MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 2830–2837 Vol. 16, No. 6
0270-7306/96/$04.00+0

Ste12 and Mcm1 Regulate Cell Cycle-Dependent Transcription of *FAR1*

L. J. W. M. OEHLEN, J. D. McKINNEY, AND F. R. CROSS*

The Rockefeller University, New York, New York 10021

Received 19 July 1995/Returned for modification 16 August 1995/Accepted 19 March 1996

The transcripts of many genes involved in *Saccharomyces cerevisiae* mating were found to fluctuate during the cell cycle. In the absence of a functional Ste12 transcription factor, both the levels and the cell cycle pattern of expression of these genes were affected. *FUS1* and *AGA1* levels, which are maximally expressed only in G_1-phase cells, were strongly reduced in *ste12*$^-$ cells. The cell cycle transcription pattern for *FAR1* was changed in *ste12*$^-$ cells: the gene was still significantly expressed in G_2/M, but transcript levels were strongly reduced in G_1 phase, resulting in a lack of Far1 protein accumulation. G_2/M transcription of *FAR1* was dependent on the transcription factor Mcm1, and expression of a gene with Mcm1 fused to a strong transcriptional activation domain resulted in increased levels of *FAR1* transcription. The pattern of cell cycle-regulated transcription of *FAR1* could involve combinatorial control by Ste12 and Mcm1. Forced G_1 expression of *FAR1* from the *GAL1* promoter restored the ability to arrest in response to pheromone in *ste12*$^-$ cells. This indicates that transcription of *FAR1* in the G_1 phase is essential for accumulation of the protein and for pheromone-induced cell cycle arrest.

Exposure of haploid *Saccharomyces cerevisiae* cells to the mating factor secreted by the opposite mating type elicits a developmental pathway that prepares cells for mating. The response includes arrest in the G_1 phase of the cell cycle and the transcriptional induction of a large set of genes that contribute to successful mating. The mating factor signal transduction pathway includes a seven-transmembrane receptor, a heterotrimeric G protein, and a series of functionally associated kinases (for reviews, see references 5 and 43). The last two of this series of kinases, Kss1 and Fus3, are proteins that are highly homologous to each other and to other members of the MAP kinase family. Both Fus3 and Kss1 are able to phosphorylate the transcription factor Ste12 (5, 16). Ste12 can bind to specific pheromone-responsive elements (PREs) in the promoters of many genes that are involved in mating. Ste12 has some low levels of activity in the absence of pheromone and thus supports the basal transcription of these genes. Its activity, however, is strongly enhanced when cells are exposed to the mating factor (14, 17, 41 and reviewed in reference 43). Some *ste12* mutants allow normal basal transcription but are defective in pheromone-induced transcriptional activation (25). The failure of cells with these mutant *ste12* alleles to mate shows that transcriptional induction is essential for conjugation.

Pheromone efficiently arrests cells at a specific point in G_1 phase, called START, and thus prevents the transition from G_1 to S phase. In the absence of pheromone, this transition is catalyzed by a kinase which consists of a stable catalytic subunit (encoded by the *CDC28* gene) and an unstable stimulatory subunit (encoded by one of the three known *CLN* genes, *CLN1*, *CLN2*, and *CLN3*). There is accumulating evidence that pheromone arrests the cell cycle at START by interfering with the activity of the Cln-Cdc28 kinase(s). Critical components for cell cycle arrest are encoded by the *FUS3* and *FAR1* genes (8, 15). Fus3 phosphorylates Far1 in response to pheromone (37, 45), and phosphorylated Far1 can associate with and has been reported to inhibit Cln1-Cdc28 and Cln2-Cdc28 kinase complexes (37, 38, 45). This is likely to be part of the mechanisms by which pheromone interferes with the execution of START.

