a second gene, *SSD1*. The *SSD1* locus is polymorphic because different strains can contain different versions of *SSD1* (Sutton et al. 1991a). In *SSD1-v* backgrounds, the *sit4-102* mutation does not result in a temperature-sensitive phenotype and deletion of *SIT4* results in viability but with a slow growth rate. In *ssd1-d* or Δ*ssd1* backgrounds, the *sit4-102* mutation causes a temperature-sensitive phenotype and deletion of *SIT4* results in inviability. Because deletion of *SSD1* in a wild-type *SIT4* strain results in only subtle phenotypic alterations (Sutton et al. 1991a), *SSD1-v* either plays a minor role in the *SIT4* pathway or regulates a pathway that can partially function in place of the *SIT4* pathway.

In this paper we show that *SIT4* is required for the normal late G_1 accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs. Is *SSD1* involved in this process? Following release from α-factor arrest, isogenic *SIT4 SSD1-v1* and *SIT4* Δ*ssd1* strains accumulate *CLN2* RNA at very similar rates (M.J. Fernandez-Sarabia, A. Sutton, and K.T. Arndt, unpubl.). However, a minor involvement of *SSD1* in G_1 cyclin RNA accumulation might be obscured by the major role played by *SIT4*. For example, at least one functional *CLN* gene (*CLN1*, *CLN2*, or *CLN3*) is required for the late G_1 accumulation of *CLN1* and *CLN2* RNAs (Cross and Tinkelenberg 1991). However, deletion of *CLN3* by itself causes almost no difference in *CLN1* and *CLN2* RNA levels. The requirement of *CLN3* for *CLN2* RNA accumulation is seen only in the absence of *CLN1* and *CLN2* function (Cross and Tinkelenberg 1991).

Currently, there is only weak evidence for the involvement of *SSD1* in the accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs. When shifted to the nonpermissive temperature, a *sit4-102 ssd1-d1* strain arrests with a 1n or near 1n DNA content. The lack of DNA synthesis in the absence of both *SIT4* and *SSD1-v* function is the result of the lack of G_1 cyclin function because heterologous expression of *CLN2* allows DNA replication in these cells. In contrast, an isogenic *sit4-102 SSD1-v1* strain is viable and is able to replicate its DNA at 37°C. Moreover, Δ*sit4 SSD1-v* strains are also viable. Because viability requires G_1 cyclin function, *SSD1-v* provides a pathway for G_1 function independently of *SIT4*. Therefore, either *SSD1-v* can positively regulate accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs or *SSD1-v* allows cell cycle progression at lower than normal levels of G_1 cyclin function (which, in the absence of SIT4 function, would be provided mostly by *CLN3*). Therefore, in Figure 7, the *SSD1-v* arrow that leads to the black box for *CLN1*, *CLN2*, and *HCS26* RNA accumulation includes a question mark.

Heterologous expression of *CLN2* (Fig. 6A), *HCS26*, or both *CLN2* and *HCS26* (M.J. Fernandez-Sarabia, A. Sutton, and K.T. Arndt, unpubl.) does not give viability in the absence of both *SIT4* and *SSD1* function. Although expression of *CLN2* from the *S. pombe ADH* promoter allows a cell to complete or almost complete DNA synthesis in the absence of *SIT4* and *SSD1* function, most of the cells are blocked for bud initiation (Fig. 6B,C). In *S. cerevisiae*, bud initiation normally occurs at or very close to the initiation of S phase. Therefore, *SIT4* and *SSD1* are required for at least one essential function in

late G_1 or G_1/S in addition to their requirement for G_1 cyclin function. This additional function or functions is indicated in Figure 7.

Targets of SIT4

At what level in the pathway for the accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs does SIT4 function? The experiments presented in this report show that the requirement of *SIT4* for the late G_1 accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs is at least partly via *SWI4*. Because *SWI4* is probably a direct DNA-binding factor for transcription of *CLN1*, *CLN2*, and *HCS26* (Nasmyth and Dirick 1991; Ogas et al. 1991), 1991), the simplest model would be that *SIT4* is required for the accumulation of *SWI4* RNA, which would in turn induce *CLN1*, *CLN2*, and *HCS26* RNA accumulation. In this model, *SIT4* would be required for accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs indirectly and only through *SWI4*. However, if *SWI4* RNA accumulation and *CLN1*, *CLN2*, and *HCS26* RNA accumulation are mutually interdependent (i.e., *SWI4* could be part of the positive feedback loop if *CLN1*, *CLN2*, and *HCS26* positively regulate not only themselves but also *SWI4*), then *SIT4* could function at either level — directly for *SWI4* RNA accumulation and/or directly for *CLN1*, *CLN2*, and *HCS26* RNA accumulation.

