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Role of Swi4 in Cell Cycle Regulation of *CLN2* Expression

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Expression of the *Saccharomyces cerevisiae CLN1* and *CLN2* genes is cell cycle regulated, and the genes may be controlled by positive feedback. It has been proposed that positive feedback operates via Cln/Cdc28 activation of the Swi4/Swi6 transcription factor, leading to *CLN1* and *CLN2* transcription due to Swi4 binding to specific sites (SCBs) in the *CLN1* and *CLN2* promoters. To test this proposal, we have examined the effects of deletion either of the potential SCBs in the *CLN2* promoter or of the *SWI4* gene on *CLN2* transcriptional control. Deletion of a restriction fragment containing the identified SCBs from the promoter does not prevent cell cycle regulation of *CLN2* expression, although expression is lowered at all cell cycle positions. A promoter containing a 5.5-kb plasmid insertion or an independent 2.5-kb insertion at the point of deletion of the SCB-containing restriction fragment also exhibits cell cycle regulation, so involvement of unidentified upstream SCBs is unlikely. Neither Swi4 nor the related Mbp1 transcription factor is required for cell cycle regulation of the intact *CLN2* promoter. In contrast, Swi4 (but not Mbp1) is required for correct cell cycle regulation of the insertion/deletion promoter lacking SCB sites. We have extended previous genetic evidence for involvement of Swi4 in some aspect of *CLN2* function: a mutant hunt for *CLN2* positive regulatory factors yielded only *swi4* mutations at saturation. Swi4 may bind to nonconsensus sequences in the *CLN2* promoter (possibly in addition to consensus sites), or it may act indirectly to regulate *CLN2* expression.

Start in the budding yeast cell cycle is the time in late G_1 at which cells become committed to cell cycle progression (29). Start is controlled by the Cdc28 protein kinase, which is activated at Start by the G_1-acting cyclin homologs Cln1, Cln2, and Cln3 (23, 30). Cln1 and Cln2 are cyclically expressed in the cell cycle; transcript and protein levels peak around the time of Start (34, 36). Cln3 levels vary little in the cell cycle (34, 35). The strong increase in *CLN1* and *CLN2* transcription around the time of Start requires the activity of Cdc28 and at least one of the three Cln proteins (9, 11), suggesting that a positive feedback loop may control their transcription.

The Swi4/Swi6 transcription factor activates transcription of the *HO* endonuclease gene at Start (1, 6, 16, 22, 25). Swi4 and Swi6 are components of a complex that binds to a consensus sequence (SCB) repeated 10 times in the *HO* promoter (1, 21, 28). The SCB sequence is sufficient to confer cell cycle-regulated expression (6). Swi4 is the DNA-binding component of the Swi4/Swi6 complex, and Swi6 binds to Swi4 (28). It was proposed that Swi4/Swi6 regulates *CLN1* and *CLN2* transcription (24, 26): there are SCB consensus sequences in the *CLN1* and *CLN2* promoters, and genetic interactions between *SWI4*, *SWI6*, *CLN1*, *CLN2*, and *CLN3* were observed. Swi4/Swi6 complexes bind specifically to these sequences from the *CLN2* promoter in vitro (24, 26). Reductions in *CLN1* and *CLN2* RNA levels were observed in *swi4* mutant strains (24, 26).

The Mbp1 protein is related to Swi4 (18); it interacts with Swi6 to make the DSC1/MBF transcription factor (10, 18–20), which functions to regulate transcription of genes involved in DNA synthesis, by binding to the MCB consensus; the MCB site is sufficient to confer cell cycle-regulated expression. It was proposed that Swi4 and Mbp1 might cooperate in regulating *CLN1* and *CLN2* transcription (18).

We report analysis of the requirement for Swi4-binding sites, Mbp1, and Swi4 for regulating *CLN2* transcription.

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MATERIALS AND METHODS

Plasmid constructions. Standard methods were used throughout (2). JM206 contained *cln2-delxs* (9) sequences from 1.2 kb 5′ to the initiation codon to the *Hin*dIII site at position 1852 (9, 14), cloned in RS306 (*Sal*I to *Hin*dIII) (33). The *Sal*I site was from the pBR322-based cloning vector in which *CLN2* was initially isolated (15). p*cln2*::*URA3* was constructed by digesting JM206 with *Nru*I and *Bcl*I and ligating in a *Sma*I-*Bam*HI fragment containing *URA3*. The pBR322 sequence was removed from JM206 by PCR amplification of an *Xho*I-*Sph*I fragment containing *CLN2* promoter sequence; this fragment was cloned into *Xho*I-*Sph*I-digested JM206 to produce FC206-1. The PCR primers used were FCPR1, TTACTCGAGCACGATGCGTCCGGCGTAGA; and CLN2 d273N, ATTATTCTCGAGTTAAACAAGTAGCCAGAGG CT. FC206-2 (containing the *cln2-delNS* promoter) was constructed by digesting FC206-1 with *Nru*I and *Sph*I, blunting the ends with T4 DNA polymerase, and ligating. The resulting plasmid was shown by restriction mapping to contain an approximately 100-bp deletion, with loss of the *Nru*I and *Sph*I sites. FC206-4 was constructed in several steps. First, a *GPD* upstream activation sequence-TATA box fragment (3) was produced by PCR amplification from a plasmid (pT7T3 19u-HIS2) provided by D. Lew, containing 400 bp of the *GPD* promoter and TATA box. The oligonucleotides used were the T3 primer, priming in the vector 5′ of the *GPD* sequence, and FCPR3 (TTATTAGCATGCAAGACTAACTATAAAAGTA GA). The amplification introduced a synthetic *Sph*I site 82 nucleotides 3′ to the putative *GPD* TATA box. The amplification product was digested with *Sma*I (cutting in the polylinker 5′ to *GPD* sequences) and *Sph*I and cloned into *Nru*I-*Sph*I-digested FC206-1, to produce FC206-3. A fragment starting with an *Sph*I site 14 nucleotides 5′ of the *CLN2* transcriptional start site at position 553 (data not shown) and ending at the *Hin*dIII site in the *cln2-delxs* coding sequence (at position 1852) was produced by PCR amplification with primer FCPR2 (TTATTAGCATGCAATAGATAATATCAAGGAC AA) and the T7 primer priming in the RS306 polylinker,

