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Mcm1 Is Required To Coordinate G_2-Specific Transcription in *Saccharomyces cerevisiae*

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In the budding yeast *Saccharomyces cerevisiae*, *MCM1* encodes an essential DNA-binding protein that regulates transcription of many genes in cooperation with different associated factors. With the help of a conditional expression system, we show that Mcm1 depletion has a distinct effect on cell cycle progression by preventing cells from undergoing mitosis. Genes that normally exhibit a G_2-to-M-phase-specific expression pattern, such as *CLB1*, *CLB2*, *CDC5*, *SWI5*, and *ACE2*, remain uninduced in the absence of functional Mcm1. In vivo footprinting experiments show that Mcm1, in conjunction with an Mcm1-recruited factor, binds to the promoter regions of *SWI5* and *CLB2* at sites shown to be involved in cell cycle regulation. However, promoter occupation at these sites is cell cycle independent, and therefore the regulatory system seems to operate on constitutively bound Mcm1 complexes. A gene fusion that provides Mcm1 with a strong transcriptional activation domain causes transcription of *SWI5*, *CLB1*, *CLB2*, and *CDC5* at inappropriate times of the cell cycle. Thus, Mcm1 and a cooperating, cell cycle-regulated activation partner are directly involved in the coordinated expression of multiple G_2-regulated genes. The arrest phenotype of Mcm1-depleted cells is consistent with low levels of Clb1 and Clb2 kinase. However, constitutive *CLB2* expression does not suppress the mitotic defect, and therefore other essential activities required for the G_2-to-M transition must also depend on Mcm1 function.

During a cell division cycle, the temporal order of DNA replication, nuclear division, and cytokinesis can be correlated with the periodic association of cdc2/Cdc28 protein kinase(s) with different types of cyclins (36). Oscillations in the synthesis and degradation of individual cyclins coordinate the different subprograms required for ordered cell division. In the budding yeast *Saccharomyces cerevisiae*, the so called G_1 cyclin Cln3 controls the transcriptional induction of two additional G_1 cyclin genes, *CLN1* and *CLN2*, as well as genes necessary for the initiation of DNA synthesis (13a, 51). Cln1 and Cln2 trigger events important for bud formation (6, 12). Clb5 and Clb6, whose activation depends on functional G_1 cyclins, mainly direct the initiation of DNA synthesis (17, 44). In conjunction with Clb3 and Clb4, they also control spindle formation. Finally, Clb1 and Clb2, the last cyclins to be activated during an *S. cerevisiae* division, are necessary for the initiation of mitosis (4, 19, 47), although they can contribute to the execution of earlier cell cycle events (41, 46).

Cyclins are unstable proteins whose regulated degradation is thought to be required for cell cycle progression. Thus, cyclins represent some of the few known cell cycle-specific proteins that need to be resynthesized during every cell division (19, 46). Consequently, transcriptional control mechanisms are most likely to contribute to cell cycle progression by directing periodic expression of cyclin genes. This has in fact been demonstrated for all known cyclin genes in yeast cells with the exception of *CLN3*. Knowledge of the identity and the mode of action of the relevant transcription factors regulating cyclin gene expression is therefore paramount to understanding the molecular mechanisms that govern the tight temporal activation pattern of different cyclin families. Whereas the factors and signals that guide *CLN1/2* and *CLB5/6* transcription have been studied to a considerable extent, this cannot yet be said for the other two families of B-type cyclins (for a review, see reference 28).

CLB1 and *CLB2* fall into a class of transcription units whose expression is confined to a period from late S phase to M phase. Apart from *CLB1* and *CLB2*, several other known genes exhibit a similar temporal expression pattern: *SWI5*, *ACE2*, and *CDC5* (14, 26, 37). *SWI5* encodes a transcription factor essential for the transcriptional activation of the *HO* endonuclease gene, whereas Ace2, a transcription factor related to Swi5, guides the expression of genes necessary for cell separation (14). *CDC5* encodes a protein kinase whose inactivation leads to an arrest after completion of anaphase (26). The similarities in the transcriptional pattern are further emphasized by the fact that these genes require an active Clb kinase for persistent expression (4) (unpublished data). The surprising observation that mitotic kinase activity is necessary for its own transcriptional induction has led to the proposal that a positive feedback loop operates during cell cycle progression from G_2 into mitosis (4). An important question concerning this model is whether the similarity of regulation between *SWI5*, *CLB1/2*, and *CDC5* is the consequence of *CLB* regulation rather than a reflection of more general regulatory features, such as the use of the same or similar transcription factors.

With regard to the potential *cis*- and *trans*-acting factors that direct G_2-specific transcription, our current understanding is based almost exclusively on studies with *SWI5*. A 55-bp element that is necessary and sufficient for conferring periodic transcription was identified in the *SWI5* promoter. In vitro, a general transcription factor, Mcm1, was found to bind within this region as a partner of an as yet genetically unidentified protein, called Sff (for Swi5 transcription factor). The analysis of *SWI5* promoter mutants showed a good correlation of their ability to form an Mcm1-Sff ternary complex in vitro and to

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TABLE 1. Yeast strains used

Strain	Relevant genotype
GA127	α *mcm1*::*LEU2* Sp*adh-MCM1c*::*URA3*
GA131	**a** *mcm1*::*LEU2* Sp*adh-MCM1c*::*URA3*
GA135	**a** *mcm1*::*LEU2 GAL1-MCM1c*::*URA3*
GA229	**a** *mcm1*::*LEU2 GAL1-UR-MCM1c*::*URA3*
GA231	**a** *mcm1*::*LEU2 GAL1-UR-MCM1c*::*URA3 clb3*::*TRP1 clb4*::*HIS3*
GA504	α *mcm1*::*LEU2 GAL1-UR-MCM1c*::*URA3 rad9*::*URA3* Sp*adh-CLB2*::*TRP1*
GA503	**a** *mcm1*::*LEU2 GAL1-UR-MCM1c*::*URA3 clb3*::*TRP1 clb4*::*HIS3 rad9*::*URA3* Sp*adh-CLB2*::*TRP1*
GA522	α *mcm1*::*LEU2 GAL1-UR-MCM1c*::*URA3 ADH1-CLB2*::*URA3*
GA567	**a** *mcm1*::*LEU2 GAL1-UR-MCM1c*::*URA3 ADH1-CLB2*::*URA3 rad9*::*URA3*
K1534	**a** *bar1*
K1990	**a** *cdc28-4*
K2949	**a** *clb1*::*URA3 clb2*::*LEU2 GAL-CLB2*::*URA3*
K4083	**a** *cdc34-2*

exhibit upstream activation sequence (UAS) activity in vivo. From this strong but still indirect evidence, it was postulated that Mcm1 might be essential for *SWI5* transcription by providing an anchor for Sff (32). Mcm1 is a founding member of the so-called MADS box family of DNA-binding proteins. Like its close mammalian relative, SRF, it regulates gene expression by recruiting coregulatory proteins that participate in both transcriptional activation and repression (48, 49). The current view of how Mcm1 might function has been obtained primarily by studying its role in the determination of mating specificity (for a review, see reference 15). Accordingly, the specificity of transcriptional regulation lies mainly, if not exclusively, with the associated factors rather than with Mcm1 itself. There is evidence that Mcm1 is also important for genes involved in many other cellular processes, such as regulation of arginine metabolism (35). Indeed, a general search for Mcm1 binding sites revealed that many potential promoter regions, including the 5′-flanking region of *CLB2*, contained sequences able to interact with the protein in vitro (29). Originally, *MCM1* was also identified not as a gene important for mating but as a gene required for minichromosome maintenance (34). Even if this phenotype was somewhat suggestive of a role in DNA replication, this proposal has yet to acquire further evidence.

The distinct temporal overlap in the expression of *SWI5*, *CLB1*, *CLB2*, and *CDC5* raised the question of whether the mechanisms suggested for *SWI5* regulation might have been adopted as a more general mode for cell cycle-controlled gene activity in G_2. To clarify this important issue, we provided genetic evidence that Mcm1 is crucial for *SWI5*, *CLB1*, *CLB2*, and *CDC5* expression. To demonstrate that this is due to direct interactions of Mcm1 with the respective promoters, we performed in vivo footprinting studies on *CLB2* and *SWI5*. In addition, we studied how a hyperactive Mcm1 product might influence the transcriptional pattern of these genes. These experiments also addressed the question of when during the cell cycle Mcm1 complexes occupy these promoters. Finally, we evaluated the cell cycle arrest phenotype of Mcm1-depleted cells. We found that it was consistent with the primary defect in cell cycle progression being due to low expression of G_2- and M-specific genes.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study are shown in Table 1. All strains were isogenic or closely related to strain W303-1A (**a** *leu2 trp1 ura3 his3 ade2 can1* [from R. Rothstein]). The *cdc34-2* and *cdc28-4* mutant strains were described by Amon et al. (4). The disruption of the *MCM1* gene was described by Primig et al. (40). Other strains were obtained by sporulating and dissecting the appropriate diploid parents or by standard yeast transformation procedures (45).