Because the predicted SIT4 protein is very similar to the catalytic subunit of mammalian type 1 and type 2A phosphatases and because SIT4 has phosphatase activity in vitro (M.J. Fernandez-Sarabia, A. Sutton, and K.T. Arndt, unpubl.), SIT4 most probably functions as a protein phosphatase to activate the late G_1 accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs. What is the target of the SIT4 phosphatase in this pathway? One possibility is that the SIT4 substrate in this pathway is an RNA polymerase II general transcription factor. Mutations in *SIT4* were originally identified by their ability to increase transcription of the *HIS4* gene in the absence of the normal DNA-binding transcriptional activation factors (GCN4, BAS1, and BAS2) (Arndt et al. 1989). Strains containing these original *sit4* transcriptional suppressor alleles have slightly altered length and levels of *URA3* RNA and slightly altered length (but normal levels) of *LEU4* RNA. However, these same *sit4* strains have normal lengths and levels of *ACT1*, *PGK*, *ILV2*, *FUS1*, and *GAL1* RNAs (Arndt et al. 1989). In addition, the absence of *SIT4* function (a Δ*sit4 SSD1-v1* strain at 30°C or a *sit4-102 ssd1-d1* strain at 37°C) causes no effect or very little effect for *ACT1*, *CLN3*, *URA3*, and *SWI6* RNAs (see Figs. 1, 2, 3, and 7), *HIS4* RNA (when GCN4, BAS1, and BAS2 are functional), or *CDC28* RNA (A. Sutton and K.T. Arndt, unpubl.). Because the major transcriptional effects of mutations in *SIT4* are somewhat specific for the accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs, SIT4 might dephosphorylate either an RNA polymerase II factor that functions for transcription of a subset of genes (e.g., *SWI4*, *CLN1*, *CLN2*, and *HCS26*) or a direct DNA-binding factor that functions to activate *SWI4* transcription in late G_1.

As an alternative to the modulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNA accumulation by a direct involvement of *SIT4* in the transcription process, SIT4 could affect G_1 cyclin accumulation indirectly by the potential positive feedback loop. Here, SIT4 could regulate the stability of *CLN* RNA, the stability of CLN proteins, or the translation of *CLN* RNA. Because the late G_1 increase in the accumulation of *CLN1* and *CLN2* RNAs requires *CLN1* and *CLN2* themselves (and therefore probably CLN1 and CLN2 proteins by translation of *CLN1* and *CLN2* RNA) (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991), the rate of protein synthesis (which is related to the growth rate of the cell) could regulate the rate of accumulation of *CLN1* and *CLN2* RNAs. Further experiments will be required to determine the precise substrate (or substrates) that requires dephosphorylation by SIT4 for normal accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs.

Regulation of Start

The results in this report show that *SIT4* is required in late G_1 for the normal accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs. The *SIT4* protein phosphatase is well positioned to regulate the accumulation of these RNAs because *SIT4* undergoes cell-cycle dependent associations with two high molecular mass proteins, p155 and p190 (Sutton et al. 1991a). The SIT4 protein exists mostly as free monomeric SIT4 in G_1 cells. Very close to late G_1, SIT4 associates in separate complexes with p155 and p190 and remains associated with these proteins until about mid- or late mitosis (Sutton et al. 1991a). Because p155 and p190 are most likely regulatory subunits of SIT4, this cell cycle-dependent association of SIT4 with p155 and p190 probably gives a cell cycle-dependent regulation of the in vivo activity of SIT4 toward specific cell cycle-regulated substrates. Therefore, SIT4 is well positioned to regulate cell cycle-dependent processes (such as the accumulation of *SWI4*, *CLN1*, *CLN2*, and *HCS26* RNAs) that occur in late G_1.

What regulates the association of p155 and p190 with SIT4? Because both p155 and p190, but not SIT4, are phosphorylated in vivo (Sutton et al. 1991a), the association of p155 and p190 with SIT4 may be regulated by the phosphorylation state of p155 and p190. Therefore, the signals (whose possibilities are indicated in Fig. 7) that control Start could regulate this process by the phosphorylation of p155 and/or p190. In the future, reagents specific for p155 and p190 will be essential to better understand the individual functions of SIT4 during late G_1.

