MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5701–5707 Vol. 16, No. 10
0270-7306/96/$04.00+0
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The Transcription Factor Swi5 Regulates Expression of the Cyclin Kinase Inhibitor p40^{SIC1}

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Received 21 March 1996/Returned for modification 22 April 1996/Accepted 25 July 1996

DNA replication in budding yeast cells depends on the activation of the Cdc28 kinase (Cdk1 of *Saccharomyces cerevisiae*) associated with B-type cyclins Clb1 to Clb6. Activation of the kinase depends on proteolysis of the Cdk inhibitor p40^{SIC1} in late G_1, which is mediated by the ubiquitin-conjugating enzyme Cdc34 and two other proteins, Cdc4 and Cdc53. Inactivation of any one of these three proteins prevents p40^{SIC1} degradation and causes cells to arrest in G_1 with active Cln kinases but no Clb-associated Cdc28 kinase activity. Deletion of *SIC1* allows these mutants to replicate. p40^{SIC1} disappears at the G_1/S transition and reappears only after nuclear division. Cell cycle-regulated proteolysis seems largely responsible for this pattern, but transcriptional control could also contribute; *SIC1* RNA accumulates to high levels as cells exit M phase. To identify additional factors necessary for the inhibition of the Cdk1/Cdc28 kinase in G_1, we isolated mutants that can replicate DNA in the absence of Cdc4 function. Mutations in three loci (*SIC1*, *SWI5*, and *RIC3*) were identified. We have shown that high *SIC1* transcript levels at late M phase depend on Swi5. Swi5 accumulates in the cytoplasm during S, G_2, and M phases of the cell cycle but enters the nuclei at late anaphase. Our data suggest that cell cycle-regulated nuclear accumulation of Swi5 is responsible for the burst of *SIC1* transcription at the end of anaphase. This transcriptional control may be important for inactivation of the Clb/Cdk1 kinase in G_2/M transition and during the subsequent G_1 period.

DNA replication in budding yeast cells depends on the activation of Cdk1/Cdc28 kinase associated with six different B-type cyclins, Clb1 to Clb6. Clb-associated Cdk1 kinase is regulated by transcription of cyclin genes, by proteolysis of cyclin proteins, and by accumulation of inhibitory proteins. Due largely to the onset of *CLB5* and *CLB6* transcription in late G_1 (*CLB1* to *CLB4* RNAs do not appear until later), DNA replication is normally triggered by the appearance of active Clb5 or Clb6/Cdc28 kinases (10, 26). In the absence of Clb5 and Clb6, Clb1 to Clb4 trigger replication. Normally, DNA replication is simultaneous with bud formation, but in the *clb5 clb6* mutant, DNA replication is delayed relative to bud emergence (26). Clb/Cdc28 kinase activity is also regulated by changes in cyclin stability (1) and by binding of the inhibitory protein p40^{SIC1}. p40^{SIC1} does not inhibit Cdc28 kinase associated with Cln cyclins which also appear in late G_1 and are necessary for activating *CLB* gene transcription, turning off Clb proteolysis, and triggering p40^{SIC1} proteolysis (19, 22, 25). p40^{SIC1} protein accumulates to high levels in G_1, disappears at the G_1/S transition, and does not reaccumulate until cells complete nuclear division and reenter G_1. To understand how p40^{SIC1} regulates the activity of cyclin B/Cdc28 kinases, we need to know how p40^{SIC1} accumulation is regulated. It has been demonstrated that the appearance of Cln1 and Cln2/Cdc28 kinases in late G_1 leads to the rapid proteolysis of p40^{SIC1} (25). This instability of p40^{SIC1} persists until cells undergo anaphase (24). It is not understood why it becomes stable in the subsequent G_1 period. p40^{SIC1} proteolysis depends on three genes: *CDC4*, *CDC34*, and *CDC53* (25). *CDC34* encodes an E2-type ubiquitin-conjugating enzyme (11). The functions of the Cdc4 and Cdc53 proteins are currently not understood. Mutants with temperature-sensitive alleles of *CDC4*, *CDC34*, and *CDC53* fail to degrade p40^{SIC1} at the restrictive temperature and arrest in G_1 with little or none of the Clb/Cdc28 kinase activity needed for entry into S phase (25). The Cln/Cdc28 kinases, in contrast, are active during this arrest (32), and cells duplicate their spindle pole bodies and form buds—events that are not dependent on Clb/Cdc28 kinases. Deletion of the *SIC1* gene allows these mutants to activate the Clb kinases and to enter S phase (25).

To investigate the role of Cdc4 in cell cycle progression, we set out to isolate mutations that would allow the *cdc4-1* mutants to activate the Clb kinases and thereby enter S phase. We isolated mutations in three genes, *RIC1* to *RIC3*, that allow cells to partially overcome the replication defect of a *cdc4-1* mutant. *RIC2* corresponds to *SIC1*, and *RIC1* corresponds to *SWI5*, which encodes a G_1-specific transcription factor first identified because of its role in activating the *HO* endonuclease gene (29). Our data suggest that in addition to controlling the stability of p40^{SIC1}, transcriptional control of the *SIC1* gene contributes to reaccumulation of p40^{SIC1} protein during G_1. *SIC1* transcripts are present at a low level throughout the cycle, but they accumulate transiently to high levels as cells exit from mitosis. We have shown that Swi5 and the related factor Ace2 both bind, in vitro, to specific sites within the *SIC1* gene promoter. However, while Swi5 has a major role in transiently activating *SIC1* during anaphase, Ace2 plays a minor role.

MATERIALS AND METHODS

Strains and media. All yeast strains were derivatives or were backcrossed at least three times to W303 (*MAT***a** *HML*α *HMR***a** *ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1*). The strains used in this study are listed in Table 1. Cells were grown in yeast extract-peptone medium (YEP) supplemented with 2% glucose (YEPD) or raffinose (YEPR) unless otherwise stated.