Growth conditions. Yeast cells were grown in YEP medium supplemented with 2% glucose (YEPD), 2% galactose (YEPgal), 2% raffinose (YEPraf), or 1% galactose and 1% raffinose (YEPgal+raf). The *cdc28-4* strain was grown to log phase (optical density at 600 nm [OD_{600}] of 0.3) at 23°C in YEPraf. Cell cycle arrest was achieved by shifting the temperature to 37°C for 150 min. After arrest was observed, cells were spun down and resuspended in YEPgal warmed to 37°C. Mcm1-VP16 synthesis was induced for 180 min at 37°C. *cdc34-2* strains were handled similarly except that α-factor (3 μg/ml) was used to synchronize cells prior to the temperature shift. After 180 min, the pheromone-arrested cells were spun down, washed with YEP medium, and resuspended in fresh medium at 37°C. *GAL1-UR-MCM1c* strains were grown in YEPgal+raf at 30°C to log phase. After the medium was changed to YEPD or YEPgal+raf, the culture was split. Nocodazole (15 μg/ml) was added to one of the cultures, and both cultures were incubated in YEPD or YEPgal+raf at 30°C. Samples were taken at the indicated times. For protein kinase assays, cells were synchronized with pheromone for 1 h in galactose- and for an additional hour in glucose-containing medium. After the release into glucose medium, samples were taken at 30-min intervals for Clb kinase assays, fluorescence-activated cell sorting (FACS), and in situ immunofluorescence analyses.

Plasmid constructions. Yeast expression constructs were derivatives of the plasmids described by Gietz and Sugino (20). To construct the ubiquitin-Mcm1c fusion, a *Bam*HI-*Pst*I fragment containing the core region of *MCM1* (codons for amino acids [aa] 1 to 98) was generated by PCR with the following two primers: 5′-TATATGGATCCGGACATATGTCAGACATCGAAGAAGG and 5′-GAGGACTGCAGTCAATCAGGGGCGTTAAGACAGGCC. The fragment was cut with restriction enzymes and ligated with an *Eco*RI-*Bam*HI fragment of the *GAL1* promoter into YIplac211. In the *Bam*HI site between the *GAL1* promoter and the *MCM1* sequence, a *Bgl*II-*Bam*HI PCR fragment that contained sequences encoding ubiquitin and a C-terminal extension for recognition by N-recognin (52) was inserted. The resulting plasmid was called pGA2067. The PCRs were performed with AAGAAGAATTCTAGATCTCTCCCTCCCAC and GGCTGCGCAACTGTTGGG as the primers.

For the construction of an Mcm1-VP16 fusion, an *Eco*RI-*Kpn*I fragment containing the *GAL1* promoter and part of the *MCM1* coding region (aa1 to 154) excised from plasmid pGA1774 (a *TRP1* derivative of plasmid pGA1752 [2]) was cloned into YIplac204 and YIplac211 together with a *Kpn*I-*Pst*I PCR fragment containing the coding region for the 76 C-terminal amino acids of VP16 (50). The following oligonucleotides were used as primers: AATTTCATATGGTAC CCC-CGACCGATGTCA and AAAACTGCAGCCTAGGATCCACCGTACT CGTCAATTCC. The construction of the Max-VP16 fusion protein was described by Amin et al. (1). The coding region for this fusion protein was ligated with the *GAL1* promoter in YIplac204. The construction of the Sp*adh-CLB2* and *ADH1-CLB2* plasmids is documented in Amon et al. (3) and Schwob et al. (43).

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated as described by Cross and Tinkelenberg (11) except that the nucleic acids were dissolved at room temperature instead of at 65°C. RNAs were separated on formaldehyde-agarose gels and transferred to Genescreen filters (DuPont) as recommended by the manufacturer. The blots were processed by the method of Church and Gilbert (10), and RNA signals were quantified by densitometry with a Hoefer Scientific Instruments GS300 scanning densitometer. The DNA fragments labeled by random priming and used as hybridization probes were similar to those described by Fitch et al. (18), Amon et al. (4), and Kitada et al. (26).

Gel retardation assay. Protein extracts were obtained as described by Pfeifer et al. (39). The probes were prepared from a subcloned serum response element (SRE) sequence (24) by labeling isolated fragments with [α-^{32}P]dATP and Klenow polymerase. The polyacrylamide gel electrophoresis and DNA-binding reaction conditions were those of Zinck et al. (55).

Genomic footprinting. In vivo dimethyl sulfate (DMS) protection assays followed the procedure described by Saluz and Jost (42) with minor modifications. Cells were grown as 600-ml cultures to an OD_{600} of 2 to 3, harvested, and resuspended in 2 ml of medium. After 5 μl of DMS was added and incubated with the cells for 5 min at room temperature, the reaction was stopped by addition of 30 ml of TNEB (10 mM Tris-HCL [pH 8], 1 mM EDTA, 40 mM NaCl, 100 mM β-mercaptoethanol). Genomic DNA was isolated from spheroblasts, purified by several ethanol precipitations, finally resuspended in TE, and stored at −70°C. The following end-labeled oligonucleotides were used as primers: ATGCCCATGCTATGAGATGCTAGCTGT (*CLB2*, top strand), TCG AATATGTTTACATATTGAGCCCGTTTAGGAAAGT and ATTAGGTCG ACCACTACTCCTTCTAATCAAACACG (*CLB2*, bottom strand), TTGCAG ACGATCCTCTATAGTACTCAA (*SWI5*, top strand), and TTCATAGCAGC ATTGTTGGAAATATCT (*SWI5*, bottom strand). Primer extension reactions were carried out for 40 cycles (1 min at 94°C, 2 min at 65°C, and 3 min at 72°C) in standard buffer (16.6 mM NH_4SO_4, 67 mM Tris-HCl [pH 8.8], 8.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mg of bovine serum albumin per ml), with 8 U of *Taq* polymerase, 1 ng of ^{32}P-end-labeled oligonucleotide, and 300 μM deoxynucleoside triphosphate mix. The DNA was then precipitated by addition of CTAB (hexadecyltrimethylammonium bromide), purified by two ethanol precip-

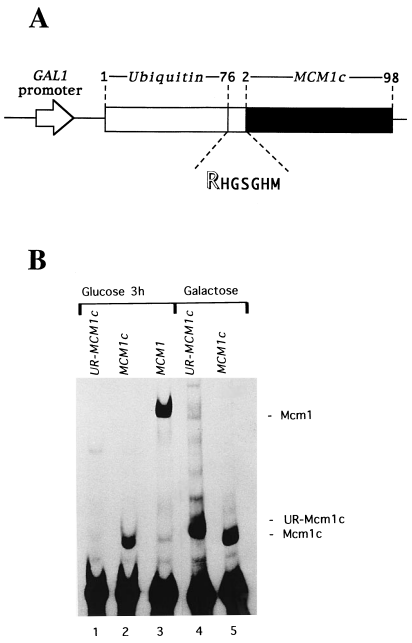


FIG. 1. Conditional *S. cerevisiae MCM1* expression system. (A) Schematic illustration of the conditional *MCM1* expression construct *GAL1-UR-MCM1c*. The core domain of *MCM1* (encoding aa 1 to 98) was fused to a sequence encoding a short peptide sequence which possesses an N-terminal arginine codon (5). A ubiquitin moiety precedes this destabilizing residue. The transcription of this construct was driven by a *GAL1* promoter. (B) Characterization of the *UR-MCM1* expression system by a gel retardation assay. *mcm1*Δ cells carrying the *GAL1-UR-MCM1c* construct (GA229) or a *GAL1-MCM1c* construct (GA136) were grown with galactose (lanes 4 and 5) or with glucose for 3 h (lanes 1 and 2). Lane 3, protein extract of log-phase wild-type cells (W303-1B). Retardation of a serum response element-containing probe was used to quantify the relative amounts of UR-Mcm1c, Mcm1c, and Mcm1.

itations, and finally resuspended in 5 μl of 0.1 M NaOH–2 mM EDTA. Samples were analyzed on 8% polyacrylamide gels containing 8 M urea.

Other techniques. Flow cytometric DNA quantification was performed on a Becton Dickinson FACScan as described by Lew et al. (30). In situ immunofluorescence, photomicroscopy, and Western (immunoblot) analysis were performed as described by Amon et al. (4). H1 kinase assays with immunoadsorbed Clb2 protein were done as described by Surana et al. (46).