Mating appears to be a highly regulated developmental pathway, which occurs only in G_1-phase cells: cells arrest very specifically at START in response to pheromone, and when cells are forced to arrest outside of G_1, they fail to mate (39). This specificity is presumably required to ensure the correct ploidy of the zygote. Mechanisms that may contribute to achieve the G_1 specificity for pheromone-induced arrest include the cell cycle regulation of Far1 levels (32, 33) and the repression of pheromone signal transduction activity as cells execute START (35). Although maximal *FAR1* transcript levels are found both in pre-START G_1- and G_2/M-phase cells, the accumulation of significant Far1 protein levels is restricted to G_1 phase, at the time at which its function is required for pheromone-mediated cell cycle arrest (32). It has been found that Far1 is stable in G_1-phase cells and unstable at other cell cycle positions (32, 33) making it likely that regulated instability of Far1 contributes to the regulation of *FAR1* function. In this study we investigated the mechanism and functional significance of the transcriptional control of *FAR1* and other genes involved in mating.

MATERIALS AND METHODS

Yeast strains and plasmids. The genotypes of the strains used in this study are given in Table 1. Most strains were isogenic to BF264-15D (*trp1 1a leu2-3,112 ura3 ade1 his2*). Strains K2944 (provided by K. Nasmyth) and K3080 and GA231 (provided by G. Ammerer) are isogenic to W303. Strains were constructed by standard techniques (4). The *ste12*::*LEU2* disruption allele and the *GAL1-VP16-MCM1* construct were as described previously (1, 19). For some experiments the auxotrophic marker was switched to another auxotrophic marker (*URA3* or *TRP1*) by using swap plasmids that will be described elsewhere. Other mutant alleles for various loci and constructs for overexpression of *CLN3* and *FAR1* have been described previously (33, 35).

Growth conditions and synchronization procedures. Cells were grown in YEP medium or synthetic dropout medium as described previously (35) at 30°C, unless indicated otherwise. Cell cycle synchronization was achieved by several different protocols. *cdc15-2* cells and *cln1*$^-$ *cln2*$^-$ *cln3*$^-$ cells with a functional cyclin gene expressed from the *GAL1* promoter were synchronized as described previously (35). Selection of cells at different cell cycle positions based on cell

* Corresponding author. Mailing address: The Rockefeller University, Box 237, 1230 York Ave., New York, NY 10021. Phone: (212) 327 7685. Fax: (212) 327 7923.

TABLE 1. Strains used

Strain[a]	Genotype
BF264-15D derivatives	
1608-21C	*MAT***a** *bar1 cln1*::*TRP1 cln2*Δ *cln3*Δ *leu2*::*LEU2*::*GAL1*::*CLN3*
1316-1A	*MAT***a** *bar1 cln1*Δ *cln2*Δ *cln3*Δ *ste12*::*LEU2 leu2*::*LEU2*::*GAL1*::*CLN3*
1255-5C	*MAT***a** *bar1*
1255-5C VP16-MCM1	*MAT***a** *bar1 trp1*::*TRP1*::*GAL1-VP16-MCM1*
1255-5C ste12::LEU2	*MAT***a** *bar1 ste12*::*LEU2*
1255-5C ste12::LEU2 VP16-MCM1	*MAT***a** *bar1 ste12*::*LEU2 trp1*::*TRP1*::*GAL1-VP16-MCM1*
1255-5C GAL1FAR1	*MAT***a** *bar1 far1*::*URA3 leu2*::*LEU2*::*GAL1*::*FAR1*
1255-5C ste12::L>U GALFAR1	*MAT***a** *bar1 ste12*::*URA3 leu2*::*LEU2*::*GAL1*::*FAR1*
W303-1a derivatives	
W303-1a	*MAT***a**
W303-1a ste12::LEU2	*MAT***a** *ste12*::*LEU2*
GA231	*MAT***a** *clb3*::*TRP1 clb4*::*HIS3 mcm1*::*LEU2 GAL-UbiR-MCM1-98*::*URA3*
K3080	*MAT***a** *clb1*$^-$ *clb3*::*TRP1 clb4*::*HIS3 clb2-VI*
K3080 VP16-MCM1	*MAT***a** *clb1*$^-$ *clb3*::*URA3 clb4*::*HIS3 clb2-VI trp1*::*TRP1*::*GAL1-VP16-MCM1*
K2944-1B	*MAT***a** *bar1*::*ura3-ns cdc15-2*