Linkage analysis. To demonstrate linkage between mutations isolated in the screen and the *SIC1* and *SWI5* genes, mutants were crossed with K4163 and D69, respectively, and sporulated, and the phenotype of spores from at least 10 tetrads was analyzed by fluorescence-activated cell sorting (FACS) with a FACScan.

BrdU incorporation assay. Stationary-phase cells of strains D6 and D8 were

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TABLE 1. List of the strains and their genotypes used in this study

Strain	Relevant genotype
D6	*MAT***a** *cdc4-1 TRP1 GPD-HPV-TK-URA3*$_7$
D8	*MAT*α *cdc4-1 HIS3 GPD-HPV-TK-URA3*$_7$
D50	*MAT***a** *cdc4-1 TRP1 GPD-HPV-TK-URA3*$_7$ *ric1-1*
D52	*MAT***a** *cdc4-1 TRP1 GPD-HPV-TK-URA3*$_7$ *ric3-1*
D58	*MAT***a** *cdc4-1 TRP1 GPD-HPV-TK-URA3*$_7$ *ric2-1*
D69	*MAT***a** *cdc4-1 HIS3 swi5::URA3*
K1993	*MAT***a** *cdc15-2*
K1998	*MAT***a** *swi5::URA3*
K3659	*MAT***a** *cdc15-2 ace2::HisG-URA3-HisG*
K3772	*MAT***a** *ace2::HisG-URA3-HisG*
K3773	*MAT***a** *swi5::LEU2 ace2::HisG-URA3-HisG*
K3774	*MAT***a** *cdc15-2 swi5::LEU2*
K3995	*MAT*α *cdc4-1 HIS3*
K3996	*MAT***a** *cdc4-1 TRP1*
K4163	*MAT***a** *cdc4-1 sic1::HIS3*
K4719	*MAT***a** *SIC1-HA*$_4$
K4877	*MAT***a** *SIC1-HA*$_4$ *swi5::URA3*

mutagenized by ethyl methanesulfonate to 50% survival and plated on YEPD at 25°C (2,500 cells per 13-cm-diameter plate). To minimize the background signal coming from cells which had passed the Cdc4 step before the temperature shift but had not replicated yet, colonies were grown for 4 to 5 days (i.e., until more than 90% of the cells in a colony were in the G_1 stationary phase). The colonies were then replica plated onto nitrocellulose filters (Schleicher & Schuell) on fresh YEPD plates containing 200 μg of bromodeoxyuridine (BrdU) per ml and incubated at 37°C until more than 50% of the cells were released from stationary phase (checked by budding). In the wild-type cells, budding and replication happen almost simultaneously. We reasoned that the mutants with an abolished requirement for Cdc4 function should replicate by the time 50% of the cells have budded. The incubation time on YEPD-BrdU plates was kept short to minimize the background of mitochondrial replication. BrdU incorporation into DNA was detected by a filter assay as described earlier (5), except that the first incubation step with sorbitol, EDTA, and dithiothreitol was done at 37°C.

Mutants positive in the BrdU filter assay were rechecked for the replication phenotype in the following way: patches were grown until cells were mostly in stationary phase (overnight) on YEPD plates at 25°C. They were then inoculated into fresh YEPD at 37°C and analyzed by FACScan after 4 h.

Isolation and analysis of RNA from synchronous cultures. YEPR-grown wild-type (K4719) and *swi5* (K4788) cells were synchronized by centrifugal elutriation as described previously (26). In the *cdc15* release experiment, *SWI5 ACE2* (K1998), *swi5*Δ (K3774), and *ace2*Δ (K3659) cultures were grown in YEPD at 25°C until they reached an optical density at 600 nm of 0.2. They were then transferred to 37°C for 2.5 h (i.e., until they were arrested [dumbbell shaped]) and then released from the block by addition of an appropriate amount of YEPD at 4°C so that the resulting temperature was 25°C. Cultures were grown further at 25°C, total RNA was isolated, and 10 μg was subjected to Northern (RNA) transfer as described previously (4).

For detection of the *SIC1* transcript, membranes were probed with the 0.7-kb *Asp* 718-*Nde*I fragment of *SIC1*. *CMD1* and *PCL1* transcripts were used as internal controls for loading and for the cell cycle progression, respectively. Quantitations were done with a Molecular Dynamics PhosphorImager.

In vitro DNA-binding assays. Purification of Swi5 (6×His-tagged Swi5 fusion protein) and Ace2 (glutathione *S*-transferase–Ace2 fusion protein), gel retardation assays, and DNase I footprinting were performed as described previously (7). DNA probes were end labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP (9,000 Ci/mol). The *SIC1* probe for gel retardation was a 221-bp *Asp* 718-*Taq*I fragment from the *SIC1* promoter. The *HO* probe was a 208-bp *Bgl*II-*Pvu*II fragment from plasmid M1403. Plasmid M1403 contains a 46-bp region from the *HO* promoter, including Swi5 binding site B, cloned into the pIC19R vector; thus, the *HO* probe also contains plasmid sequences. The *CTS1* probe was a 206-bp *Eco*RI-*Hin*fI fragment from plasmid M1818 (7) that contains two Ace2 binding sites. The *SIC1* probe for DNase I footprinting was a 461-bp *Eco*RI-*Asp* 718 fragment from the *SIC1* promoter, labeled at the *Asp* 718 end.