followed by digestion with *Sph*I and *Hin*dIII. This fragment was cloned into *Sph*I-*Hin*dIII-digested FC206-3, to produce FC206-4. This construction positions the *GPD* TATA box (3) at the same spacing relative to the *CLN2* initiator as is observed between the putative *CLN2* TATA box and initiator. The *Bam*HI site in the FC206-3 polylinker (derived from RS306) was removed by *Eco*RI and *Xba*I digestion, Klenow fragment fill-in, and ligation, to produce FC206-5. FC206-6 was produced by digesting both YRp7 (2) and FC206-5 with *Bam*HI and *Sph*I and ligating appropriate fragments to replace the *GPD* insert from FC206-5 with YRp7. FC206-8 was produced similarly by recombining *Nru*I- and *Sph*I-digested FC206-1 and YRp7. FC206-13 was produced by ligating the polylinker from M13mp19 from *Bam*HI to *Sph*I to *Bam*HI- and *Sph*I-digested FC206-5. FC206-14 was produced by ligating an *Sal*I restriction fragment containing *HIS3* and the bacterial kanamycin resistance gene (from pJA50, provided by S. Elledge) into *Sal*I-digested FC206-13. FC206-7a, -7b, -7c, and -7d were produced by ligating a synthetic restriction fragment containing three copies of the synthetic SCB sequence described elsewhere (6), bounded by *Bam*HI and *Sph*I sites (sticky ends protruding), to *Bam*HI- and *Sph*I-digested FC206-5. This synthetic restriction fragment was produced by phosphorylating and hybridizing two oligonucleotides: FCPR5, GATCCACGAAAATCGATCCACGAAAATCGATCCAC GCGAAAACATG; and FCPR6, TTTTCGTGGATCGAT TTTCGTGGATCGATTTTCGTG. The putative *CLN2* TATA box was removed from JM206 in two steps. First, JM213 was constructed by PCR amplification from JM206 by use of CLN2d243 (TTATTACTCGAGGCAATCGACTCTGGTAA CTAT) and CLN2p619 (CAACATTTAAAACGTAATCAT) followed by *Bcl*I-*Xho*I digestion and subcloning into *Bcl*I-*Xho*I-digested JM206. This replaced material from the JM206 polylinker to the 3′ border of the TATA region (see Fig. 1, 2, and 3) with an *Xho*I site. An *Xho*I fragment containing the 5′ CLN2 promoter region up to the 5′ border of the TATA region was produced by PCR amplification with CLNN2d273N (see above) and CLN2d613 (TTATTACTCGAGCGGAAAT-CATCGCGAAATTTG); this fragment was cloned into JM213 to produce JM217, in which the TATA region was replaced by an *Xho*I site. The TATA box deletion was recombined with the YRp7 insertions in FC206-6 and FC206-8 (see above) by exchanging the *Sph*I-*Bcl*I fragment of JM217 for the *Sph*I-*Bcl*I fragments of FC206-6 and FC206-8, resulting in clones MH206-9 and MH206-10. To make a *CLN2* promoter mutant starting at −334, a product of PCR amplification with FCPR7 (TTATTAGCATGCACGCAGAAACGCAGATCCGCC) and FCPR8 (AGACCTGACCATCACCACAG) as primers was produced with FC206-1 as a template and digested with *Sph*I (a site introduced by the PCR, at −334) and *Spe*I (in the *cln2* coding sequence). This fragment was cloned into *Sph*I-*Spe*I-digested FC206-6, to produce FC206-11. In this clone, YRp7 sequence was juxtaposed to position −334 in the *CLN2* promoter.

Yeast strains and introduction of promoter mutations. All strains were isogenic with BF264-15D. *cln1-del*, *cln2-delxs*, *cln3-del*, and the *GAL1*::*CLN3* and *GAL1*::*CLN1* expression cassettes were all described previously (8, 9, 30).

The *swi4*::*LEU2* construct replaced the internal *Bam*HI fragment of *SWI4* with *LEU2*; this construct was provided by B. Andrews and was introduced into the BF264-15D background by one-step gene replacement (29a). The *swi4*::*LEU2* allele was initially introduced into a *cln1 cln2-delxs cln3* p*GAL1*::*CLN3* background; while such strains were viable, they were slow growing and highly abnormal morphologically; it also proved difficult to synchronize these strains (29a). Therefore, the *swi4*::*LEU2* allele was backcrossed three times into an isogenic *cln1 cln2 cln3* p*GAL1*::*CLN1* background; *GAL1*::*CLN1* completely rescued the growth rate and partially rescued cell morphology and synchronizability of *swi4*::*LEU2* strains (data not shown). Backcrossed *swi4*::*LEU2* strains were compared with isogenic *SWI4* siblings from the same backcross. The *mbp1*::*URA3* construct (18) replaced an internal *Eco*RI fragment of *MBP1* with *URA3*.

The *cln2*::*URA3* allele was introduced by one-step gene disruption in a strain (1315-23D) of genotype *cln1-del CLN2 cln3-del leu2*::*LEU2*::*GAL1*::*CLN3*. Ura$^+$ transformants in which *cln2* was disrupted became galactose dependent for viability. One such strain, 1315-23Db, was chosen for further work. The wild-type *cln2* promoter, the *cln2-delNS* promoter, and the *cln2-delNS/SCB* promoters were introduced into 1315-23Db by cotransformation of linear fragments containing the mutant promoters driving the *cln2-delxs* nonfunctional coding sequence and a *TRP1* episomal vector. Trp$^+$ transformants were tested on 5-FOA to identify Ura$^-$ derivatives. On blot hybridization, these Ura$^-$ derivatives all proved to contain the indicated promoter driving the *cln2-delxs* coding sequence.

The TATA deletion constructs in JM217, MH206-9, and MH206-10 and the YRp7-substituted promoters in FC206-6, FC206-8, and FC206-1 were introduced by duplicative integration by digestion of the plasmids with *Bcl*I (cutting in the *cln2-delxs* coding region) and transformation of *ura3 cln1-del cln2-delxs cln3-del leu2*::*LEU2*::*GAL1*::*CLN3* yeast to Ura$^+$. Following integrative duplication, popouts were selected on 5-FOA and screened by Southern blot hybridization to identify yeast strains in which the mutant promoter had replaced the wild type.

Strain 1531-8B (*cln1-del cln2*::*URA3 cln3-del leu2*::*LEU2*::*GAL1*::*CLN3 his3 HIS2*) was constructed by mating and tetrad analysis. This strain was transformed with *Xho*I- and *Eco*RI-digested FC206-14, and His$^+$ transformants were selected. Transformations in which *cln2-delNS/HIS3/kan*^{R} had replaced *cln2*::*URA3* were identified by a Ura$^-$ phenotype.

Cell cycle synchronization. *cln1 cln2 cln3 GAL1*::*CLN3* strains were synchronized in G_1 by incubation in raffinose and released into synchronous cell cycles by galactose addition, as described elsewhere (9).