Materials and methods

Strains and media

Yeast strains are shown in Table 1. Yeast cultures were grown, as indicated, on either YEPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose), or synthetic complete (SC) medium (Sherman et al. 1989) containing all amino acids and uracil at 0.1 g/liter (except leucine at 0.2 g/liter) and adenine at 0.075

Table 1. S. cerevisiae *strains*

Strain	Genotype	Source
AY926	*MAT*α *ssd1-d2 ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	Sutton et al. (1991a)
CY11	*MAT*α *cdc25-1 ura3 leu2 trp1 can1*	B. Futcher
CY12	*MAT*α *cdc28-13 his4-580 lys2° trp1-1 tyr1°*	B. Futcher
CY146	*MAT*α Δ*sit4-2* (::*HIS3*) [*sit4-102* on *LEU2*/cen] *ssd1-d2 ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	Sutton et al. (1991a)
CY248	*MAT***a** Δ*sit4-2* (::*HIS3*) *SSD1-v1 ura3 leu2-3 his3*	Sutton et al. (1991a)
CY279	*MAT*α Δ*sit4-2* (::*HIS3*) *SSD1-v1 ura3 leu2-3 his3 trp1-1*	Sutton et al. (1991a)
CY738	*MAT***a** Δ*sit4-2* (::*HIS3*) [*sit4-102* on *URA3*/cen] *ssd1-d1 ura3 leu2-3, 112 his3 trp1 lys2-801 ade2-1 can1-100*	this study
CY1176	*MAT***a** Δ*sit4-2* (::*HIS3*) [wt *SIT4* on *LEU2*/cen] *ssd1-d1 ura3 leu2-3, 112 his3 trp1 ade2-1 can1-100*	this study
CY1178	*MAT***a** Δ*sit4-2* (::*HIS3*) [*sit4-102* on *LEU2*/cen] *ssd1-d1 ura3 leu2-3, 112 his3 trp1 ade2-1 can1-100*	this study
CY1231	*MAT***a** *pGAL:SIT4 SSD1-v1 ura3-52 leu2-3 his3-Δ200 lys2-801*	this study
CY1266	*MAT***a** *pGAL:SIT4* Δ*ssd1::LEU2 ura3-52 leu2-3 his3-Δ200 lys2-801*	this study
CY1381	*MAT*α *cln1::TRP1 ssd1-d2 ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	E. Elion
CY1386	*MAT***a** *cln2::TRP1* Δ*SSD1::LEU2 ura3 leu2 his3 trp1*	this study
CY1393	*MAT***a** Δ*sit4-2* (::*HIS3*) [*LEU2*/cen plasmid] *SSD1-v1 CLN2-HA ura3 leu2-3,112 his3 ade2-1*	this study
CY1394	*MAT***a** Δ*sit4-2* (::*HIS3*) [wt *SIT4* on *LEU2*/cen] *SSD1-v1 CLN2-HA ura3 leu2-3,112 his3 ade2-1*	this study
CY1471	*MAT***a** Δ*sit4-2* (::*HIS3*) *SSD1-v1 ura3 leu2 his3 trp1*	this study
CY1507	*MAT***a** Δ*cln3::URA3 ssd1-d2 ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	M. Linskens and B. Futcher
CY1518	*MAT***a** *cdc15-2 ssd1-d2 ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	K. Nasmyth
CY1765	*MAT***a** Δ*swi4::HIS3 ssd1-d1 ura3-52 leu2Δ1 his3-Δ200 lys2-801 ade2-101 his3Δ124lacZ*	JO57-6B of Ogas et al. (1991)
CY1784	*MAT***a** Δ*cln3::LEU2 SSD1-v1 ura3-52 leu2-3 his3-Δ200 lys2-801*	this study
CY1901	*MAT*α Δ*swi6::LEU2 ssd1-d1 ura3-52 leu2Δ1 his3-Δ200 lys2-801 ade2-101*	J. Ogas
CY2189	*MAT*α Δ*sit4-2* (::*HIS3*) [*sit4-102* on *URA3*/cen] *ssd1-d1 ura3 leu2 his3 trp1 lys2-801 ade2* [*TRP1*/cen]	this study
CY2192	*MAT*α Δ*sit4-2* (::*HIS3*) [*sit4-102* on *URA3*/cen] *ssd1-d1 ura3 leu2 his3 trp1 lys2-801 ade2* [*S. pombe ADH:CLN2* on *TRP1*/cen]	this study

g/liter. For plasmid selection, the appropriate amino acid or uracil was omitted.