RESULTS

Screen for suppressors of *cdc4* mutation. To identify genes required for the inhibition of the Clb/Cdc28 kinase in *cdc4* mutants, we set out to identify mutations that allow a *cdc4-1* strain to grow at the restrictive temperature (35°C). After mutagenesis of 5×10^6 cells of the *cdc4-1* strains (K3995 and K3996), only 108 colonies grew at 35°C, and only 8 of them contained recessive suppressor mutations. Contrary to our expectations that the recessive suppressors would be due to a loss of an inhibitory function, genetic analysis showed that all eight recessive mutations were *cdc4* alleles. Eight dominant mutations were tested in a similar manner and were also found to be tightly linked to the *CDC4* locus. These results suggest that there may be more than one essential target for Cdc4.

Screen for mutants which replicate DNA in the absence of Cdc4 function. Having established that it is difficult or impossible to suppress the proliferative defect of *cdc4* mutants by loss-of-function mutations in other genes, we instead set out to identify mutations that merely allow *cdc4-1* mutant cells to replicate DNA at the restrictive temperature. We used strains containing seven copies of the herpes simplex virus thymidine kinase expressed from the constitutive *GPD* promoter, which allowed us to measure the incorporation of BrdU into DNA. *cdc4-1* strains were mutagenized by ethyl methanesulfonate plated onto YEPD plates at 25°C, replica plated onto a nitrocellulose filter on fresh YEPD plates containing BrdU, and incubated at the restrictive temperature for *cdc4-1*. After screening of approximately 170,000 colonies, 274 mutants capable of incorporating BrdU were examined by FACScan to determine their ability to replicate DNA (see Materials and Methods). Ten mutants showed a strong ability to replicate DNA at a nonpermissive temperature for *cdc4-1*, and genetic analysis showed that these mutants form three allelism groups, *RIC1* to *RIC3* (for replicates in *cdc4*). We identified six alleles of *RIC1*, two alleles of *RIC2*, and two alleles of *RIC3*. The FACS profiles of representative mutants from each group are shown in Fig. 1A. The mutations allow about one-half of the *cdc4* mutant cells to fully replicate their genomes.

Since by this stage it had been shown that deletion of *SIC1* enables a *cdc4-1* strain to replicate DNA (see reference 25 and Fig. 1A), *SIC1* was a likely candidate for one of the *RIC* genes. The various *ric* mutants were crossed to a *sic1*Δ *cdc4* double mutant strain (K4163), and the resulting spores were tested by FACScan for the ability to replicate DNA at the nonpermissive temperature. We found that *RIC2* (two alleles) corresponds either to *SIC1* itself or to a closely linked gene. Interestingly, the *sic1* mutation is partially dominant in terms of the DNA replication phenotype. As shown in Fig. 1B, a diploid that is heterozygous *SIC1*/*sic1*Δ but homozygous *cdc4*/*cdc4*, is able to replicate DNA at the nonpermissive temperature. This suggests that the *SIC1* gene product is haplo-insufficient, which is consistent with the observation that p40^{SIC1} acts stoichiometrically in binding to and inhibiting the Clb/Cdk1 kinase (16, 25).

It was found accidentally that overexpression of *CLB5* from the *GAL* promoter is toxic in *swi5* mutant cells (reference 9 and data not shown), as it is in a *sic1* mutant. This suggested that the Swi5 transcriptional activator plays a role, either directly or indirectly, in regulating Clb5 kinase expression or activity. Therefore, we tested whether a *swi5* null mutation suppresses the DNA replication defect associated with *cdc4*. The FACS analysis in Fig. 1A shows that a *swi5* deletion confers a *ric*$^-$ phenotype. To determine whether the *ric1* and *ric3* mutant strains contain mutations in *SWI5*, genetic crosses were performed. Linkage analysis demonstrated that all 48 spores from 12 tetrads from the cross of *ric1-1 cdc4-1* with *swi5*Δ *cdc4-1* (D69) had the parental (replicating) phenotype. We concluded that *RIC1* corresponds to *SWI5*.

There are quantitative differences in the ability of *sic1* and *swi5* to suppress the DNA replication defect caused by *cdc4*. A *sic1* deletion allows more than 90% of the *cdc4-1* mutant cells to complete S phase, while a deletion of *SWI5* enables only 50% of the cells to complete S phase at 37°C (Fig. 1A). The