DNA and RNA analysis. DNA and RNA extraction and analysis were as described elsewhere (2, 9, 17).

Mutant hunt. A strain of genotype *cln1 CLN2 cln3* p*URA3/GAL1*::*CLN3* was mutagenized with ethyl methanesulfonate as described elsewhere (32) and plated on yeast extract-peptone-galactose plates at 100 to 200 colonies per plate. When colonies were grown, they were replica plated to yeast extract-peptone-dextrose medium, and colonies unable to propagate on glucose medium at 38°C were identified. Those strains that were unable to propagate solely because of inviability on glucose (unrelated to expression of *GAL1*::*CLN3*) were identified by their ability to grow on 5-fluoro-orotic acid plus galactose medium (4) and discarded. We established a quantitative criterion for tightness of these mutants: less than 1 viable colony on glucose medium per 10^4 viable colonies on galactose medium. This criterion was important in excluding a large number of leaky mutants. Mutants were mated to the wild type, and 2:2 segregation of the mutant phenotype was established (32). This procedure also generated complementation testers for the mutants. The mutant hunt was carried out at 38°C in the hope of identifying temperature-sensitive alleles of the complementation groups. No tight temperature-sensitive alleles were recovered; however, we observed that the *swi4* alleles recovered (see below) were generally leakier at 30°C than at 38°C.

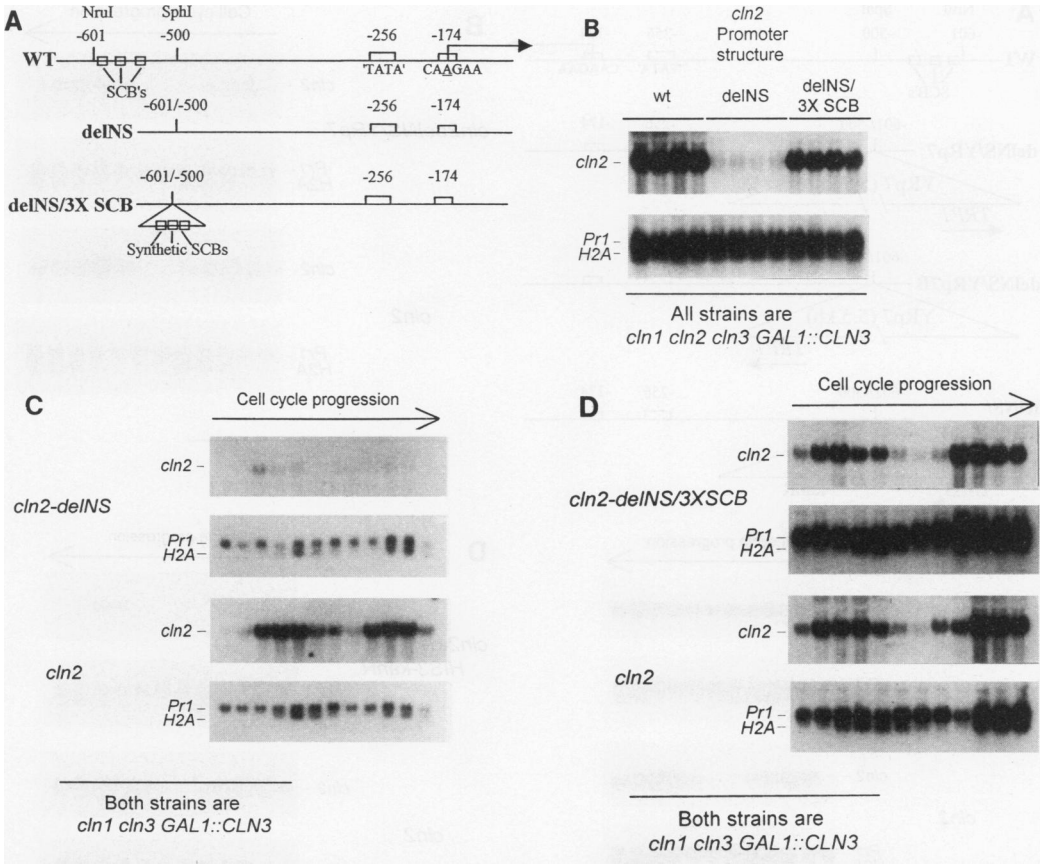

FIG. 1. Expression of *cln2* from a promoter lacking SCB elements. (A) Structures of wild-type and mutant promoters. The *Nru*I-*Sph*I fragment containing the identified SCBs (24, 26) was deleted in *cln2-delNS* (second line). In *cln2-delNS/3XSCB* (third line), three synthetic SCB sequences were inserted at the point of this deletion. These promoters were substituted for the wild-type promoter, driving the defective reporter *cln2* coding sequence from the *cln2-delxs* allele (9). (B) Four independently derived clones with either the wild-type promoter, the *cln2-delNS* promoter, or the *cln2-delNS/3XSCB* promoter were tested for *cln2* RNA levels in asynchronous culture. *Pr1*, protein 1 mRNA; *H2A*, histone H2A mRNA. Strains were isogenic and were *cln1 cln2-delxs cln3 GAL1*::*CLN3*. (C) *cln1 cln2-delxs cln3 GAL1*::*CLN3* strains were blocked in G_1 by incubation in raffinose medium and then released into synchronous cell cycles by addition of galactose (9). In this experiment, one strain (1315-23D-1, lower panels) contained the wild-type *cln2* promoter, and the other (1315-23D-2, upper panels) contained the *cln2-delNS* promoter. These strains were isogenic. Samples were extracted every 12 min. RNA blots were probed with *cln2* probe or with a probe detecting both protein 1 (*Pr1*) and histone H2A mRNA (27). The former is cell cycle constitutive and serves as a loading control; the latter is highly expressed in S phase. In all experiments reported here, the first and second cycles of bud emergence corresponded roughly with the rise in histone H2A mRNA levels (data not shown). (D) The experiment was performed exactly as in C, except that a strain containing *cln2-delNS/3XSCB* was used.

RESULTS

Deletion of Swi4-binding sites. Three potential SCB elements were identified in the *CLN2* promoter (24, 26, 28), within a 101-bp *Nru*I-*Sph*I restriction fragment (14) (Fig. 1). We deleted this fragment from the chromosome, producing the *cln2-delNS* allele (Fig. 1). We did this in a *cln1-del cln2-delxs cln3-del GAL1*::*CLN3* or *GAL1*::*CLN1* background, in order to assay *CLN*-dependent *cln2* transcription (the nonfunctional *cln2-delxs* coding sequence contains a deletion in the cyclin homology region but produces a properly regulated transcript [9]). The deletion resulted in a strong defect in *CLN2* transcription in asynchronous culture (Fig. 1). Introduction of three tandem SCB sequences (6) in place of the deleted fragment restored a high level of *cln2* expression (Fig. 1), consistent with the idea that low expression from the *cln2-delNS* promoter was due to loss of SCB elements from the promoter.