RESULTS

Establishment of a conditional Mcm1 expression system. To address the role of Mcm1 in cell cycle-specific processes, we established a conditional Mcm1 expression system. We constructed a strain in which the endogenous *MCM1* gene was deleted (40) and replaced by a truncated but functional *MCM1* sequence expressed from the repressible *GAL1* promoter. Although these cells eventually stopped growing on glucose-containing medium, the long phenotypic lag prevented a clear diagnosis of the cellular defects caused by the absence of Mcm1 (32) (data not shown). To overcome this problem, we fused a ubiquitin-encoding sequence to the core region of *MCM1* (*MCM1c*) (40) so that a highly destabilizing amino acid (arginine) was now directing ubiquitin-dependent degradation of the mature protein (Fig. 1A) (5, 38). This strain (GA229) is referred to herein as the *GAL1-UR-MCM1c* strain, and its artificial Mcm1 product is named UR-Mcm1c.

As predicted by the N-end rule hypothesis (52), we found that functional UR-Mcm1c rapidly disappeared upon repression of the *GAL1* promoter. For example, in a gel retardation assay, we were unable to detect binding activity in extracts from cells that had been transferred to glucose-containing medium for 3 h (Fig. 1B, lane 1). In contrast, nonubiquitinated Mcm1c activity was still observed under these conditions (Fig. 1B, lane 2). Although UR-Mcm1c was relatively unstable in comparison to wild-type Mcm1c, its expression from the *GAL1* promoter nevertheless sufficed to fulfill the known biological functions of Mcm1; *GAL1-UR-MCM1c* cells were viable at all temperatures and mating competent in both haploid cell types (data not shown). After a shift to glucose-containing medium, however, cells stopped proliferating after one or two cell divisions while exhibiting morphological changes normally associated with low Clb kinase activity (see below) (47).

Expression of *SWI5*, *CLB1*, *CLB2*, *CDC5*, and *ACE2* depends on Mcm1. To test whether loss of Mcm1 function could be correlated with a cell cycle-specific transcriptional defect, we analyzed the expression pattern of several genes in our *GAL1-UR-MCM1c* strain (GA229). Previous evidence (32) suggested that transcription of *SWI5* depends on a ternary protein-DNA complex containing Mcm1 and Sff. However, an essential contribution of Mcm1 has never been proven genetically. We found that *SWI5*-specific RNA was present in cells grown on galactose but the level was drastically diminished after the cells were transferred to glucose-containing medium (Fig. 2A). To ensure that galactose- and glucose-grown cells were compared at the same cell cycle stage, we treated the cultures in a parallel experiment with nocodazole. This drug blocks cells in the G_2/M phase, generating a situation which should maintain high expression levels of G_2-specific genes. Even under these conditions, we observed a significant difference in the level of *SWI5* RNA when we compared Mcm1-plus and Mcm1-depleted cells (Fig. 2A).

We repeated the Northern analysis with probes specific for *CLB1*, *CLB2*, and *CLB3*. Cells grown in galactose- or galactose/nocodazole-containing medium expressed significant levels of *CLB1* and *CLB2* mRNA, whereas cells lacking Mcm1 because of glucose addition to the growth medium displayed only low or undetectable levels of *CLB1* and *CLB2* mRNA (Fig. 2B and C). In contrast, *CLB3* expression seemed to be unaffected by growth conditions, confirming that its mode of regulation is different from that of the other two mitotic cyclin genes (18). It should be noted, however, that *CLB1* transcription is considerably lower in cells that express only the core of Mcm1 than in cells with full-length Mcm1. Since a similar effect has been noticed previously for α-specific genes (7, 9), this observation suggests that there is a role for the nonessential C terminus of Mcm1 in transcription of *CLB1*. There are also differences in *SWI5* and *CLB2* RNA levels in untreated and nocodazole-treated cells. Since prolonged incubation of cells with nocodazole affects the survival of the cells, we suggest that these promoters are slightly sensitive to suboptimal growth conditions. The specificity of all the effects was evidenced by probing for the expression of *ACT1*, a gene expected to be largely unaffected by Mcm1 depletion, whereas the dependency on Mcm1 was also shown for *ACE2* gene expression, which temporally overlaps *SWI5* transcription (data not shown).

Contemplating that Mcm1 might have a more global role in G_2-specific transcriptional activation, we investigated the effect of its depletion on another essential cell cycle-regulated gene, *CDC5*. Cells deficient in Cdc5 function arrest in a late step of mitosis, as they fail to exit anaphase. As shown in Fig. 2D, the *CDC5* transcript level was indeed markedly lower in *GAL1-UR-MCM1c* cells transferred to glucose medium either with or without nocodazole treatment. Interestingly, repression of *CDC5* transcription seems to cause first-cycle arrest (27), providing another reason why G_2-specific regulation of transcription could be important for cell cycle progression.

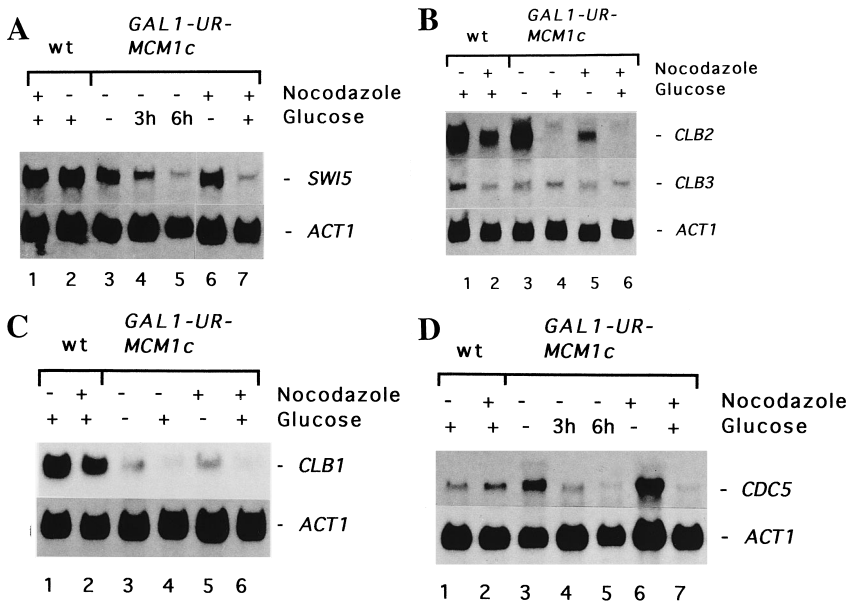


FIG. 2. Expression of *SWI5*, *CLB1*, *CLB2*, and *CDC5* is dependent on Mcm1. Northern blots were done with *SWI5* probe (A) with a *CLB2* and a *CLB3* probe (B), with a *CLB1* probe (C), and with a *CDC5* probe (D). The constitutively expressed *ACT1* served as an internal control. *GAL1-UR-MCM1c* cells were grown on either galactose (lane 3) or for 3 h on glucose (lane 4). In addition, the *SWI5* and *CDC5* RNA levels were determined in cells incubated on glucose for 6 h (A and D, lane 5). RNA from a log-phase wild-type strain was used as a control (A, lane 2; B, C, and D, lane 1). Nocodazole-treated *GAL1-UR-MCM1c* cells were grown on galactose (A and D, lane 6; B and C, lane 5) or for 3 h on glucose (A and D, lane 7; B and C, lane 6). RNA from nocodazole-arrested wild-type cells (W303-1A) was loaded as indicated (lanes wt). The low level of *CLB1* RNA found in *GAL1-UR-MCM1c* cells (GA229) suggests that the UR-Mcm1c protein cannot activate this promoter to full activity.

Constitutive expression of *CLB2* does not suppress the G_2-specific transcription defect of *mcm1* cells. Since an active Clb kinase is necessary for G_2-specific transcription and since *CLB1* and *CLB2* are among the promoters affected by low Clb kinase levels, we investigated whether expressing *CLB2* with an Mcm1-independent promoter would be sufficient to overcome the transcriptional block in other G_2-specific genes. We introduced plasmids that contained the *CLB2* coding region fused either to the *ADH1* promoter or to the *Schizosaccharomyces pombe adh* promoter into the *GAL-UR-MCM1c* strain (3, 43). After these cells were transferred to glucose-containing medium, we ensured by Western blot analysis and protein kinase assays that Clb2 protein was made and sequestered into an active kinase (Fig. 3A; also see Fig. 9A). We found that Clb kinase activity could not rescue cells from the lethal consequences of Mcm1 depletion. This may not be surprising, considering the rather promiscuous role of this transcription factor. However, Northern blot analysis also showed that the Mcm1-independent expression of Clb2 did not prevent the decrease in *SWI5* and *CDC5* RNA levels (Fig. 3B). Thus, with regard to these promoters, Mcm1 complexes might function downstream of the mitotic kinase and perhaps represent one of its direct targets.