[a] Strains are isogenic derivatives of BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*) or W303-1a (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1*).

size was accomplished by centrifugal elutriation (11). In short, cells grown in YEPD medium (4) (1 liter; optical density at 660 nm of ~1.0) were quickly cooled by the addition of crushed ice (~0.5 liter). Cells were then harvested by centrifugation and resuspended in ice-cold phosphate-buffered-saline solution (PBS). The suspension was sonicated to disperse clumps and then pumped, at a pump speed of 50 ml/min, into a 4°C Beckman JE5.0 elutriator rotor spinning at 3,500 rpm. Cells in the rotor were equilibrated for 10 min with ice-cold PBS and subsequently 400-ml fractions in ice-cold PBS were collected at the indicated flow rates between 70 and 210 ml/min. A portion of the cells was filtered (cellulose-acetate, 45-mm diameter, 0.45-μm-pore size) and resuspended in 15 ml of ice-cold PBS. Samples were then taken for Northern (RNA) analysis and microscopic examination. Cell size was determined electronically with a Coulter counter.

Northern and Western blot (immunoblot) analysis. Procedures for mRNA analysis were carried out as described previously (32). DNA restriction fragments excised from low-melting-point agarose gels or fragments generated by PCR were used as probes. Probes were labeled by random-prime labeling with a Prime-It kit (Stratagene). Fragments for making probes were, for *AGA1*, a 0.5-kb *Spe*I-*Mlu*I fragment from plasmid pAGA1 (provided by P. Lipke) (40); for *STE12*, an ~0.6-kb *Sac*I-*Bam*HI fragment (19); for *SST2*, a 745-bp PCR fragment (with ATCCAGATTCTGAGTCGCACA and TAGTAGGAAAGTGT TCCGATA as PCR primers); for *STE2*, a 715-bp *Sal*I-*Eco*RV fragment; for *MFA2*, a PCR fragment containing almost the entire open reading frame (with ATGCAACCGATCACCACT and CAGTTCAAAACTCTCCAC as PCR primers [34]); and for *H2A*, an ~0.4-kb PCR fragment (with CCGGTGGTAAAG GTGGTAAAG and TGGCAGACTTCTTTGGCAACA as primers [36]). Other fragments used in hybridizations have been described previously (9, 32).

Sample preparation, gel electrophoresis, and electroblotting for Western analysis were done as described previously (32). Blots were blocked with a blocking solution containing PBS, 5% Nonidet P-40 detergent, 0.1% Tween 20 detergent, 0.5% bovine serum albumin, and 2.3% dried low-fat milk. Incubation with crude anti-Far1 antiserum (32) and washes of the blots were done in PBS with 0.2% Tween 20. Proteins were detected by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham).

RESULTS

Transcription of many genes involved in mating is cell cycle regulated. It has been shown previously that the basal expression of *FAR1*, *FUS1* (involved in cell fusion), and *STE2* (the α-factor receptor) is cell cycle regulated (32, 35, 46). In order to see whether cell cycle regulation is a general property for genes involved in mating, we looked at the transcript levels of a number of other genes that affect different aspects of the mating response, such as mating-factor production (*MFA2* [34]), agglutination (*AGA1* [40]), and recovery from arrest (*SST2* [12]). For this purpose, cells with a temperature-sensitive (ts) *cdc15* allele were grown at the permissive temperature, arrested in late M phase by a shift to a restrictive temperature, and then released from the block by being shifted back to the permissive temperature. The transcript levels for many genes were found to fluctuate during the cell cycle (Fig. 1). Although there were some differences in the strength and timing of cell cycle-regulated transcription, a general pattern of expression was that transcript levels were high in late M/early G_1 phase followed by a decrease as cells entered S phase (indicated by