Surprisingly, *cln2* expression from the *cln2-delNS* promoter is cell cycle regulated, with timing similar to that of the wild type (Fig. 1). *cln2* expression from the synthetic SCB-containing promoter was also cell cycle regulated (Fig. 1), at a much higher level of expression.

Cell cycle regulation of *cln2* transcription from the promoter with the SCBs deleted could be due to additional unidentified SCB elements farther upstream than the regions sequenced (14, 24). To attempt to insulate the *cln2-delNS* promoter from potential upstream regulatory sequences, we inserted most of the YRp7 plasmid (a 5.5-kb pBR322 derivative containing *TRP1* [2]) in the chromosome in place of the 101-bp *Nru*I-*Sph*I fragment containing the identified SCBs (*cln2-delNS/YRp7*

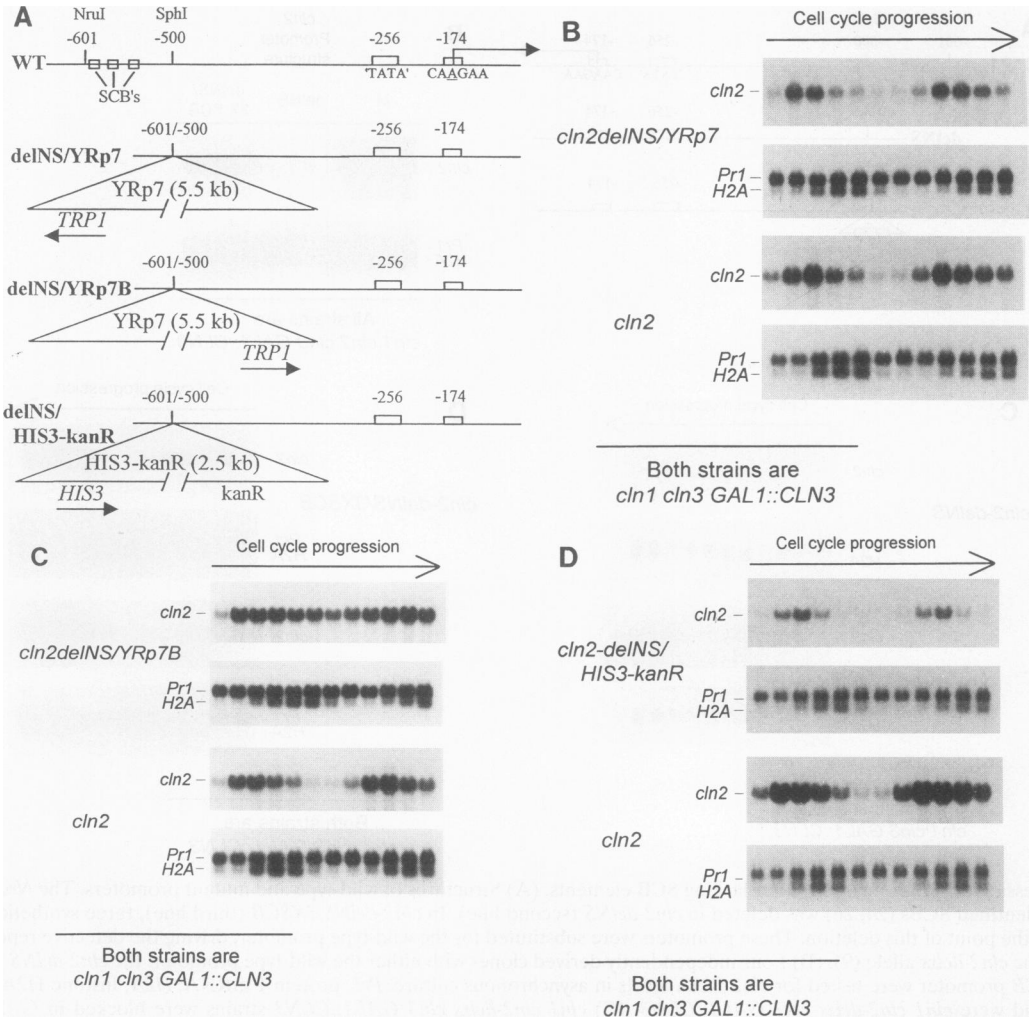

FIG. 2. Substitution of heterologous sequences for SCBs in the *cln2* promoter yields regulated expression. The experiments were done exactly as described for Fig. 1B. (A) Structures of mutant promoters. YRp7 (second and third lines; the third is orientation B): almost the complete 5.6-kb YRp7 plasmid (*TRP1* gene in pBR322 [2]) was inserted at the indicated position. *HIS3-kan*^{R} (fourth line): a 2.5-kb cassette containing *HIS3* linked to the kanamycin resistance gene (from plasmid pJA50, provided by S. Elledge) was inserted at the indicated position. These promoters were substituted for the wild-type promoter, driving the defective reporter *cln2* coding sequence from the *cln2-delxs* allele (9). (B) One strain contained the *cln2-delNS/YRP7* promoter. (C) One strain contained the *cln2-delNS/YRP7B* promoter. (D) One strain contained the *cln2-delNS/HIS3-kan*^{R} promoter. Abbreviations are as for Fig. 1.

[Fig. 2]). This deletion/insertion would place any unidentified upstream SCB elements 5.5 kb farther upstream from the *cln2* transcriptional start site than they are in the wild-type promoter. This promoter directs cell cycle-regulated *cln2* expression (Fig. 2). RNA from this promoter was correctly initiated, as shown by primer extension analysis (data not shown). We constructed a promoter in which YRp7 was inserted in the same position as in *cln2-delNS/YRp7* but in the opposite orientation (Fig. 2). Cell cycle regulation of *cln2* expression was observed with this promoter as well, although this promoter showed less of a drop in *cln2* RNA levels late in the cell cycle (Fig. 2D).

We recombined these deletion/insertion promoters with the intact *CLN2* coding sequence and examined the function of the mutant gene. *CLN2-delNS/YRp7* and *CLN2-delNS/YRp7B* resulted in complete viability in a *cln1 cln3* background; cell volume (a sensitive indicator of *CLN* gene function [7, 15, 34]) was similar in these strains to that in *cln1 CLN2 cln3* controls (data not shown).