An Mcm1-VP16 fusion deregulates *CLB1*, *CLB2*, and *CDC5* transcription. So far, our experiments demonstrated that Mcm1 was needed for high levels of *CLB1*, *CLB2*, and *CDC5* transcription. They did not resolve the issue of whether the requirement for Mcm1 actually reflected its direct interaction with the *CLB1*, *CLB2*, and *CDC5* promoters. Since in most cases Mcm1 seems to be able to bind promoter DNA but unable to activate transcription without an appropriate protein partner, we tried to construct a fusion in which Mcm1 was directly connected to a strong activation domain. If Mcm1 were involved in the activation of *CLB1*, *CLB2*, and *CDC5* by recruiting a regulated ternary complex factor such as Sff, such a partner-independent Mcm1 fusion variant should now stim-

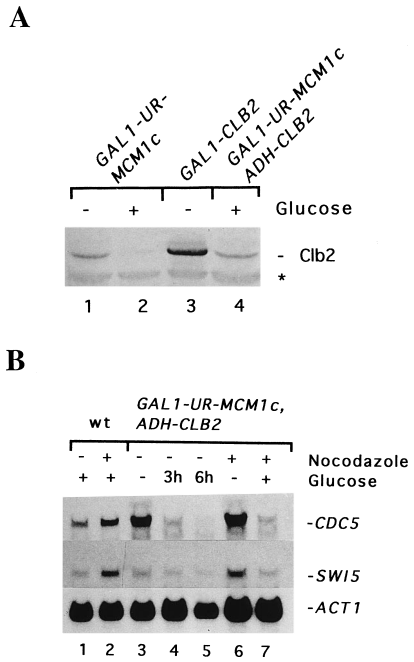


FIG. 3. In Mcm1-depleted cells, the constitutive expression of *CLB2* does not suppress the decrease in G_2-specific transcription. (A) Western analysis with Clb2-specific antibodies. Strains were grown on either galactose or glucose as described in the legend to Fig. 2. The Clb2-specific signal is indicated; the star signifies a cross-reacting protein. Strains are GA229 (lanes 1 and 2), K2949 (lane 3), and GA504 (lane 4). (B) Northern analysis of *CDC5* and *SWI5*. Lanes 1 and 2, RNA from a wild-type strain (W303-1A); lanes 3 to 7, RNA from a *GAL1-UR-MCM1c rad9* Sp*adh-CLB2* strain (GA504).

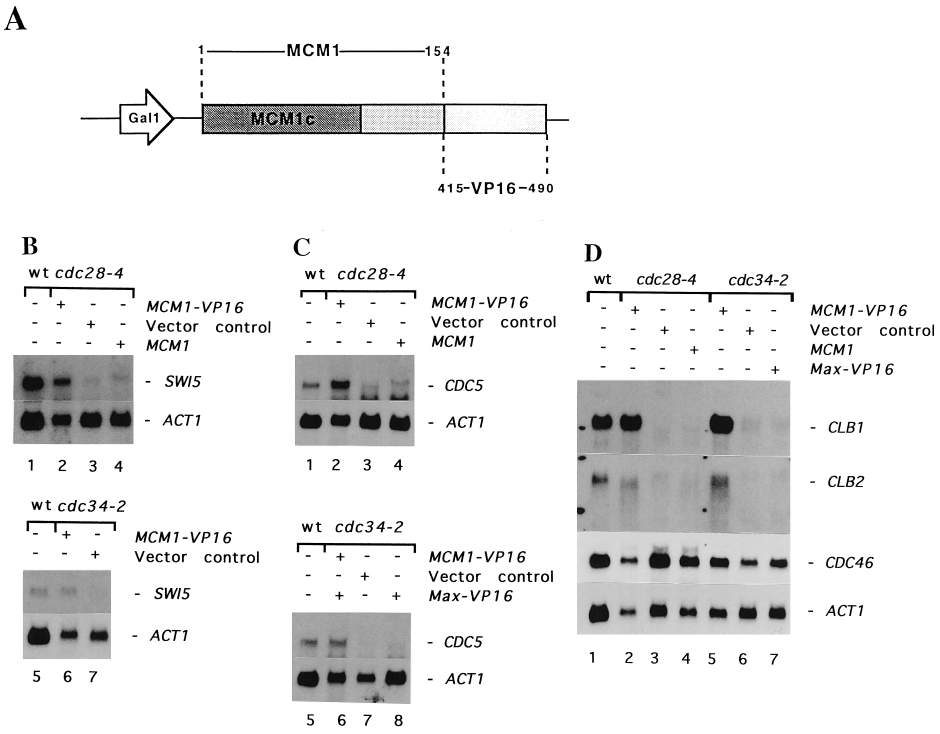


FIG. 4. Mcm1-VP16 fusion protein activates expression of *SWI5*, *CLB1*, *CLB2*, and *CDC5* in G_1. (A) *MCM1-VP16* fusion construct. The coding region for aa 1 to 154 of *MCM1* (shaded region) was fused to the sequence encoding the C-terminal 76 aa of VP16. This construct was driven by the *GAL1* promoter. The darkly shaded area indicates the core region of Mcm1. (B, C, and D) Strains carrying either the *cdc28-4* or the *cdc34-2* allele transformed with the *GAL1-MCM1-VP16* construct (B and C, lanes 2 and 6; D, lanes 2 and 5), the empty vector (B and C, lanes 3 and 7; D, lanes 3 and 6), a *GAL1-MCM1* construct (lanes 4), or the Max-VP16 construct (D, lane 7; C, lane 8) were arrested in G_1. After 2.5 h, Mcm1-VP16 and Max-VP16 synthesis was induced by addition of galactose. Northern blot analysis was done with probes specific for *SWI5*, *CLB1*, *CLB2*, *CDC46*, *CDC5*, and *ACT1*. *ACT1* was used as an internal control. RNA from log-phase wild-type cells served as an additional control (B and C, lanes 1 and 5; D, lane 1). *CDC46* was included because its promoter contains a potential Mcm1 binding site that might have influenced its mode of regulation (23).

ulate the expression of *CLB1*, *CLB2*, and *CDC5* at inappropriate times during the cell cycle. We replaced part of the nonessential C-terminal domain of Mcm1 with aa 415 to 490 of the viral activator VP16 (50) and expressed the fusion gene from the *GAL1* promoter (Fig. 4A). The construct was integrated into the genome of either *cdc28-4* or *cdc34-2* mutant strains. Under nonpermissive conditions, *cdc28-4* cells arrest at a point before they become committed to the cell cycle program (*START*), whereas *cdc34-2* cells arrest in late G_1, unable to initiate DNA replication (21, 47). Thus, both mutations cause cells to remain at cell cycle stages that do not display *SWI5*, *CLB1*, *CLB2*, or *CDC5* transcription (4).

Cells containing either the *MCM1-VP16* or one of several control constructs were grown in raffinose at the permissive temperature. After the shift to 37°C, production of Mcm1-VP16 was induced with galactose. To obtain a more uniform arrest of the *cdc34-2* strain, cells were synchronized by treatment with α-factor, washed, and then shifted to 37°C. Northern analysis of the galactose-treated cells revealed that *SWI5*, *CLB1*, *CLB2*, and *CDC5* RNA levels were markedly elevated in Mcm1-VP16-expressing cells (Fig. 4B, C, and D). In contrast, Max-VP16, a fusion protein of VP16 and the unrelated mammalian transcription factor Max (1), and high levels of wild-type Mcm1 itself failed to induce these transcripts. Similarly, cells transformed with an empty vector displayed only background levels of *SWI5*, *CLB1*, *CLB2*, and *CDC5* RNAs (Fig. 4C and D). The *MCM1*-independent transcription unit *ACT1* was used as a control and found to be unaffected by *MCM1-VP16* expression. We propose that Mcm1 controls the different Clb-dependent promoters directly by binding to the promoters of these genes.

Mcm1/Sff binding motifs are present in the *CLB1* and *CLB2* promoters. Since our results with the Mcm1-VP16 fusion suggested a direct involvement of Mcm1 for *CLB1*, *CLB2*, and *CDC5* promoter activity, we expected to find motifs with similarity to known cognate Mcm1 sites. These sites are related to the CCA/T_6GG core motif characteristic of the group of DNA-binding proteins to which Mcm1 belongs (54). Unfortunately, information on the *CDC5* upstream region is limited, and no cognate Mcm1 sites were recognized within the published sequence (27). In contrast, we could identify one binding motif in the *CLB1* 5′-flanking region at position −506. More important, we found several candidate sites in a region from 550 to 760 bases upstream of the start of the *CLB2* reading frame. Some of the same sites (positions −690, −562, and −543 from the initiation ATG) were pointed out as putative Mcm1 binding sites by Kuo and Grayhack (29), who found that Mcm1 can bind in vitro to a large DNA fragment overlapping the upstream region of *CLB2*.