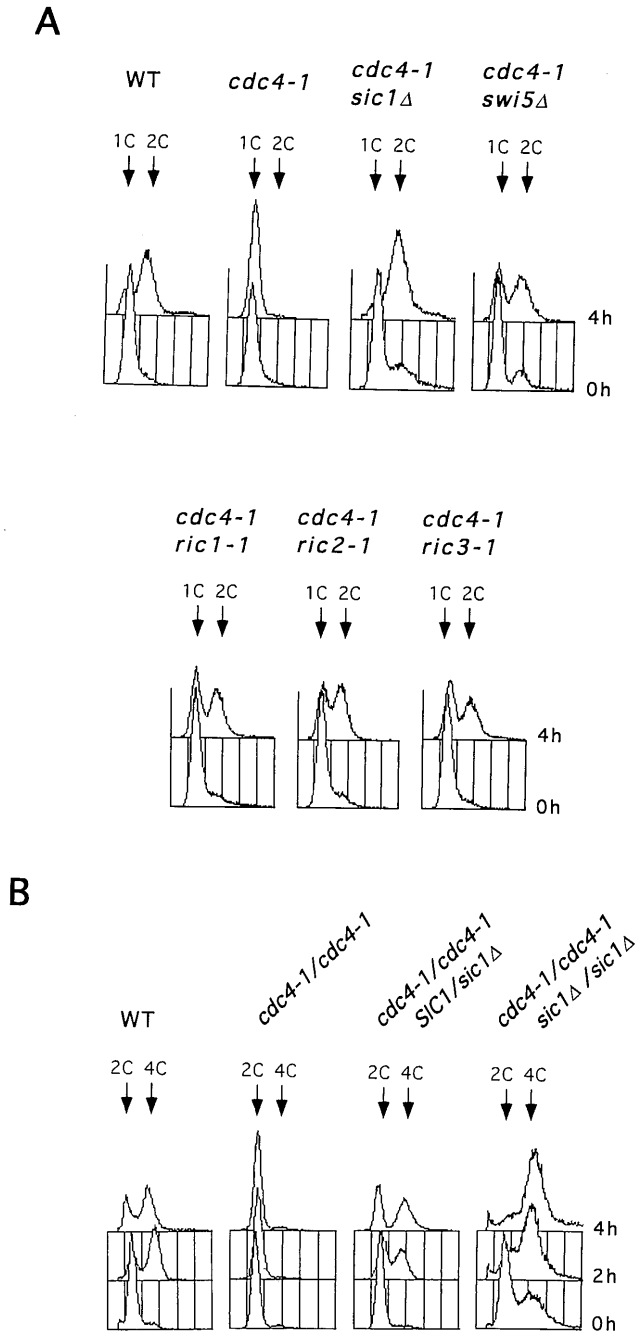

FIG. 1. Mutations in *RIC1*, *RIC2*, and *RIC3* genes as well as deletions of *SIC1* and *SWI5* partially suppress a *cdc4-1* mutation. Stationary-phase cells from 1-day-old patches grown at 25°C were inoculated into fresh YEPD at 37°C. Samples for FACScan were taken 0, 2, and 4 h after temperature shift. (A) DNA content of wild-type (WT [K699]), *cdc4-1* (K3996), *cdc4-1 sic1*Δ (K4163), *cdc4-1 swi5*Δ (D69) *cdc4-1 ric1-1* (D50), *cdc4-1 ric2-1* (D58), and *cdc4-1 ric3-1* (D52) strains. (B) DNA content of diploid of wild-type (WT), *cdc4-1*/*cdc4-1*, *cdc4*/*cdc4 SIC1*/*sic1*, and *cdc4*/*cdc4 sic1*/*sic1* strains.

same is true for the effect on cell morphology. After 4 h at the nonpermissive temperature, all of the *cdc4-1* single mutants have multiple elongated buds, presumably because Cln/Cdc28 kinases which stimulate bud emergence are active during this arrest and cause this phenotype (1, 32). Multiple buds were not seen in *sic1*Δ *cdc4-1* mutants when cells were arrested at 37°C; most cells arrest with a dumbbell shape. When *swi5*Δ *cdc4-1* mutants were shifted to 37°C, we found a mixture of dumbbell-shaped cells with single buds and cells with multiple elongated buds.

Mutations at the *RIC3* locus proved to have no obvious phenotype in a *CDC4*$^+$ background, and, therefore, we have not attempted to clone the gene.

***SIC1* transcription is regulated by Swi5.** *SWI5* encodes a transcription factor needed for the expression of the *HO* endonuclease involved in mating-type switching (29, 30). Recent work suggests that it is also needed for the transcription of *EGT2*, whose transcripts appear as cells exit mitosis (15). *SWI5* is transcribed during G_2 and M phases, but Swi5 protein stays in the cytoplasm and does not accumulate in nuclei until the end of mitosis, when Clb-associated kinases are destroyed by cyclin proteolysis. Swi5 protein is rapidly degraded upon its entry into nuclei, with the result that Swi5 protein accumulates to high levels in nuclei only transiently as cells exit from mitosis (17). *SIC1* as well as *EGT2* is transcribed at the time Swi5 enters the nucleus (8, 25). To test whether Swi5 is involved in the transcription of *SIC1*, we compared *SIC1* transcript levels in *SWI5* and *swi5*Δ cycling cultures. In the *swi5*Δ mutant, the level of *SIC1* RNA is decreased to 50% of that of the wild-type RNA (Fig. 2). We also tested whether *SIC1* transcription depends on *ACE2*, which encodes a related transcription factor that also enters nuclei only at the end of mitosis. *SIC1* transcripts are reduced to about 80% of the wild-type level in *ace2*Δ cells. However, deletion of both *SWI5* and *ACE2* genes reduced *SIC1* RNA levels to 20% of that of the wild type, suggesting that Swi5 and Ace2 share the task of activating *SIC1*, as they do for *EGT2* (15).

To test whether Swi5 is required specifically for the accumulation of *SIC1* RNAs at the end of mitosis, we compared *SIC1* transcript levels of *SWI5* and *swi5*Δ strains synchronized by centrifugal elutriation (Fig. 3). The basal levels of *SIC1* transcripts were similar, but the transient accumulation at the M-to-G_1-phase transition was reduced by two- to threefold in *swi5*Δ cells (zero-minute time point after elutriation and 140 to 170 min later in the subsequent M/G_1 phases). In the *swi5* mutant, a small increase in *SIC1* expression is still seen at the M/G_1 period of the cell cycle, and it is likely that Ace2 is responsible for this activation. We could not, however, test the effect of an *ace2* mutation by using centrifugal elutriation to synchronize cells, because *ace2* strains are too clumpy and do not separate into single cells. We, therefore, compared the *SIC1* transcript levels after release from a cell cycle arrest in late mitosis due to a temperature-sensitive *cdc15-2* allele. In this case, deletion of *SWI5* abolished cell cycle-regulated *SIC1*

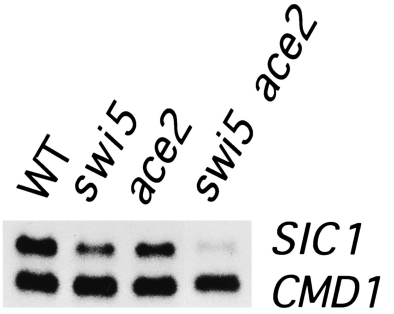

FIG. 2. *SIC1* transcript is regulated by *Swi5*. Results are from Northern blot analysis of the *SIC1* mRNA levels of exponentially growing wild-type (WT [K699]), *swi5*Δ (K1998), *ace2*Δ (K3772), and *swi5*Δ*ace2*Δ (K3773) cultures. *CMD1* RNA serves as an internal loading control.