We were concerned that sequences present in YRp7 might have an unanticipated impact on *CLN2* regulation. Therefore, we constructed a *cln2* promoter with an *HIS3/kan*^{R} cassette inserted in place of the *Nru*I-*Sph*I fragment. This 2.5-kb insertion has no DNA sequences in common with YRp7. The *cln2-delNS/HIS3-kan*^{R} promoter showed significant cell cycle regulation, albeit at a low level of expression (Fig. 2C). Thus, although the different insertions have subtly different properties, cell cycle regulation of *CLN2* is observed with all three.

In these constructs, YRp7 or *HIS3/kan*^{R} is inserted between positions −601 and −500 (Fig. 2). There are no sequences matching the SCB consensus (24, 26) between −500 and the initiation site (14). Therefore, it appears unlikely that any SCB sequences are required for cell cycle control of *CLN2* transcription, although sequences in the SCB-containing −600 to −501 restriction fragment clearly contribute strongly to the overall level of *CLN2* expression. Thus, there is probably another element in the minimal −500 promoter that is capable of providing cell cycle-regulated expression. Preliminary re-

sults (data not shown) suggest that further deletion of this promoter to −334 reduces but does not eliminate cell cycle regulation of *CLN2* without affecting initiation site selection. We cannot rule out the possibility that upstream SCBs that can act through large insertions of heterologous DNA are involved, nor can we rule out the possibility that there are Swi4-binding sites in the sequence between −500 and the initiation site that are not recognized by looking for the SCB consensus (24, 26). Another possibility is that some or all of the residual control of *CLN2* RNA abundance in these promoter mutants is posttranscriptional. However, substitution of the *GPD* upstream activation sequence-TATA region (3) for the *CLN2* promoter-TATA region resulted in completely cell cycle-constitutive expression of *CLN2* RNA in an α-factor block-release experiment (data not shown), suggesting that there is no significant contribution of posttranscriptional control to cell cycle regulation of *CLN2* mRNA abundance. However, because of the very low level of expression of *cln2* RNA from this construct, we were unable to determine if correct initiation sites were employed; thus, this result cannot be fully evaluated.

Evaluation of a homologous sequence in the *CLN1* and *CLN2* promoters. We noted a homologous region between the *CLN1* and *CLN2* promoters, including potential TATA box elements (Fig. 3). Deletion of this element resulted in abnormal transcript mobility on gel electrophoresis, as expected for deletion of a functional TATA box (13): the major transcript migrated faster than wild-type *cln2* RNA (data not shown). However, strong cell cycle regulation of the faster-migrating transcript was still observed (Fig. 3A). This element might still provide cell cycle control if the control were redundant with control from the SCB sequences. Therefore, we combined the deletion of this sequence with the deletion/insertion of YRp7 described above. Effective regulation of these double-mutant promoters, with timing similar to that of the wild type, was observed (Fig. 3), despite the abnormality of electrophoretic mobility of *cln2* transcripts produced. For both of the YRp7 constructs, a slowly migrating transcript that did not show appropriate cell cycle regulation was produced upon TATA box deletion.

Cell cycle regulation of *CLN2* transcription in the absence of Swi4. We constructed *cln1 cln2-delxs cln3* p*GAL1*::*CLN1* strains that were either *SWI4* or *swi4*::*LEU2*. The *swi4*::*LEU2* allele deletes the coding sequence for amino acids 115 to 835 (1), disrupting the DNA-binding domain (amino acids 36 to 155 [28]). We observed significant cell cycle regulation of *cln2* RNA in *swi4*::*LEU2* strains (Fig. 4). This was true despite an overall reduction in cell cycle synchrony in the *swi4*::*LEU2* strain, as indicated by the histone H2A control for degree of cell cycle synchrony (Fig. 4) and by decreased synchrony of bud emergence in *swi4*::*LEU2* strains compared with *SWI4* controls (data not shown).

Surprisingly, when we tested the *cln2-delNS/YRp7* construct in a *swi4*::*LEU2* strain, we found that deletion of *SWI4* largely eliminated cell cycle regulation of this promoter in the first cell cycle following release (Fig. 4B). This result was unanticipated, since this promoter mutation was designed to eliminate Swi4-binding sites. The relevance of this observation to control of *CLN2* transcription from the intact promoter by Swi4 is unclear.

The delayed rise in *CLN2* transcription observed in the *swi4*::*LEU2 cln2-delNS/YRp7* strain in this experiment was reproducible (three strains tested). We do not have an explanation for this at present.

Cell cycle regulation of *CLN2* in the absence of Mbp1. The Mbp1 transcription factor is related to Swi4 and has been proposed to cooperate with Swi4 in *CLN2* regulation (18). We tested *mbp1*::*URA3* strains for regulation of transcription of *CLN2* either from the intact promoter or from the *delNS/YRp7* promoter. The *mbp1*::*URA3* strains showed regulation similar to that of the *MBP1* strains (Fig. 5). Thus, at least in *SWI4* strains, Mbp1 is not required for *CLN2* regulation. However, it may be required in the absence of Swi4 (18). *CLN1* cell cycle regulation was shown to be normal in *mbp1* strains (18).

A mutant hunt for factors essential for *CLN2* function. *cln1 CLN2 cln3* strains, but not *cln1 CLN2 CLN3* strains, were inviable in the absence of *SWI4* (24), supporting the idea (24, 26) that Swi4 might be an essential activator of *CLN2*. To ask whether there were other genes that could mutate to give this phenotype, we mutagenized a strain of genotype *cln1 CLN2 cln3 GAL1*::*CLN3* and identified 34 mutants that were inviable when *GAL1*::*CLN3* was turned off by a switch from galactose to glucose (see Materials and Methods).

Seven mutations fell into the *erc1* complementation group. *erc1* mutants exhibited no detectable defect in *CLN2* expression, nor did they display a cell cycle-specific arrest, but rather lysed after multiple cell cycles on glucose medium (data not shown). *erc1* mutations also result in lethality in *cln1 cln2 CLN3* strains (data not shown). Thus, Erc1 is probably not specifically involved in *CLN2* function.

We isolated 27 mutants that gave a G_1 arrest phenotype. Fourteen of these were in the *cln2* gene itself. The remaining 13 mutants were all in the same complementation group (*erc2*), which we identified as *swi4*: (i) *erc2-1* failed to complement *swi4*::*LEU2* and failed to recombine with *swi4*::*LEU2* in meiotic analysis; and (ii) a low-copy-number plasmid that complemented *erc2-1* contained *SWI4* on the basis of partial sequence analysis, and rescuing activity mapped to the *SWI4* region of the plasmid; the insert in this plasmid was shown to map to the *erc2-1* locus (data not shown).