If the same transcription factor complexes that bind to the *SWI5* promoter are also involved in the activation of *CLB1* and *CLB2* transcription, one might expect sequence similarities beyond Mcm1 binding motifs. The analysis of *SWI5* promoter mutants suggested that the Sff recognition site in *SWI5* should be contained within the motif AGGTAAACAA (32). Indeed, in the regions flanking two of the proposed sites in the *CLB2* upstream region, one can find the motifs AGGTCAACA and ATGTAAACA. The upstream region of *CLB1* also contains similar motifs flanking the potential Mcm1 binding site (for a comparison of all the relevant sequences, see Fig. 5). An extensive promoter analysis of the *CLB2* promoter showed that

SWI5	-324	ACTTTAA**CCTGTTTAGG**__AAAA**AGGTAAACAA**TAACAATA	-286
CLB2-I	-697	ATAGCGA**CCGAATCAGG**____AAA**AGGTCAACAA**CGAAGTTC	-660
CLB2-III	-736	GCACTTT**CCTAAACGGG**CTCAAT**ATGTAAACAT**ATTATACA	-776
CLB1	-523	ACAACCG**CCCAAAGAGG**__AAAA**ACATCAACAA**TCAAGAAG	-485
CLB1'	-500	TGTTTTT**CCTCTTTGGG**____CGG**TTGTAAACAA**AAGGTCGT	-537
consensus		**CC a/t GG GT$^{C}_{A}$AACAA**	

FIG. 5. Comparison of putative Mcm1/Sff binding sites using motifs found in the 5′-flanking regions of *SWI5*, *CLB2*, and *CLB1*. For the single Mcm1 binding site found in *CLB1*, two potential alignments are shown (CLB1 and CLB1′), whereas CLB2-I and CLB2-III are two completely separate elements found in the *CLB2* promoter.

the region containing these sites is essential and sufficient for the regulated transcriptional activation of *CLB2* (33).

In vivo, the *CLB2* and *SWI5* UASs are constitutively occupied by an Mcm1 complex. In vivo footprinting studies were performed for two reasons. First, we wanted to obtain evidence that the sites in the *CLB2* promoter are indeed important for Mcm1 binding in living cells. Second, we were interested in whether cell cycle regulation of the promoters was due to differential binding of any of the transcription factors. Genomic DMS protection patterns were established at the *SWI5* promoter, comparing wild-type and Mcm1-depleted cells. As shown in Fig. 6, distinct footprints that cover the predicted contact sites for Mcm1 can be found within the *SWI5* UAS. On one of the DNA strands, an additional nucleotide is protected at the proposed Sff binding motif. All these interactions are missing in *mcm1* mutant cells (for a summary, see Fig. 6E). Clear differences between Mcm1-plus and -minus cells were also found within the *CLB2* promoter (Fig. 6C and D). Mcm1-dependent signals were identified at positions −690, −718, and −748, giving more strength to the argument that Mcm1's action on the promoter is direct. In one of the proposed Mcm1/Sff binding regions (MCE-I + SFRE-I [Fig. 6D and E]), the concomitant loss of the protection signals across the Mcm1 binding site and the potential Sff binding site is particularly striking. The data demonstrate that Mcm1 directs the binding of a ternary complex factor to a consensus sequence of *CLB2* and *SWI5*. Not only are these results in complete agreement with the in vitro evidence for *SWI5* (32), they also emphasize the importance of Sff recruitment by Mcm1 in vivo.

Since we were able to detect protection of the different binding sites by using DNA isolated from asynchronous cultures, we suspected that transcription factors might be bound to the promoters throughout the cell cycle. To confirm this notion, we compared the pattern in cells arrested with mating pheromone in G_1 with that in cells that were treated with nocodazole (Fig. 7). We found no significant differences in the protection pattern even when these cells were arrested in phases of the cell cycle which display either very low or high levels of *SWI5* and *CLB2* RNAs. Therefore, the ternary complex forms a stable, cell cycle-independent target structure that could receive specific activating or repressing modifications as cells pass through different stages of the cell cycle.

Morphological phenotype of Mcm1-depleted cells. Loss of Mcm1 function clearly depresses the transcription of G_2-specific genes. The question is how severely this phenomenon affects cell cycle progression and whether it accounts for the morphological phenotypes observed in Mcm1-depleted cells. At 4 h after glucose addition, most *GAL1-UR-MCM1c* cells stopped proliferating (data not shown). At this stage, many buds exhibited an unusual elongated cell morphology (Fig. 8B). In situ immunofluorescence studies with antitubulin antibodies revealed that the majority of cells contained a bipolar spindle of short to medium size, with the nucleus localized at the neck of the bud (Fig. 8C, D, and E). Prolonged growth on glucose resulted in some rebudding even if the cells contained only one nucleus. Flow cytometry analysis demonstrated that cells accumulate with a *2N* DNA content after the shift to nonpermissive conditions (Fig. 8F). After more extended time in glucose, the signal sometimes drifts towards >*2N* DNA content, but the cause for this phenomenon has not been investigated in detail yet. We believe that it is most likely a consequence of cell growth and a defect in cell separation, but we cannot exclude that it is due to rereplication. Overall, the observed phenotype was highly reminiscent of that of cells arrested at the G_2-to-M transition because of the lack of functional B-type cyclins Clb1 and Clb2 (41, 47). Nevertheless, the spindle morphology seemed to be not quite uniform enough and perhaps too leaky for a definite conclusion.

Since Clb3 and Clb4 can partially substitute as mitotic cyclins, and since these genes are not under the control of Mcm1 (see above), we repeated the experiments in a *GAL-UR-MCM1c* background lacking functional *CLB3* and *CLB4*. Indeed, when this strain was depleted of Mcm1, the morphological phenotype was now more pronounced and uniform. Cells soon started to exhibit highly elongated buds. Even as the cells rebudded, the DNA content, as measured by FACS analysis, remained at *2N*, except that prolonged arrest led to a drift in the signal to higher DNA content. Antitubulin staining revealed that the cells could not construct a bipolar spindle, although a spindle pole body plaque and astrotubular structures were clearly visible (Fig. 8G and H), which is exactly the phenotype described for a *clb1 clb2 clb3 clb4* mutant strain (4, 18).

When *CLB2* was expressed from a constitutive promoter in Mcm1-depleted cells, one could observe the formation of prominent bipolar spindles (Fig. 9C). The cells also had a more rounded appearance than *mcm1 clb3 clb4* mutant cells. Consistent with our in vitro H1 kinase assays (Fig. 9A), this observation indicated that the Clb2 kinase was functional and active in vivo. Interestingly, this kinase activity did not seem to prevent rebudding, even though most cells still contained only one major DNA mass that colocalized with the spindle (Fig. 9D). Thus, the kinase activity might not be persistent in individual cells. As suggested by FACS analysis, the DNA replication pattern of *mcm1* cells that lack *CLB3* and *CLB4* is very similar to that of *mcm1* cells that express *CLB2* constitutively. In principle, this observation could be due to an active checkpoint control that monitors the integrity of DNA replication. However, the introduction of a mutation that disrupts at least one of the checkpoint controls, such as *rad9*, changed neither the apparent DNA content nor the nuclear morphology in these strains (Fig. 9B). Only rarely did we observe cells with a normal anaphase spindle and separated DNA, a phenotype that could be associated with the loss of *CDC5* function. Thus, another plausible explanation for this phenotype is that additional genes necessary for chromosome separation and completion of anaphase are also under the control of Mcm1.