Deletion and disruption alleles

The deletion allele of *SIT4* (Δ*sit4::HIS3* = *sit4-2*) removes amino acids 4–308 of SIT4 (of 311 total) (Sutton et al. 1991a). The Δ*swi4::HIS3* mutation (CY1765) and the Δ*swi6::LEU2* mutation (CY1901) are from Ogas et al. (1991). The *cln1::TRP1* disruption (strain CY1381) is the same as in Hadwiger et al. (1989). The *cln2::TRP1* disruption (strain CY1386) is the same as in Xiong et al. (1991). These *cln1* and *cln2* disruptions result in reduced CLN1 and CLN2 function because a *cln1::TRP1 cln2::TRP1* Δ*cln3* strain is inviable. The Δ*cln3::URA3* mutation (CY1507) used for Figure 4, C and D, is from M. Linskens and B. Futcher (Cold Spring Harbor Laboratory) and replaces *CLN3* sequences from the *Xho*I–*Eco*RI sites with a 1.2-kb *Hin*dIII fragment of *URA3*. The Δ*cln3::LEU2* mutation (CY1784) used in Figure 4E replaces *CLN3* sequences from the *Bst*XI–*Eco*RI sites with a 2.2-kb *Sal*I–*Xho*I fragment of *LEU2*. The Δ*cln3::URA3* mutation and the Δ*cln3::LEU2* mutation are both *CLN3* null mutations and give identical phenotypes.

Plasmid construction

The *S. pombe ADH:CLN2* plasmids were prepared as follows: Plasmid c1881 [obtained from K. Nasmyth (Nasmyth and Dirick 1991)] contained the *S. pombe ADH:CLN2* construction on a 3.5-kb *Sph*I–*Pst*I fragment. This fragment was placed into the *Hin*dIII site of YCp50 (yielding pCB1342), the *Hin*dIII site of *LEU2*/cen plasmid pAB484 (yielding pCB1311), or the *Eco*RI site of *TRP1*/cen plasmid pCB1126 (yielding pCB1549). *LEU2*/cen plasmid pAB484 was prepared by replacing the *Sal*I–*Sma*I fragment (containing *URA3*) of YCp50 with a 2.2-kb *Xho*I–*Sal*I fragment containing *LEU2*. *TRP1*/cen plasmid pCB1126 was prepared by replacing the *Sal*I–*Sma*I fragment (containing *URA3*) of YCp50 with a 0.82-kb *Eco*RI–*Pst*I fragment containing *TRP1*. Expression of *CLN2* from the *S. pombe ADH* promoter in a *cln1 cln2 cln3* strain results in almost a wild-type growth rate.

The *S. pombe ADH:SWI4* plasmid was prepared as follows: A 0.7-kb *Eco*RI–*Hin*dIII fragment containing the *S. pombe ADH* promoter (Nasmyth and Dirick 1991) was placed into the *Eco*RI–*Hin*dIII backbone of YCp50, yielding pCB1481. A 3.8-kb fragment containing the *SWI4*-coding sequences [−6 to about +3888; *SWI4* open reading frame (ORF) is +1 to +3564] was then placed into the *Eco*RI site of pCB1481, yielding pCB1491. Plasmid pCB1491 fully complemented a Δ*swi4* strain for both growth rate and temperature sensitivity.

Northern analysis and probes

Northern analysis was performed as described previously (Arndt et al. 1989). The indicated amount of total RNA was loaded onto a 1% agarose gel containing 6% formaldehyde, 0.02 M morpholinopropanesulfonic acid, 0.005 M sodium acetate, and 0.001 M

EDTA (final pH of 7.0). The gels were blotted onto BioTrans nylon membranes. The probes were the 1.3-kb *ClaI–EcoRI* fragment of *CLN1*, the 0.75-kb *XhoI–HindIII* fragment of *CLN2*, the 2.2-kb *HpaI–XhoI* fragment of *CLN3*, the 0.6-kb *AluI* fragment of *ACT1*, the 1.2-kb *HindIII* fragment of *URA3*, the 2.2-kb *BamHI* fragment of *SWI4*, and the 2.9-kb *HindIII–BglII* fragment of *SWI6*. The probe for *HCS26* was a DNA fragment obtained from a plasmid containing sequences from ~10 bp upstream to ~10 bp downstream of the *HCS26* ORF [obtained by polymerase chain reaction (PCR) by J. Ogas (University of California, San Francisco)]. The blots were washed twice (15 min each) at 24°C using 2× SSC, 0.1% SDS, and twice (15 min each) at 65°C using 0.1× SSC, 0.1% SDS.

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SIT4 protein phosphatase is required for the normal accumulation of SWI4, CLN1, CLN2, and HCS26 RNAs during late G1.

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