An *erc2-1* (*swi4*) *cln1 CLN2 cln3 GAL1*::*CLN3* strain gave first-cycle arrest as unbudded G_1 cells upon shift to glucose (Fig. 6B and data not shown). *cln1 CLN2 cln3 erc2-1 GAL1*::*CLN3* strains transformed with *CLN1* on a low-copy-number plasmid were essentially inviable on glucose medium (data not shown), consistent with the idea that Swi4 is required for efficient *CLN1* expression (24, 26). *erc2-1 cln1 cln2 CLN3* strains were viable (data not shown), further confirming the specificity of this mutation to *CLN1* and *CLN2* function (however, *swi4* null mutations result in lethality in a *cln1 cln2 CLN3* background [24]).

Since this mutant hunt was carried to saturation (7 *erc1* mutations, 13 *swi4* mutations, and 14 *cln2* mutations, with all mutants accounted for), there are probably few or no other genes that are essential for viability in a *cln1 CLN2 cln3 GAL1*::*CLN3* strain on glucose but are not essential on galactose. This result increases the specificity of the conclusion that *SWI4* is required for solo function of *CLN2* (24). *swi6* deletion is nearly lethal even on galactose medium in such a strain (data not shown) (24), so mutations in this gene could not have been recovered.

We observed a deficit in *CLN2* expression in *erc2* mutant strains when *GAL1*::*CLN3* was off (in glucose medium), but not when *GAL1*::*CLN3* was on (in galactose medium) (Fig. 6A). Thus, a defect in *CLN2* expression correlated with the *erc2* mutant phenotype and, intriguingly, was efficiently rescued by *GAL1*::*CLN3* in the *erc2* mutant strains. This result is consistent with the proposal (34) that *CLN3* is an efficient transcriptional activator of *CLN1* and *CLN2*.

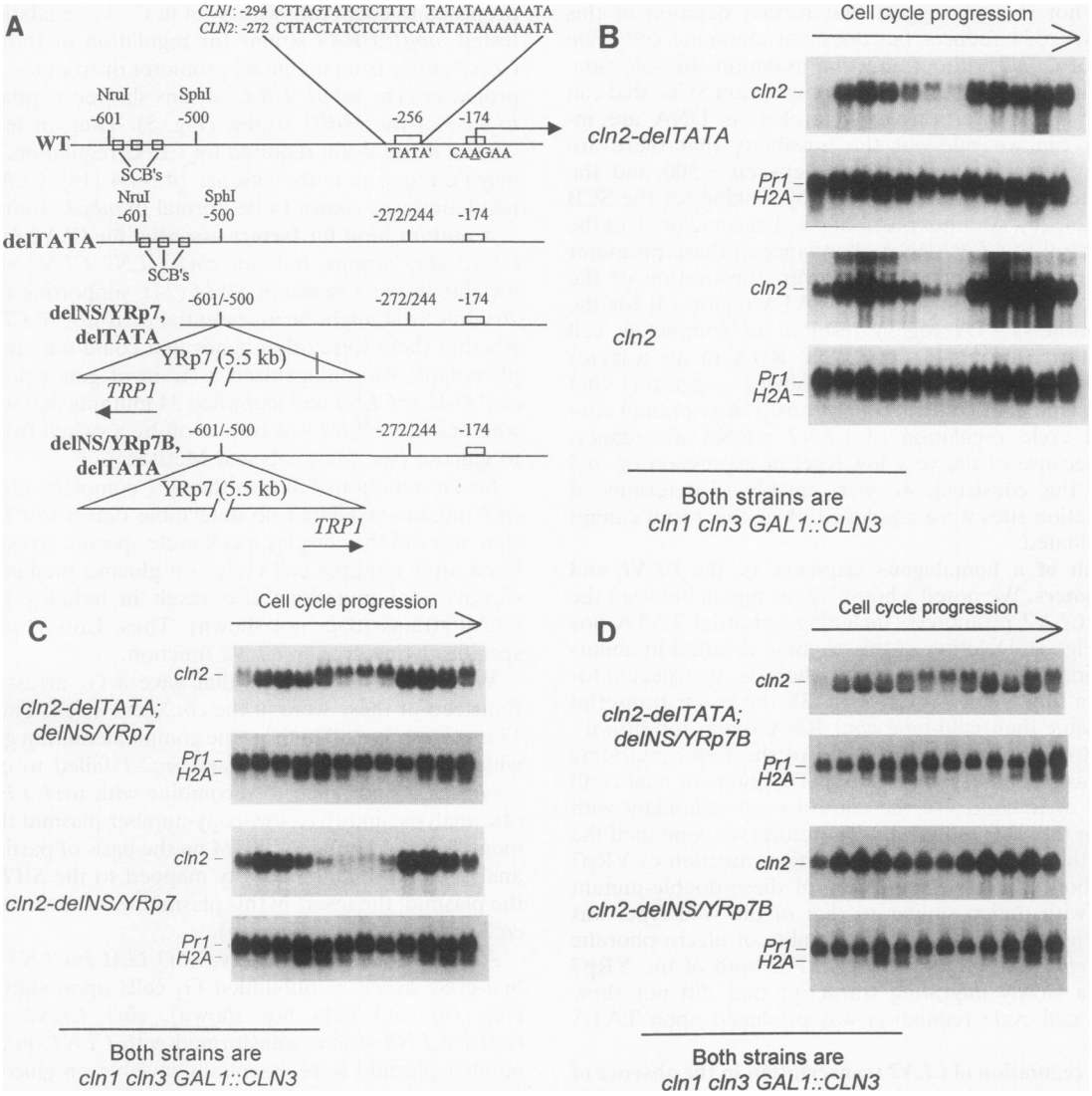

FIG. 3. A sequence homologous between *CLN1* and *CLN2* promoters is required for accurate transcription but not for cell cycle regulation of *CLN2*. (A) A region of homology between the *CLN1* and *CLN2* promoters flanking the putative TATA box is indicated. YRp7 insertions were identical to those in Fig. 2. These promoters were substituted for the wild-type promoter, driving the defective reporter *cln2* coding sequence from the *cln2-delxs* allele (9). (B) Analysis of cell cycle regulation of the *cln2-delTATA* promoter. (C) Analysis of cell cycle regulation of the double mutant promoter containing both the YRP7 insertion and the TATA deletion. (D) Analysis of cell cycle regulation of the double mutant promoter containing both the YRP7 insertion (orientation B) and the TATA deletion. The experiments were done exactly as described for Fig. 1B; abbreviations are as for Fig. 1.