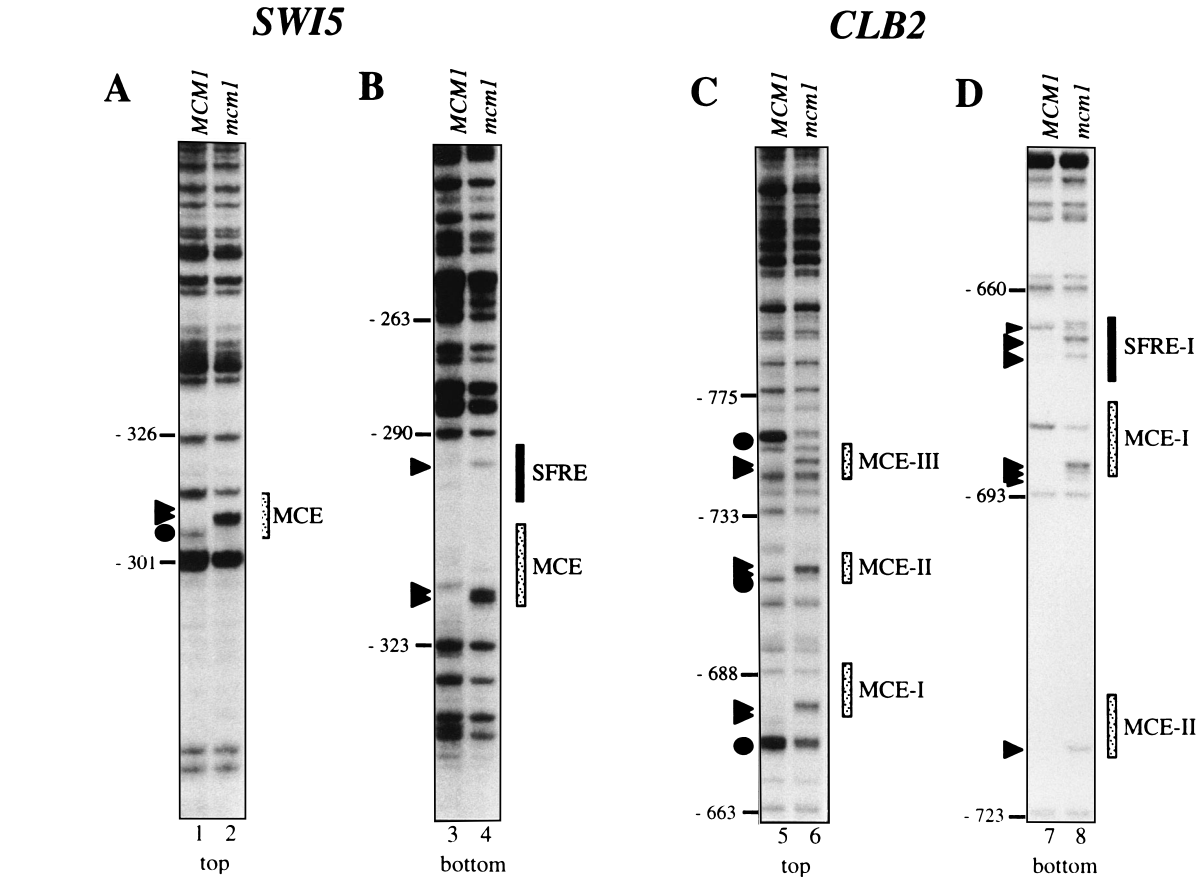


FIG. 6. Mcm1-dependent complex binds at G_2-specific promoters in vivo. (A to D) DMS-induced methylation pattern at *SWI5* and *CLB2*. Panels A and C show the patterns of the coding strands (top); panels B and D show the patterns of the noncoding strands (bottom). Lanes 1, 3, 5, and 7, DNA from strain GA231 grown on galactose; lanes 2, 4, 6, and 8, DNA from strain GA231 shifted to glucose medium for 4 h. (E) Summary of the differences found between DNAs from asynchronous cells and Mcm1-depleted cells. MCE, Mcm1 binding site; SFRE, putative Sff binding site; triangles, diminished methylation; circles, enhanced methylation.

DISCUSSION

Over the last few years, it became evident that coordinated expression of several key genes in a particular phase of the cell cycle is important for cell cycle progression. Coordinate regulation suggests an underlying common mechanism of transcriptional control. This is found in the regulation of gene expression at *START*, where regulatory complexes containing the Swi6 and Swi4 or Mbp1 factors direct cell cycle-specific transcription of several genes, including the G_1 cyclin genes *CLN1*, *CLN2*, and *HCS26*, as well as genes important for replication. The mechanism leading to coordinate expression of genes necessary for the G_2-to-M transition, however, was less clear. Pioneering studies on the *SWI5* gene raised the possibility that Mcm1 might be involved in orchestrating G_2-specific gene expression. Our work presented here provides strong evidence that this is indeed the case.

Role of Mcm1 in cell cycle progression. Mcm1's role as a transcription factor was first recognized in studies on haploid cell type determination, a process not crucial for the survival of vegetative cells. Since a deletion of the gene is lethal, the protein also has to carry out an essential function (34). Several observations indicated that one of the essential functions might be related to cell cycle-specific events. *MCM1* was isolated as a mutation responsible for a defect in minichromosome maintenance. The phenotype of this mutant suggested a role for *MCM1* in the activation of replication origins. In addition, Elble and Tye (16) reported the characterization of a temperature-sensitive allele of *MCM1* that caused a defect in cell cycle

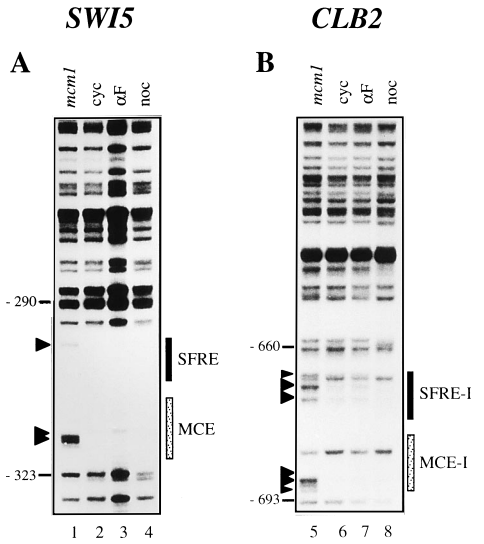


FIG. 7. Transcription factors bind to the *SWI5* promoter and to the *CLB2* promoter throughout the cell cycle. DMS protection patterns were analyzed in Mcm1-depleted cells (GA231, shifted to glucose for 4 h; lanes 1 and 2), asynchronously growing cells (GA231, grown on galactose), and cells arrested for 2 h with α-factor (K1534; lanes 3 and 7) or for 3 h with nocodazole (K1534; lanes 7 and 8). (A) *SWI5* UAS; (B) *CLB2* UAS. MCE, Mcm1 binding site; SFRE, putative Sff binding site.

progression. At the restrictive temperature, the cells arrested with a large bud and a *2N* DNA content. This phenotype essentially resembles that of mutants with special defects in DNA replication, such as *cdc9* and *cdc13* mutants. It was speculated that cells lacking functional Mcm1 arrest in G_2 because parts of the chromosomal DNA remain underreplicated, thereby activating a surveillance system (e.g., the *RAD9* pathway) (16, 22, 53). Although we cannot rule out this possibility, we offer instead the alternative explanation that Mcm1-depleted cells lack factors essential for the initiation and execution of mitosis.

Since our DNA measurements confirmed that *mcm1* cells must have replicated at least the bulk of their chromosomal DNA, they should normally be at a stage of the cycle at which the production of the mitotic cyclins has been initiated. However, our RNA measurements clearly demonstrated that *CLB1* and *CLB2* transcript levels are substantially reduced when functional Mcm1 is absent. Consistent with this observation, we found that cells lacking Mcm1 have almost undetectable Clb2-dependent H1 kinase activity. The defect in the production of active B-type cyclins was most apparent when we analyzed the effect of Mcm1 depletion in the *clb3 clb4* background. Normally, *clb3 clb4* double mutants are viable, with no severe spindle abnormalities. In the absence of Mcm1, however, the same cells completely failed to construct a bipolar spindle. This could in principle be due to two reasons: lack of Clb5/6 function (44) or lack of Clb1/2 function (18). However, since *clb3 clb4 clb5 clb6* quadruple mutants are retarded in their DNA replication, the spindle defect in *mcm1* cells should be caused by the lack of Clb1 and Clb2. Consistent with this interpretation, we also found that *clb3 clb4 mcm1* mutants were unable to repress *SWI4*-dependent transcription, as evidenced by continuous *HCS26* transcription (data not shown) (4). The inability to repress G_1 cyclins probably explains the extremely elongated bud morphology and perhaps the failure to prevent rebudding despite the lack of nuclear division (3, 31). In summary, the phenotype reported for cells unable to synthesize Clb1/2/3/4 appears to be very similar to the phenotype of Mcm1-depleted *clb3 clb4* cells.

Why did the constitutive expression of *CLB2* not suppress the *mcm1* arrest phenotype, as it did for a quadruple *clb1 clb2 clb3 clb4* mutant? One potential explanation was provided by the observation that *CDC5* transcripts are also missing in Mcm1-depleted cells. Cells deficient in the Cdc5 kinase arrest with a highly elongated spindle and the DNA separated into two distinct masses located in the prospective mother and daughter cells. Mcm1-depleted cells that overcome the cyclin deficiency by expressing Clb2 from a constitutive promoter, however, did not exhibit this phenotype. Instead, they arrested with a partially elongated mitotic spindle stretching across an undivided if slightly deformed nucleus. This phenotype is somewhat reminiscent of that of *cdc13*, *cdc16*, and *cdc23* mutant cells arrested at the nonpermissive temperature. However, a *rad9* mutation did not alter the nuclear dynamics, as it would in the *cdc13* background. This observation opens the interesting prospect that unstable proteins other than cyclins are under the control of *MCM1*. These as yet unidentified factors could be required for chromosome separation.