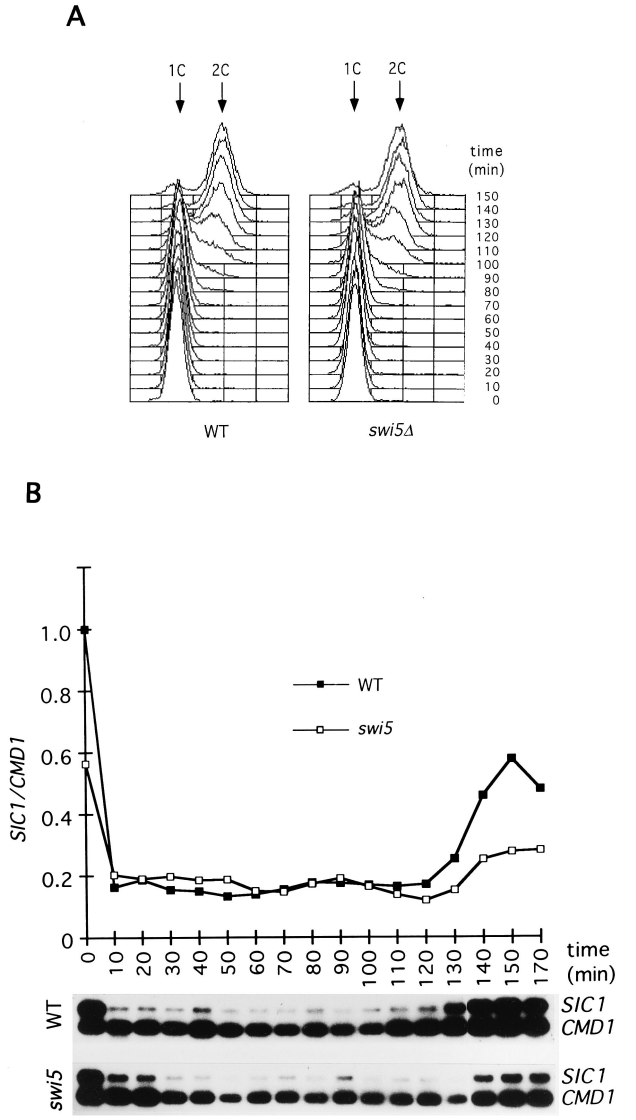

FIG. 3. Burst of *SIC1* transcription at the M/G_1 transition depends on Swi5. Wild-type (WT [K4719]) and *swi5*Δ (K4877) strains were grown in YEPR at 25°C to mid-log phase. Small G_1 cells were isolated by centrifugal elutriation and incubated in YEPD at 30°C. Samples for FACScan and Northern blotting were taken at the times indicated. (A) Cell cycle progression of cells measured by FACScan. (B) Northern blot analysis of *SIC1* mRNA levels of the wild type and the *swi5*Δ mutant. Quantitation of *SIC1* levels was done relative to an internal loading control, *CMD1*. Numbers on the *y* axes represent the *SIC1*/*CMD1* ratio, where the maximum value of the wild type at zero minutes was labeled 1.0.

RNA accumulation (Fig. 4). In *SWI5 ACE2* cells, *SIC1* RNAs accumulated during the *cdc15* arrest, increased further after the release from the block, soon afterwards fell as cells proceeded through the cell cycle, and then reaccumulated again. In contrast, in the *swi5* mutant, *SIC1* RNAs remained at a low constant level throughout this time course. Deletion of *ACE2* had a modest effect on *SIC1* RNA regulation with this protocol. The synchrony of the releases was checked by measuring the levels of the G_1 cyclin *PCL1* (*HCS26*) mRNA. We conclude that Swi5 has a major role in activating *SIC1* transcription at the end of mitosis and that Ace2 has a modest one.

Swi5 and Ace2 bind to the *SIC1* promoter in vitro. To test whether Swi5 activates *SIC1* directly by binding to its promoter, we searched for potential Swi5 binding sites in the sequences between *SIC1*'s AUG codon and the upstream gene *BOS1*. We identified two putative Swi5 binding sites, on the basis of the similarity to the DNA sequence recognized by Swi5 at the *HO* and the *CTS1* promoters (Fig. 5A). Although Swi5 and Ace2 differentially activate transcription of *HO* and *CTS1* in vivo (6), in vitro Swi5 and Ace2 each bind to both promoters with similar affinities (7).

Swi5 and Ace2 proteins purified from an *Escherichia coli* expression system were used to examine DNA binding to *SIC1* promoter sequences in vitro. When a 221-bp fragment from the *SIC1* promoter was used in a gel retardation assay with Swi5, a single protein-DNA complex was observed (data not shown). This probe contains two possible Swi5 binding sites. Thus, it was not clear whether a single molecule of Swi5 bound to this probe or whether two Swi5 molecules bound in a highly cooperative fashion. To distinguish between these possibilities, we prepared probes of similar sizes from *SIC1*, *HO* (site B), and *CTS1*. Only one molecule of Swi5 binds to this *HO* probe, while two molecules of Swi5 bind noncooperatively to *CTS1* (7). These three probes were incubated with increasing concentrations of purified Swi5 protein, and the protein-DNA complexes were analyzed by gel retardation (Fig. 5B). The mobility of the Swi5-*SIC1* protein-DNA complex was similar to that of the Swi5-*HO* complex, but quite different from that of the Swi5-*CTS1* complex. This suggests that a single molecule of Swi5 binds to the *SIC1* promoter fragment. Interestingly, the affinity of Swi5 for the *SIC1* probe is threefold higher than that for the *HO* probe.