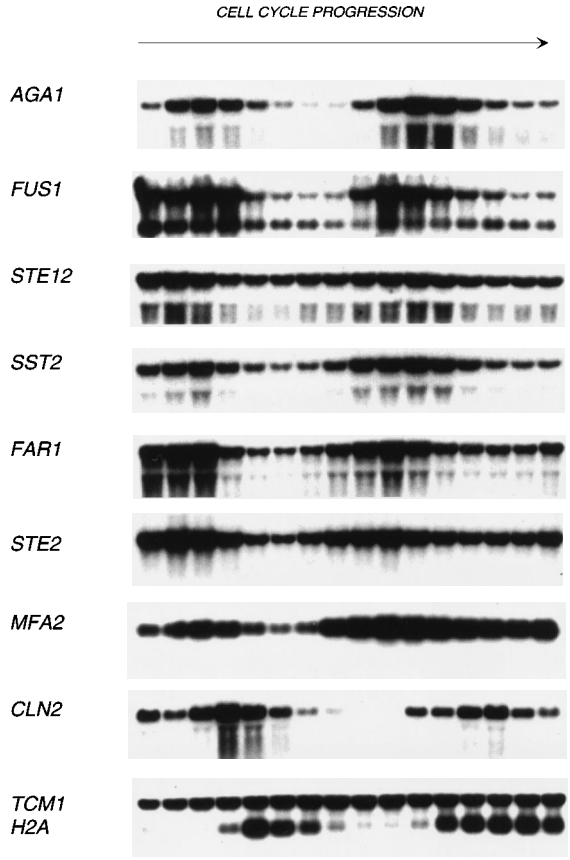

FIG. 1. Transcription of several genes involved in mating is cell cycle regulated. *MAT***a** *cdc15-2* cells (K2944-1B) were grown to early log phase at 25°C and then arrested in late M phase by shifting the culture to 36°C for 3 h. Synchronous growth was started by shifting the culture back to 25°C, and samples for Northern analysis were taken at the time of this shift and every 12 min thereafter.