DISCUSSION

Swi4 and the control of *CLN2* expression. These experiments were carried out as a test of the proposal (24, 26) that Swi4 binding to SCBs in the *CLN2* promoter was responsible for cell cycle regulation of *CLN2* expression. The results obtained present difficulties for this proposal: (i) deletion of a fragment containing the identified SCBs lowered expression overall but had little effect on cell cycle control, (ii) deletion of these sequences combined with insertion of either a 5.5-kb insertion (YRp7) or a 2.5-kb insertion (*HIS3/kan*^{R}) at the site of deletion (to increase the distance between the *CLN2* promoter and hypothetical SCBs farther upstream) also had little effect on cell cycle regulation, and (iii) deletion of Swi4 had little effect on cell cycle regulation of *CLN2*.

However, other results support the proposed involvement (24, 26) of Swi4 in *CLN2* regulation. Deletion of a fragment containing the identified SCBs strongly reduced *cln2* expression (Fig. 1). Deletion of *SWI4* nearly eliminated cell cycle regulation of a minimal *CLN2* promoter (Fig. 5). Also, a mutant hunt for essential factors specific for *CLN2* function turned up only *swi4* mutations at saturation; these mutations resulted in defects in *CLN2* expression (Fig. 6A). This mutant hunt and the observation that expression of *CLN1* or *CLN2* from heterologous promoters could rescue lethality caused by *swi4* mutations (references 24 and 26 and data not shown) argue that *CLN1* and *CLN2* expression are defective in *swi4* mutants.

This paradoxical situation could be resolved if the Swi4 requirement for *CLN1* and *CLN2* expression is strong only

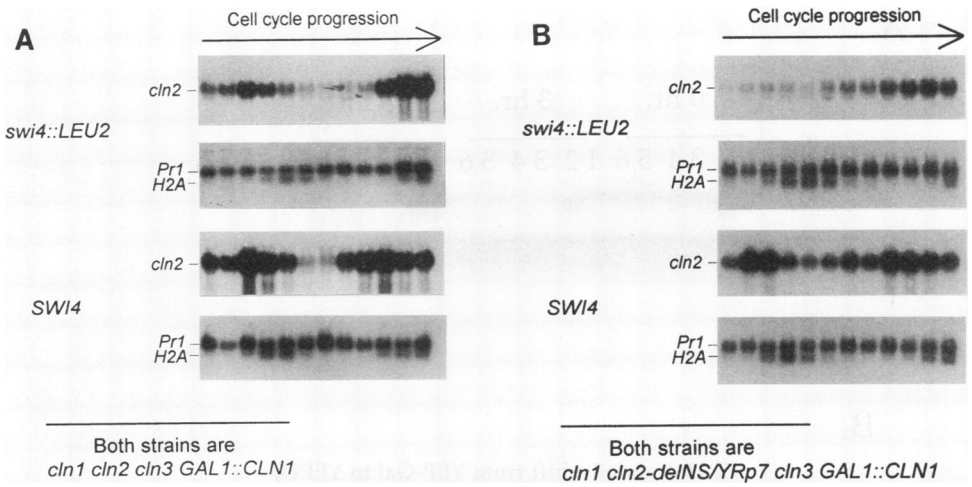

FIG. 4. Swi4 is not required for cell cycle regulation of the intact *CLN2* promoter but is required for regulation of the truncated *cln2-delNS/YRp7* promoter. (A) The experiment was done exactly as described for Fig. 1B, except that both strains contained a p*URA3/GAL1*::*CLN1* plasmid instead of *leu2*::*LEU2*::*GAL1*::*CLN3*. Both strains were *cln1-del cln2-delxs cln3-del*. The lower blots were from an *SWI4* strain, and the upper were from an *swi4*::*LEU2* strain. The strains were isogenic. (B) The experiment was done exactly as described for Fig. 1B, except that both strains contained the *cln2-delNS/YRp7* allele (Fig. 2A). Abbreviations are as for Fig. 1.

under conditions where expression of *CLN* genes is low. Thus, Swi4 might be required for attaining an initial minimum level of expression of *CLN1* and *CLN2*, but once sufficient *CLN* expression occurs, other *CLN*-dependent mechanisms might be able to drive *CLN1* and *CLN2* expression. In the experiments presented here, cell cycle progression is being driven by *CLN* overexpression from the strong *GAL1* promoter; this might eliminate an Swi4 requirement for *CLN2* expression, which would be revealed when the *GAL1*::*CLN* gene was turned off (Fig. 6).

The mode of involvement of Swi4 in the control of *CLN2* expression is not entirely clear. Neither Swi4 nor its proposed binding sites in the *CLN2* promoter are required for *CLN2* regulation. While genetic results (references 24 and 26, and see above) implicate Swi4 in *CLN2* expression and/or function, direct demonstration of a *SWI4* requirement for *CLN2* regulation requires truncation of the *CLN2* promoter (Fig. 4), and this truncated promoter contains no recognizable consensus binding sites for Swi4 (14, 24, 26). Swi4 may act at the *CLN2* promoter (either alone or together with Mbp1 [18]) by binding to nonconsensus sites as well as to the identified SCBs. Alternatively, Swi4 may act indirectly (for example, by activating expression of another transcription factor that regulates *CLN2*).

Similar paradoxical observations have been made on cell cycle regulation of the *SWI4* gene itself, which is transcriptionally regulated with a pattern similar to *CLN2* (5). The *SWI4* promoter contains binding sites for Mbp1/Swi6 (reference 12, and see below), but deletion of these sites lowers expression of *SWI4* without eliminating periodicity (12). Periodicity of *SWI4*