How direct is the effect of Mcm1 function? From our results, the work by Lydall et al. (32), and a recent deletion analysis of the *CLB2* promoter (33), there should not be any doubt that Mcm1 is directly involved in both *SWI5* and *CLB2* activation. First, in vivo footprinting reveals Mcm1-dependent in vivo protein occupancy of Mcm1 binding sites in the *SWI5* and *CLB2* promoters. Second, similar to the *SWI5* promoter, a DNA fragment encompassing the Mcm1-dependent genomic footprint in the *CLB2* promoter confers cell cycle regulation on a fused *lacZ* gene (33, 42a). Third, an autonomously active Mcm1-VP16 fusion stimulates expression of *CLB2* and *SWI5* at inappropriate stages of the cell cycle. What about other genes such as *CDC5*? Since their transcription is dependent on functional B-type cyclins, the observed expression defect could just be a consequence of low Clb kinase activity. The Mcm1-VP16 fusion experiments are also informative in this respect because the fusion construct leads to Cdc28-independent transcription of *CDC5*. Since the inappropriate expression of *CDC5* in arrested *cdc28* mutant cells cannot be due just to the presence of Clb2 kinase, it is likely that Mcm1 is directly involved in *CDC5* activation. This assumption is further supported by the observation that Mcm1-independent expression of *CLB2* does not suppress the G_2-specific transcriptional defects caused by Mcm1 depletion. Overall, our work substantiates previous speculations that an Mcm1-containing ternary complex is required for *CLB1/2* expression. This complex could thus constitute an integral part of the feedback loop supposed to sustain the expression of Clb kinase (4). Beyond this, our experiments also support a model in which Mcm1 plays a general role in the transcriptional events controlled by the mitotic kinase (Fig. 10).

What are the cell cycle-specific partners of Mcm1 and how are they regulated? The similarity of the expression obtained with *CLB2* and *SWI5* upstream regions suggests that Mcm1 might execute the same functions in the activation of these promoters. Since Mcm1 usually fulfills its role by recruiting specialized coregulators, this naturally brings up the question of their identity. Are different partners involved on G_2-specific promoters? The surprisingly similar sequence context of the Mcm1 recognition sites in *CLB1*, *CLB2*, and *SWI5* and the similarities in the appearance and behavior of the genomic footprints suggest that Mcm1 occupies these different promoters with the same partner or at least with highly related partners. A conclusive answer will require the comparison of ter-

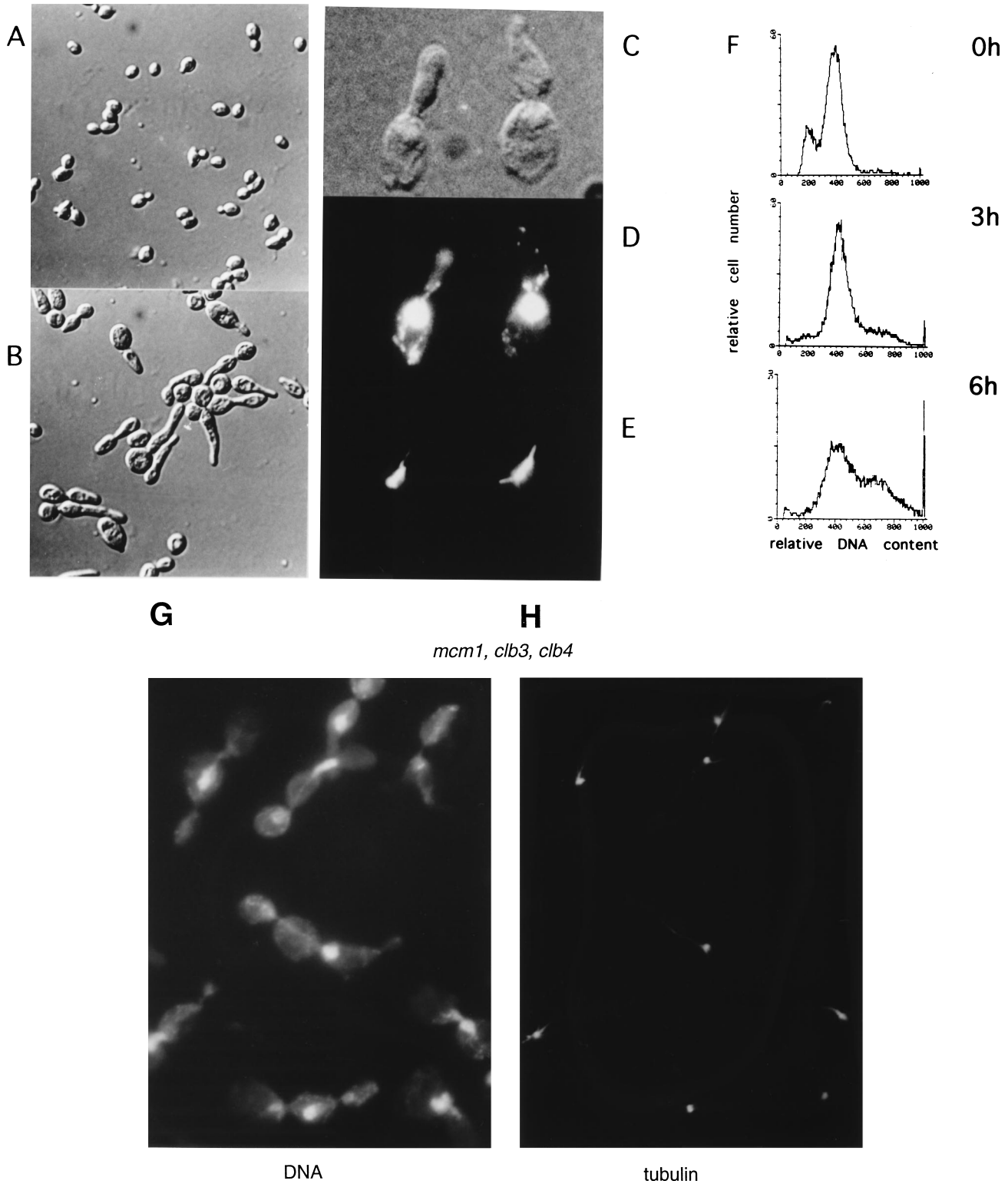
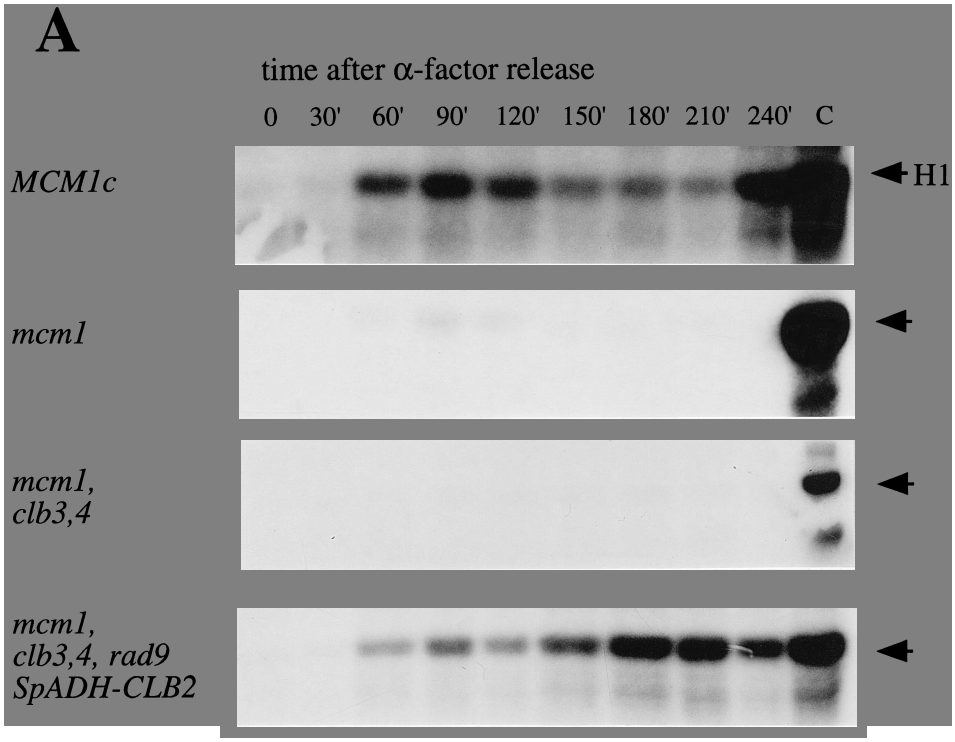


FIG. 8. Mcm1-depleted cells exhibit a defect in the G_2-to-M transition. Cells were either continuously grown on galactose (A) or shifted to glucose for 3 h (B to E) or 4 h (G and H). The cells shown in panels A to E are wild-type for B-type cyclin genes (strain GA229). Panels G and H show Mcm1-depleted *clb3 clb4* cells (strain GA231). (A, B, and C) Images obtained with Nomarski optics. (D and G) The nucleus was visualized after staining with DAPI (4′,6-diamidino-2-phenylindole). (E and H) Spindle morphology was visualized by immunofluorescence with antitubulin staining. (F) DNA content as analyzed by flow cytometry. After asynchronous GA229 cells were shifted from galactose- to glucose-containing medium, samples were taken at 3 and 6 h.