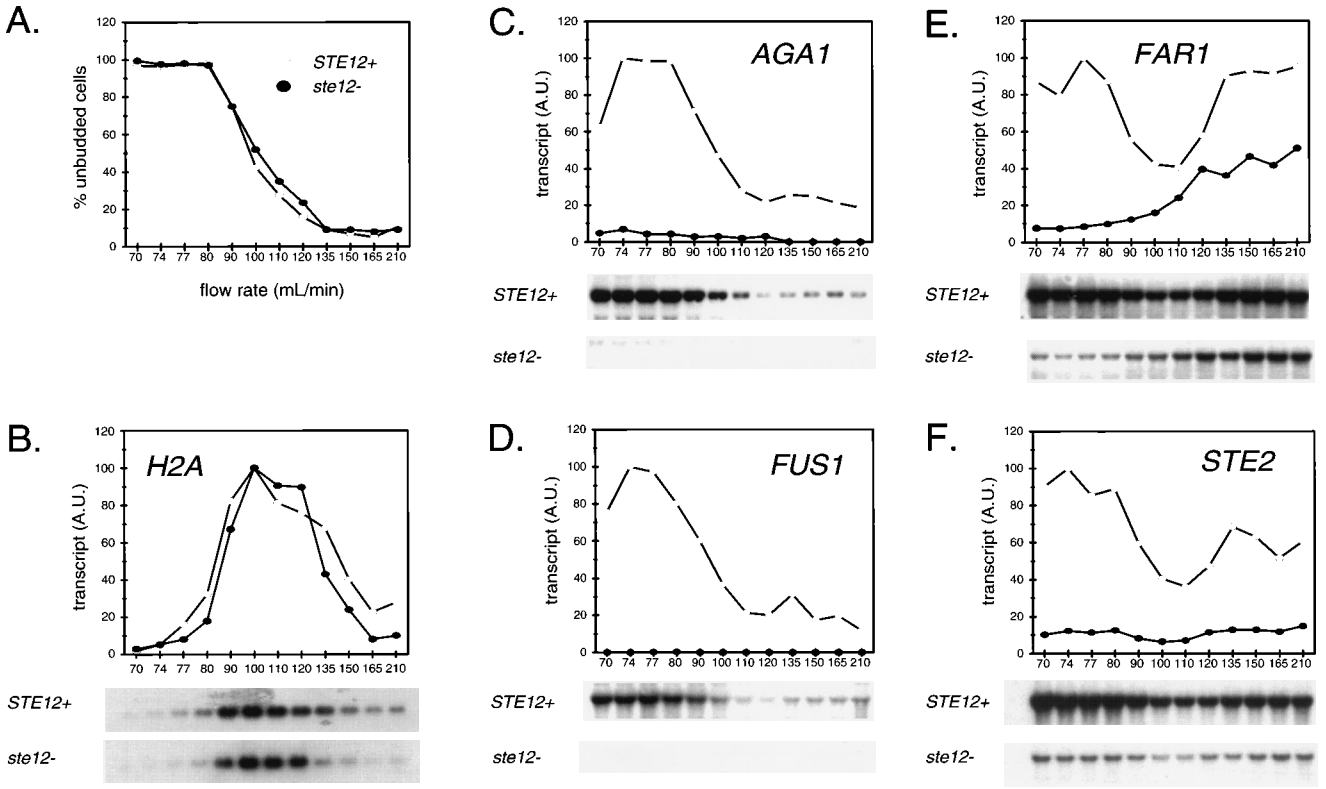

FIG. 2. Ste12 deficiency affects the cell cycle regulation of genes involved in mating. *STE12*$^+$ (1255-5C) and *ste12*$^-$ (1255-5C *ste12*::*LEU2*) cells were grown to exponential phase and then quickly chilled on ice. After concentration and sonication, cells were loaded into an elutriator rotor, and fractions were collected at the indicated flow rates. Fractions were analyzed for morphology (A) and various transcripts (B to F). Transcripts were quantified with a PhosphorImager system with *TCM1* transcripts as a loading control (data for *TCM1* are not shown). (B to F) Autoradiograph and quantified data for each transcript. The peak expression level in *STE12*$^+$ cells was arbitrarily chosen as 100 U of activity (A.U.), and other levels are expressed in relation to this value. Samples of *STE12*$^+$ and *ste12*$^-$ cells were run on the same gels, and the intensities of signals are directly comparable. Open circles, *STE12*$^+$ cells; closed circles, *ste12*$^-$ cells.

H2A transcription). In another method of synchronization, we used *cln1*$^-$ *cln2*$^-$ *cln3*$^-$ cells that were kept alive by expression of *CLN1*, *CLN2*, *CLN3*, or *CLB5* from the *GAL1* promoter. Such cells were synchronized by conditional cyclin expression as described previously (35). With these different synchronization procedures, essentially the same pattern of transcription was observed for *FAR1* and the other genes involved in mating as for the synchronized *cdc15* cells shown in Fig. 1 (data not shown). Although not all genes involved in mating were found to fluctuate (e.g., transcripts of *STE18*, which encodes the γ-subunit of the heterotrimeric G protein, were virtually constant throughout the cell cycle [data not shown]), it appears that cell cycle-regulated transcription is a common property of genes involved in mating.

***STE12* affects the cell cycle regulation of genes involved in mating.** The promoters of many genes involved in mating contain Ste12-binding PREs, which are implicated in their transcriptional induction by pheromone (27). The mechanism underlying the common patterns for cell cycle-regulated transcription of these genes might also involve PREs. To test the potential involvement of Ste12 and PREs in the transcriptional cell cycle regulation, we looked at the cell cycle pattern of transcription in cells which were devoid of Ste12. For that purpose asynchronous populations of *STE12*$^+$ and *ste12*$^-$ cells were fractionated according to cell size by centrifugal elutriation, and transcript levels were analyzed for the different size fractions.