Gel retardation experiments were also carried out with the Ace2 protein and the three promoter fragment probes. Unlike

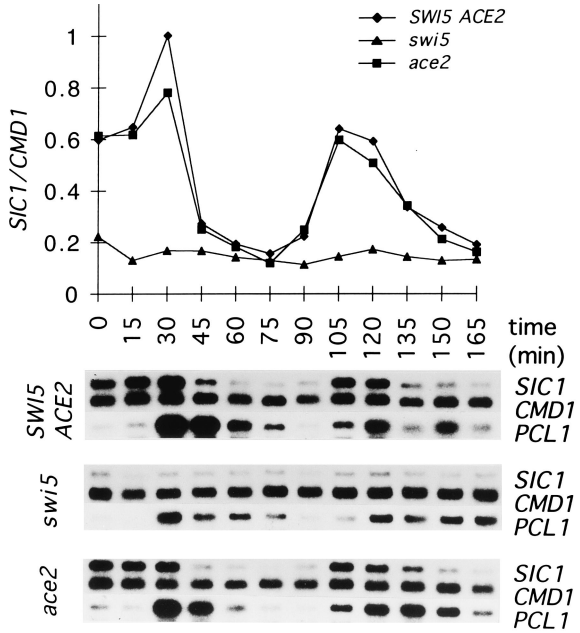

FIG. 4. *SIC1* transcript levels in *SWI5 ACE2*, *swi5*Δ, and *ace2*Δ strains after a release from the *cdc15* cell cycle block. *SWI5 ACE2* (K1993), *swi5*Δ (K3774), and *ace2*Δ (K3659) strains, all containing a *cdc15-2* mutation, were exponentially grown at 25°C, shifted to 37°C for 160 min to arrest cells in late anaphase, and then released from the *cdc15* block to 25°C. Samples for Northern analysis were taken immediately after the release (time point zero minutes) and later as indicated. Periodic expression of the *PCL1* gene confirms the synchrony of progression through the cell cycle. The *CMD1* transcript is the internal loading control. Numbers on the *y* axes represent the *SIC1*/*CMD1* ratio, where the maximum value of the wild type at 30 min was labeled 1.0.

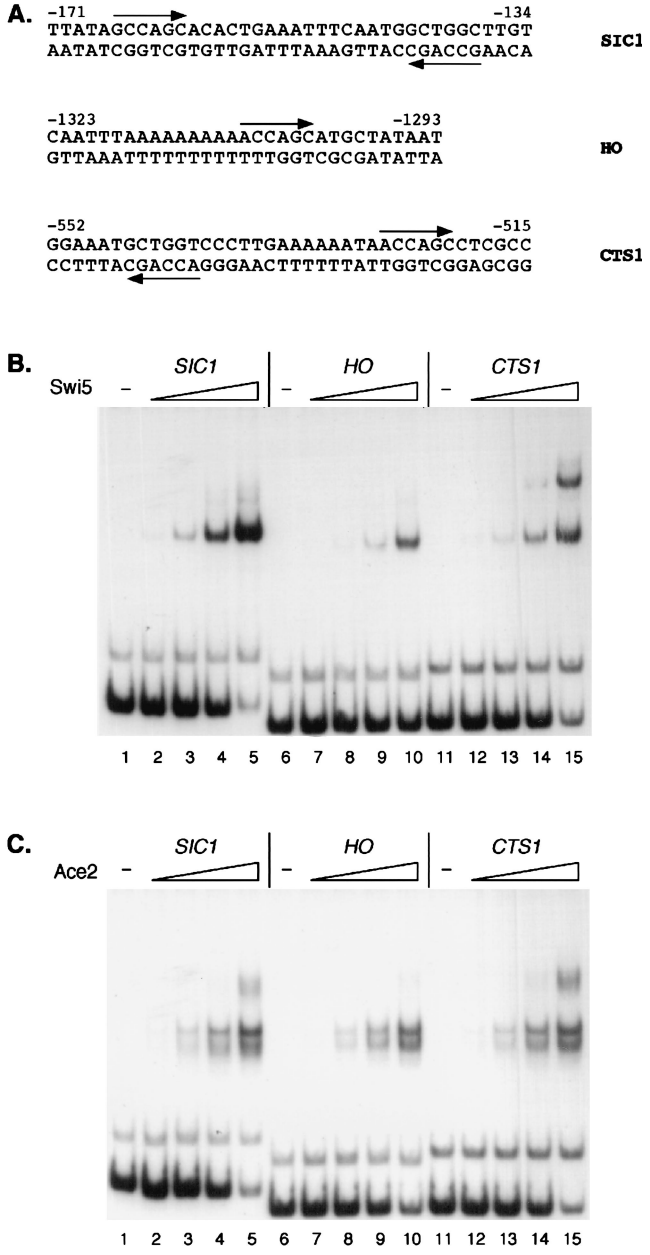

FIG. 5. Swi5 and Ace2 bind to the *SIC1* promoter. (A) The sequence of the *SIC1*, *HO* (site B), and *CTS1* promoters in the region of the Swi5 and Ace2 binding sites is shown. The numbering indicates the position relative to the translational start site. The arrows indicate the position and relative orientations of a six-nucleotide sequence conserved between the Swi5 and Ace2 binding sites. (B) In vitro binding of Swi5 to *SIC1* (lanes 1 to 5), *HO* (lanes 6 to 10), and *CTS1* (lanes 11 to 15) promoter probes. The following amounts of Swi5 were added to each binding reaction mixture: lanes 1, 6, and 11, no added protein; lanes 2, 7, and 12, 28 ng of Swi5; lanes 3, 8, and 13, 84 ng of Swi5; lanes 4, 9, and 14, 252 ng of Swi5; and lanes 5, 10, and 15, 756 ng of Swi5. (C) In vitro binding of Ace2 to *SIC1* (lanes 1 to 5), *HO* (lanes 6 to 10), and *CTS1* (lanes 11 to 15) promoter probes. The following amounts of Ace2 were added to each binding reaction mixture: lanes 1, 6, and 11, no added protein; lanes 2, 7, and 12, 8 ng of Swi5; lanes 3, 8, and 13, 24 ng of Swi5; lanes 4, 9, and 14, 72 ng of Swi5; and lanes 5, 10, and 15, 216 ng of Ace2.