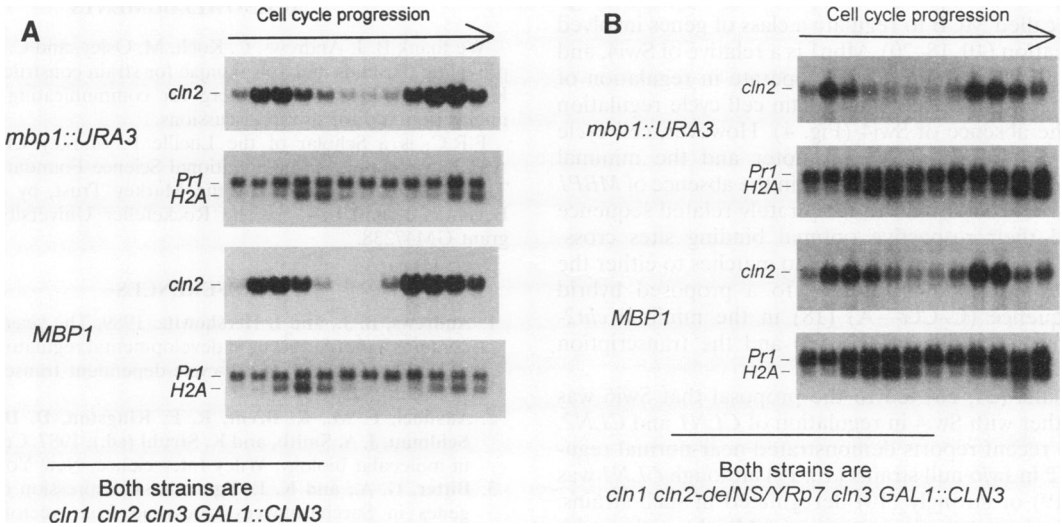

FIG. 5. Cell cycle regulation of *cln2* and *cln2-delNS/YRp7* in the absence of Mbp1. The experiments were done exactly as described for Fig. 1B, except that one strain in each experiment contained the *mbp1*::*URA3* deletion (18). (A) Both strains contained the wild-type *CLN2* promoter. (B) Both strains contained the *cln2-delNS/YRp7* promoter (Fig. 2A). Abbreviations are as for Fig. 1.

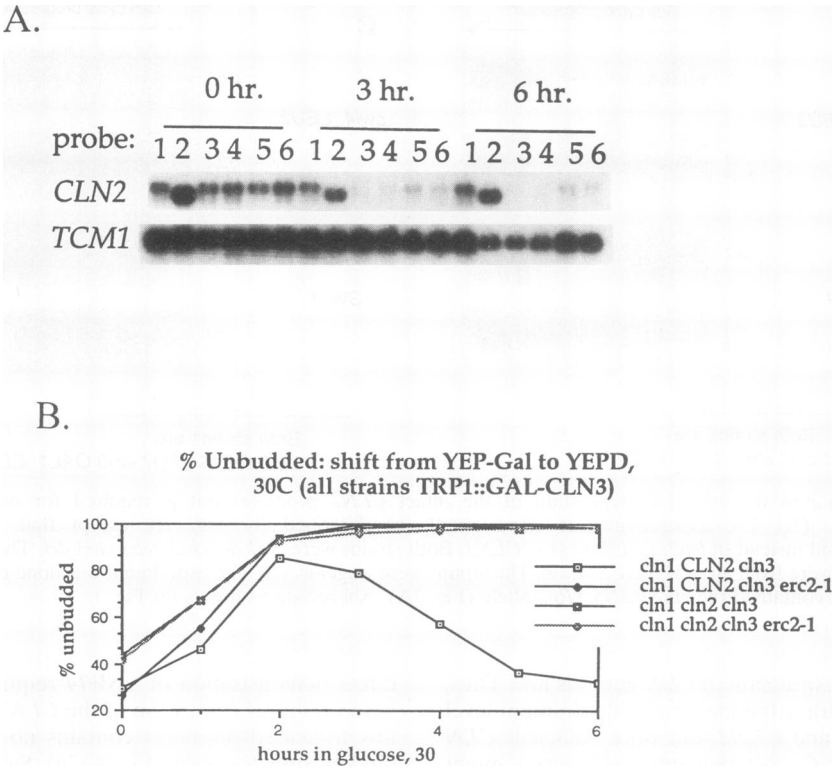

FIG. 6. Characterization of *swi4* mutations resulting in lethality in a *cln1 cln3* background. Mutant strains were isolated as described in the text. (A) Strains were grown in synthetic medium lacking uracil, at 38°C. The strains were shifted to glucose medium for the indicated number of hours, and the levels of *CLN2* transcript were analyzed. The levels of *TCM1* transcript (31) were analyzed as a control. All strains were *cln1 cln3* p*URA3-GAL1::CLN3*. Lanes: 1, *CLN2 SWI4*; 2, *cln2-delxs SWI4*; 3, *CLN2 erc2-1* (*swi4*); 4, *CLN2 erc2-1* (*swi4*); 5, *CLN2 erc2-2* (*swi4*); 6, *CLN2 erc2-3* (*swi4*). (B) All strains were *cln1 cln3 trp1::TRP1::GAL1::CLN3*. Strains were *CLN2 SWI4*, *CLN2 erc2-1* (*swi4*), *cln2 SWI4*, or *cln2 erc2-1* (*swi4*), as indicated. Log-phase cultures grown at 30°C in yeast extract-peptone-galactose were shifted to yeast extract-peptone-dextrose, and at intervals the percentage of unbudded cells (indicative of pre-Start G_1 [29]) was determined.

is dependent on SWI6 (12), however, which differs from the lack of strong dependence of *CLN2* periodicity on *SWI4* (Fig. 4).

Mbp1 and the control of *CLN2* expression. The DSC1/MBF transcription factor, composed of Mbp1 and Swi6, acts through a binding site called MCB to regulate a class of genes involved in DNA replication (10, 18–20). Mbp1 is a relative of Swi4, and Swi4 and Mbp1 were proposed to cooperate in regulation of *CLN2* (18); this hypothesis could explain cell cycle regulation of *CLN2* in the absence of Swi4 (Fig. 4). However, cell cycle regulation of the intact *CLN2* promoter and the minimal *cln2-delNS/YRp7* promoter was normal in the absence of *MBP1* (Fig. 5). Mbp1 and Swi4 bind to moderately related sequence elements, and their respective optimal binding sites cross-compete (10, 18). However, there are no matches to either the Mbp1- or the Swi4-binding sites or to a proposed hybrid consensus sequence (C–CG––A) (18) in the minimal *cln2-delNS/YRp7* promoter (between −500 and the transcription start site at −174) (Fig. 1) (14).

Genetic results (24, 26) led to the proposal that Swi6 was involved together with Swi4 in regulation of *CLN1* and *CLN2*. However, two recent reports demonstrated near-normal regulation of *CLN2* in *swi6* null strains (10, 19) although *CLN1* was moderately (19) or strongly (10) deregulated in such strains. This result is relevant to the question of Mbp1 overlap with Swi4 (18). Since Swi6 is thought to interact both with Swi4 (in the SBF transcription factor) and with Mbp1 (in the DSC1/MBF transcription factor), deletion of Swi6 might be expected to inactivate both factors; and yet *CLN2* is still cell cycle regulated in the absence of Swi6 (10, 19).

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