The average volume of the cell population in each fraction increased with increasing flow rate (data not shown). Fractions isolated at low flow rates contained almost exclusively unbudded G_1-phase cells, and fractions collected at higher flow rates were enriched in budded cells (Fig. 2A). On the basis of this and the *H2A* transcript levels (Fig. 2B), the isolated fractions represent successive stages of the cell cycle. The results from this separation process were very similar for *STE12*$^+$ and *ste12*$^-$ cells (Fig. 2A and B). As expected from the data presented above, the transcripts for various genes involved in mating were cell cycle regulated in *STE12*$^+$ cells: *FUS1* and *AGA1* levels were high in unbudded G_1-phase cells and low at later cell cycle positions (Fig. 2C and D); *FAR1* and *STE2* levels were high in unbudded cells, reduced in post-START S-phase cells, and high again later in the cycle, presumably in G_2/M-phase cells (Fig. 2E and F). In *ste12*$^-$ cells, the *FUS1* and *AGA1* levels were strongly reduced to almost undetectable levels (Fig. 2C and D), and the *STE2* and *FAR1* levels dropped also but less severely (Fig. 2E and F). The reduction in *STE2* levels seemed to be about the same at all cell cycle positions (Fig. 2F). The overall reduction in *FAR1* levels in asynchronous *ste12*$^-$ cells was about threefold (Fig. 2E and data not shown), an effect which was considerably less strong than the effect reported previously (8). An interesting change in the pattern of *FAR1* transcription was observed in *ste12*$^-$ cells: levels were markedly reduced in G_1-phase cells (about 10-fold) but only weakly in G_2/M-phase cells (about 2-fold) (Fig. 2E). This altered pattern for *FAR1* transcription and the regulation of *FUS1* and *AGA1* transcripts are consistent with the idea that

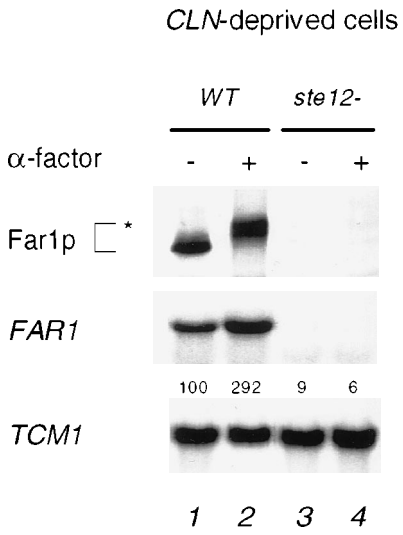

FIG. 3. G_1 expression of Far1 is Ste12 dependent. *MAT***a** *cln-GAL1*::*CLN3* cells with and without a functional *STE12* gene were grown to exponential phase on galactose medium and then shifted to raffinose medium. After 2.5 h, more than 95% of the cells were arrested as unbudded cells. A portion of the culture was treated with α-factor for 30 min (0.1 μM) before samples for Western and Northern analysis were taken. The numbers below the *FAR1* lanes reflect quantitated transcript levels, expressed in relation to the level in nonstimulated wild-type cells at the block (lane 1), which was arbitrarily chosen to represent 100 U. Phosphorylated Far1 protein is indicated with an asterisk. The strains used were 1608-21C (*WT*) and 1316-1A (*ste12*$^-$).

G_1 expression of *FAR1* and other genes involved in mating is dependent on Ste12 and that the G_2/M expression of *FAR1* is dependent on another transcription factor, which cannot stimulate *AGA1* and *FUS1* transcription.

Accumulation of Far1 is *STE12* dependent. To further test this hypothesis, we first looked at the expression levels of cells with and without *STE12* which were arrested in late G_1 phase by *CLN* deprivation. As shown in Fig. 3, *FAR1* transcript levels in G_1-arrested *ste12*$^-$ cells were about 10-fold lower than those in *STE12*$^+$ cells. Protein levels were reduced to undetectable levels in *ste12*$^-$ cells, whereas G_1-arrested *STE12*$^+$ cells contain high levels of Far1 (Fig. 3) (32). Similar transcription results were found in *STE12*$^+$ *cdc28-4* and *ste12*$^-$ *cdc28-4* cells arrested at START by a temperature shift (data not shown). A strong reduction in Far1 protein levels was also observed in asynchronous *ste12*$^-$ cells (data not shown). These data confirm that G_1-specific transcription of *FAR1* is Ste12 dependent (Fig. 2E) and indicate that G_1 expression is important for the accumulation of Far1 protein.