the results with Swi5, incubation of the highest concentration of Ace2 with the *SIC1* promoter showed two slowly migrating complexes (Fig. 5C, lane 5). The mobilities of these two bands are comparable to that seen with the *CTS1* probe (Fig. 5C, lane 15), suggesting that two molecules of Ace2 can bind *SIC1*. Additionally, Ace2 binds to all three promoter fragments with similar affinities. Although roughly threefold more Swi5 protein is required than for Ace2 to achieve a specific degree of binding in vitro, we do not know what fraction of each protein preparation is active for DNA binding. Thus, we cannot make any conclusions about the relative affinities of Swi5 and Ace2 for these sites.

To further define the regions bound by Swi5 and Ace2 at the *SIC1* promoter, DNase I footprinting was performed (Fig. 6). With Swi5, there was one region of protection that covered the downstream binding site and extended to include two residues of the upstream site. At the highest protein concentrations, several hypersensitive sites also appeared in the region 5′ of the upstream site (indicated by arrows in Fig. 6). Thus, both the gel retardation and DNase I footprinting assays identified a single binding site for Swi5 on the *SIC1* promoter spanning nucleotides −163 to −133. In contrast to the data for Swi5, the region in the *SIC1* promoter protected from DNase I digestion by Ace2 was larger, extending from nucleotide −172 to nucleotide −133. Thus, Ace2, at the highest protein concentration

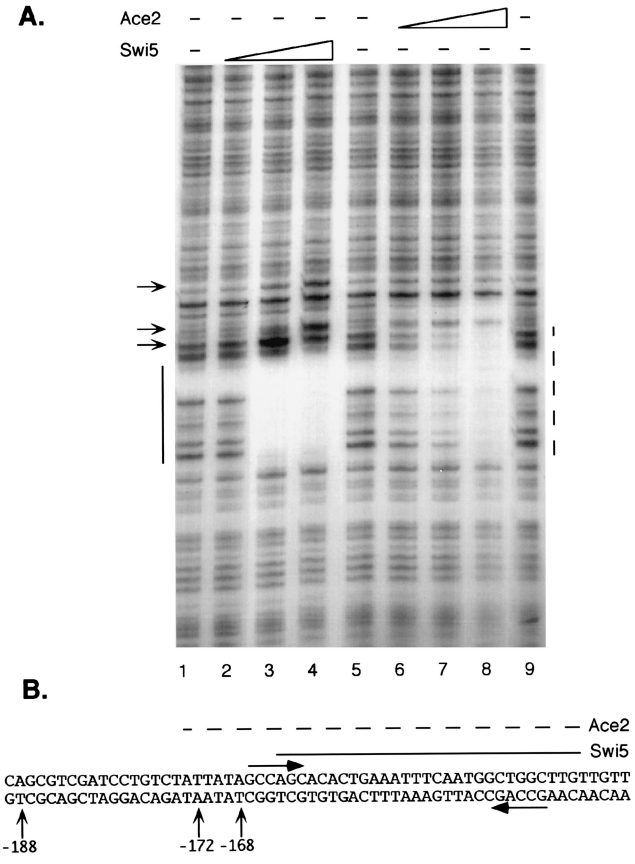

FIG. 6. DNase I footprints at the *SIC1* promoter by Swi5 and Ace2. (A) DNase I protection experiments were performed with an end-labeled fragment from the *SIC1* promoter. No protein was added to the samples in lanes 1, 5, and 9; increasing amounts of Swi5 (98 ng, 294 ng, and 882 ng) were added to the samples in lanes 2 to 4; and increasing amounts of Ace2 (24, 72, and 216 ng) were added to the samples in lanes 6 to 8. The arrows mark hypersensitive sites caused by Swi5 binding, the solid line shows the area protected by Swi5, and the dashed line shows the area protected by Ace2. (B) A summary of the DNase I protection data is presented. The hypersensitive sites and regions protected by Swi5 and Ace2 are indicated, as for panel A. The horizontal arrows indicate the two positions at which six-nucleotide sequences conserved in the Swi5 and Ace2 binding sites are present.

tested, protects both of the putative binding sites from DNase I digestion; at this protein concentration, the gel retardation assay showed that two molecules of Ace2 can bind simultaneously. We conclude that Swi5 and Ace2 can each bind to the *SIC1* promoter, but there are significant differences in how they bind.