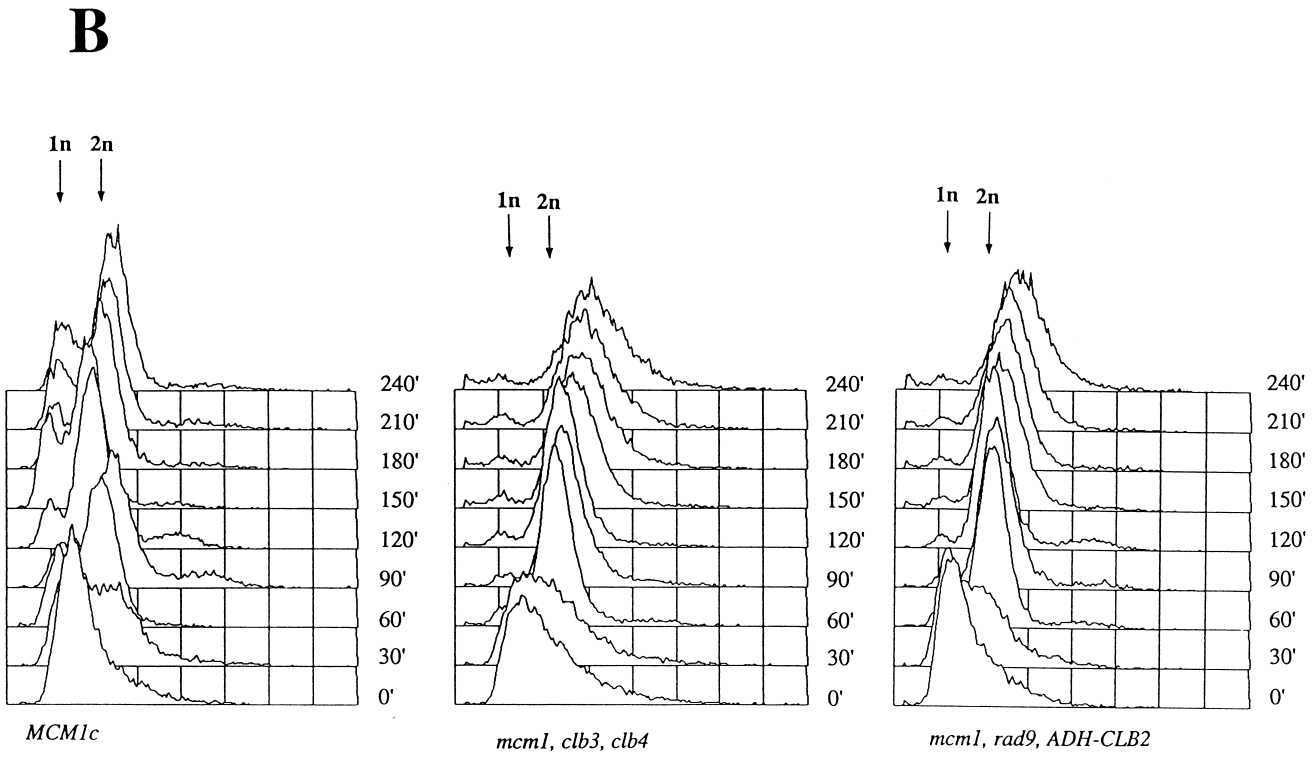


FIG. 9. Mcm1 depletion causes a defect in nuclear division. (A) H1 kinase assays with immunoabsorbed Clb2. Activities were compared with that of a standard wild-type extract (lane C). The strains were arrested with α-factor and released into glucose-containing medium for the indicated times (in minutes). Strains are GA131 (*Mcm1c*), GA229 (*mcm1*), GA231 (*mcm1 clb3 clb4*), and GA503 (Sp*adh-CLB2 rad9 mcm1 clb3 clb4*). (B) DNA contents of strains released from α-factor arrest. The strains are GA131, GA231, and GA567 (*mcm1 ADH-CLB2 rad9*). (C and D) Morphology of strain GA567 after growth on glucose for 4 h. Spindles were visualized with antitubulin antibodies, and DNA was stained with DAPI.

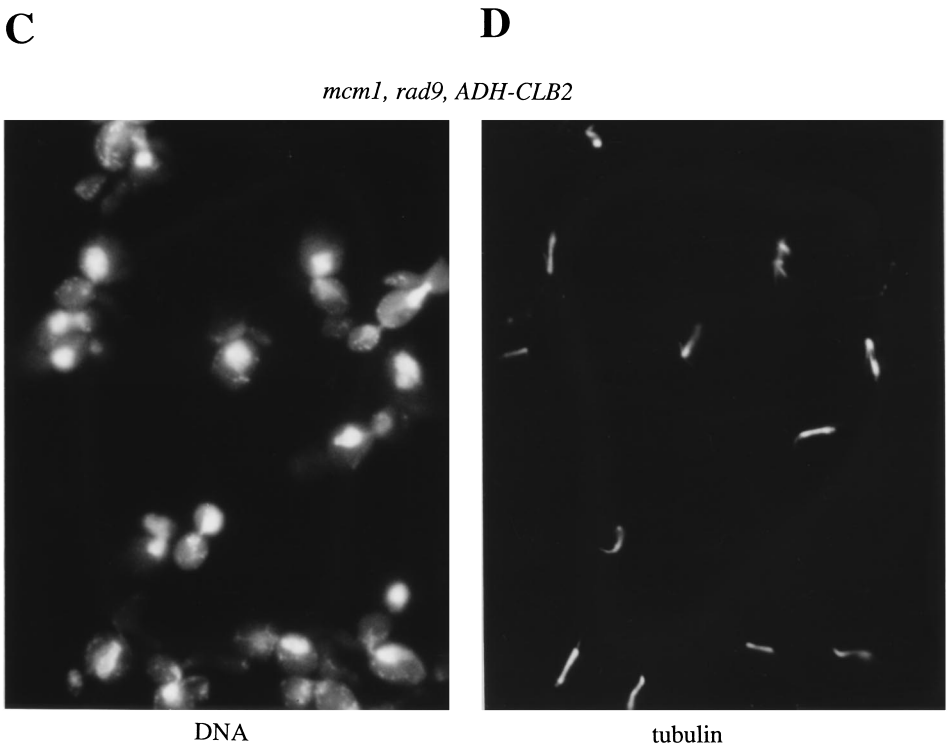


FIG. 9—*Continued.*

nary-complex factors on the different promoters and the cloning of the gene(s) encoding Sff.

How might Mcm1/Sff (or Sff relatives) be regulated? The Mcm1-VP16 expression experiments suggested that the inactivation of G_2-specific promoters in G_1 could not be due to the occlusion of Mcm1 binding sites. The in vivo footprinting data certainly confirmed this notion by revealing the continuous presence of Mcm1 footprints at the *SWI5* and *CLB2* promoters. More important, the footprint analysis showed that a ternary complex seems to bind during all phases of the cell cycle. Since the pattern is virtually identical in G_1 and G_2 cells, it is unlikely that Mcm1 replaces Sff as a partner with a structurally different repressor. Thus, the cell cycle regulatory system appears not to modulate the affinity of either Mcm1 or Sff for DNA. The events that trigger G_2-specific activation and G_1-specific repression must rather operate on the prebound complex or on targets separate from Mcm1 and Sff.

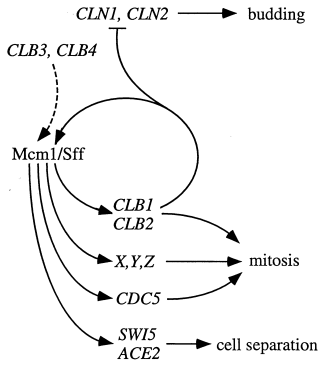


FIG. 10. Model for Mcm1 function during cell cycle progression. The cartoon accommodates previously published results (4, 32) and results from this work. Accordingly, Mcm1 recruits a special regulatory partner that determines Mcm1's G_2- and M-specific properties. In wild-type cells, activity of the transcription complex could be triggered by B-type cyclins, whose expression is independent of Mcm1. As part of a positive feedback loop, the complex might then become the target of Clb1 and Clb2 to ensure persistent expression of mitotic cyclins. Hypothetical unstable components necessary for the completion of anaphase (X, Y, and Z) and Cdc5 use the same direct regulatory system that controls transcription of *CLB1* and *CLB2*. Indirectly, Mcm1 coordinates early G_1-specific events such as cell separation by controlling the expression of *SWI5* and *ACE2*. Arrows indicate activation; the horizontal bar represents repression.

Implications for multicellular eukaryotic systems. Yeast Mcm1 and mammalian SRF proteins share important structural and functional characteristics. Apart from recruiting ternary-complex factors to specific transcriptional regulatory sites, both SRF and Mcm1 also establish nuclear control structures that are targeted by signal transducing kinases (for a review, see references 8 and 25). For SRF, the ternary complexes have primarily been shown to be targets of mitrogen-activated protein kinases to direct immediate-early gene induction at the G_0-to-G_1 transition. It will be quite interesting to see whether, by analogy to the involvement of Mcm1 in G_2-specific gene expression in *S. cerevisiae*, SRF or an SRF-related factor can also be found regulating genes involved in the progression through later steps of the mammalian cell cycle.

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