DISCUSSION

It has been known for many years that the *CDC4*, *CDC34*, and *CDC53* genes are needed for DNA replication but not for other simultaneous cell cycle events like spindle pole body duplication or bud formation. The discovery that *CDC34* encodes a ubiquitin-conjugating enzyme involved in protein degradation suggested that yeast cells must destroy proteins in order to initiate DNA replication. The study described here was initiated by the finding that Clb/Cdk1 kinases are inactive in *cdc4*, *cdc34*, and *cdc53* mutants. Our working hypothesis was that these mutants were all defective in destroying an inhibitor of Clb/Cdk1 kinases. We set out to identify this inhibitor or factors necessary for its activity by isolating mutations that allow *cdc4* mutants to overcome their G_1 arrest and enter S phase. We identified three genes, *RIC1* to *RIC3*, which are necessary to prevent DNA replication in *cdc4* mutants. *RIC2* proved to be identical to *SIC1*, which encodes the Clb/Cdk1 inhibitor recently shown to be responsible for the G_1 arrest of *cdc4*, *cdc34*, and *cdc53* mutants (25). *RIC1* proved to be identical to *SWI5*, which encodes a transcription factor responsible for activating the *EGT2* (15), *ASH1* (2), *CDC6* (21), and *RME1* (31) genes at the end of mitosis and the *HO* endonuclease gene in late G_1. We have shown that Swi5 and its related factor, Ace2, are both capable of binding to specific *SIC1* promoter sequences in vitro and that they share the task of activating *SIC1* transcription transiently at the end of mitosis. Swi5 is a much more efficient activator of *SIC1* transcription than Ace2, which is opposite to the situation at the *CTS1* promoter, where Ace2 is much more potent, even though both proteins, Swi5 and Ace2, can bind to the *CTS1* promoter in vitro. This specificity in transcriptional activation may be conferred by additional regulatory proteins (7).

Genes regulated by Swi5. *EGT2*, *ASH1*, *CDC6*, *RME1*, and *SIC1* are transcribed transiently as cells undergo anaphase. This pattern is presumably due to the transient accumulation of Swi5 and Ace2 within nuclei at this stage of the cell cycle. Both accumulate in the cytoplasm during G_2 and only enter the nuclei upon inactivation of Clb/Cdk1 protein kinases during anaphase (6, 17, 18). Swi5 is then rapidly degraded during G_1. *HO*, however, is not transcribed until late G_1 because its transcription requires, in addition to Swi5, the late G_1-specific transcription factor *SBF* (3). Thus, *HO* differs from other genes activated by Swi5 in that it does not require a high concentration of Swi5 within the nucleus when it is transcriptionally activated by *SBF*. *HO* is only transcribed in mother cells; however, this is not due to differences in the accumulation of Swi5 in mother and daughter nuclei but instead is due to the preferential accumulation of Ash1 protein in daughter nuclei (2, 28). Therefore, there is no reason to believe that, like *HO*, *SIC1* is preferentially expressed in mother nuclei. Indeed, $p40^{SIC1}$ function seems especially important for daughter cells (19).

Regulation of Sic1 by Swi5 or Ace2 may not be essential for proper timing of cell cycle progression; however, an inappropriate Sic1 level might have more subtle effects, like the ability of cells to establish prereplicative complex on the origins of replication at M/G_1 transition, a process inhibited by the Clb/Cdk1 kinase (20, 24).

Switching cell cycle states. Transcriptional regulation by Swi5 is just one aspect of the system controlling $p40^{SIC1}$ accumulation during the yeast cell cycle. $p40^{SIC1}$ is rapidly degraded during G_2 and M phases, becomes more stable as cells enter G_1, but is again rapidly degraded in late G_1 upon activation of Cdk1 kinase by the Cln1 and Cln2 cyclins (24). It is thought that phosphorylation of $p40^{SIC1}$ regulates its stability both as cells start the cell cycle and during G_2 (22, 25). It is conceivable that Cln/Cdk1 kinases target $p40^{SIC1}$ for proteolysis during late G_1 and S phases, whereas Clb/Cdk1 kinases target its proteolysis during G_2 and M phases. If this is correct, Clb/Cdk1 kinases and $p40^{SIC1}$ live lives of mutual hostility: Clb/Cdk1 inhibits accumulation of $p40^{SIC1}$, whereas $p40^{SIC1}$ inhibits Clb/Cdk1 kinases. The outcome of this battle between Cdk1 and its inhibitor switches as cells move through the cell cycle, or rather key cell cycle transitions are triggered by reversing the roles of victor and vanquished. $p40^{SIC1}$ becomes the victor during anaphase but later loses its supremacy in late G_1. Our discovery that Swi5 regulates transcription of *SIC1* fits nicely within this scheme. Swi5 is prevented from entering nuclei during S, G_2, and metaphase by phosphorylation of its nuclear localization signal by Clb/Cdk1 kinases and only accumulates within the nuclei upon the inactivation of these kinases during anaphase. This is therefore another mechanism by which Clb/Cdk1 kinases inhibit $p40^{SIC1}$ accumulation during S, G_2, and metaphase. It is another means by which changes in the outcome of the battle between $p40^{SIC1}$ and Clb/Cdk1 kinases are reinforced; reductions in the activity of Clb/Cdk1 kinases during anaphase increase *SIC1* transcription and thereby lead to yet lower Clb/Cdk1 kinase levels.

How cells trigger the transition from a state in which Clb/Cdk1 kinases hold sway to one in which $p40^{SIC1}$ rules is not understood. Activation of cyclin B proteolysis via its ubiquitination by the anaphase-promoting complex, or APC (12), presumably plays an important part. Several genes encoding kinases such as *CDC15* (23), *CDC5* (14), and *DBF2* (13) and a Ras-like GTPase, Tem1 (27), are needed for this process and for the efficient accumulation of Swi5 within nuclei, but their mode of action is not understood. More is known about how cells reverse this state of affairs in late G_1. They synthesize specialized cyclins, Cln1 and Cln2, which form complexes with Cdk1 that are only weakly or not at all inhibited by $p40^{SIC1}$ (25). It would not be surprising if yet other genes, for example, *RIC3*, were involved in deciding the outcome of the battle between $p40^{SIC1}$ and Cdk1 kinases.

ACKNOWLEDGMENTS

We thank Christian Dahmann for initial help with the screen and Ralf Jansen, Christian Koch, and Stefan Irniger for comments on the manuscript. Ralf Jansen is also thanked for data presented in Fig. 2. We are also grateful to Etienne Schwob for useful discussions.

D.J.S. was supported by National Institutes of Health grant GM48624, and K.N. was supported by the Austrian Industrial Research Promotion Fund.

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