G_2/M expression of *FAR1* is *MCM1* dependent. In addition to various PRE elements (8), the *FAR1* promoter contains a potential Mcm1 binding site (28). Since *MCM1* has been implicated in the transcription of genes expressed in G_2/M phase (1, 26, 29, 30) and since Mcm1 binds to the Mcm1 binding site in the *FAR1* promoter in vitro (10), we wanted to test the involvement of Mcm1 in *FAR1* transcription. For that purpose *mcm1*$^-$ cells, kept alive by *MCM1* expression from the *GAL1* promoter, were switched to raffinose medium to deplete *MCM1* function. To promote quick depletion of the usually stable Mcm1, an unstable form of Mcm1 was expressed from the heterologous promoter (1). In *clb3*$^-$ *clb4*$^-$ cells, Mcm1 shutoff resulted in a first-cycle arrest in G_2/M phase (1). As shown in Fig. 4A, *FAR1* expression in such cells was strongly reduced (lanes 5 and 6). Similar observations were made for *STE2* transcription, which is known to be affected by Mcm1 (Fig. 4A) (24). When galactose-grown wild-type cells were switched to raffinose medium, a drop in *FAR1* transcription was observed (data not shown). This effect could account for only about one-third of the drop of *FAR1* transcription in the Mcm1-depleted cells, making it likely that the reduction of *FAR1* transcription is largely the result of reduced Mcm1 activity (data not shown). High levels of *FAR1* were observed when wild-type cells were arrested in G_2/M by using the microtubule poison nocodazole (Fig. 4A, lanes 1 and 2). In agreement with the data presented above (Fig. 2), *ste12*$^-$ cells arrested in G_2/M by nocodazole also contained significant *FAR1* transcript levels (Fig. 4A, lanes 3 and 4). Interestingly, cells arrested in G_2/M by *CLB* deprivation (by using a *clb1*$^-$ *clb3*$^-$ *clb4*$^-$ *clb2*-ts strain [3]) also had reduced *FAR1* transcript levels, consistent with a positive role of Clb proteins in the function of Mcm1 and in *FAR1* transcription (Fig. 4A, lanes 7 and 8).

Since the depletion of Mcm1 function results in decreased transcription of *CLB1* and *CLB2* (1) and since *FAR1* transcription is reduced in cells devoid of Clb1 to Clb4 activity, we tested whether the decrease of *FAR1* transcription in Mcm1-depleted cells was an indirect result of Clb inactivation. We found that continued *CLB2* expression from either the *S. cerevisiae ADH1* promoter or the *Schizosaccharomyces pombe adh* promoter did not result in increased *FAR1* transcription in Mcm1-depleted cells (data not shown). This indicates that the observed reduction in *FAR1* transcription is not an indirect result of Clb inactivation and supports the idea that Mcm1 activity is directly required for G_2/M transcription of *FAR1*.

To further test the involvement of Mcm1 in *FAR1* transcription, we used a VP16-Mcm1 fusion protein expressed from the *GAL1* promoter (1), which has been shown to promote Mcm1-specific transcription of *CLB1*, *CLB2*, and other Mcm1-dependent genes (1, 30). VP16-Mcm1 markedly increased the expression of *FAR1* in cells which were arrested by depletion of Clb1 to Clb4 activity (Fig. 4B) and in asynchronously dividing wild-type and *ste12*$^-$ cells (Fig. 4C). These data most likely reflect a direct interaction of Mcm1 with the *FAR1* promoter. The use of promoters in which Mcm1 binding sites are mutated should further clarify whether the interaction is direct, but at present our observations, taken together with the data on Mcm1 depletion, strongly implicate Mcm1 directly in transcription of *FAR1*.

Ectopic expression of *FAR1* in *ste12*$^-$ cells restores cell cycle arrest in response to mating factor. Cells lacking *STE12* are deficient in pheromone-induced transcription and cell cycle arrest. It has been observed, however, that some components of the pheromone signal transduction pathway can be activated in the absence of *STE12*; in *ste12*$^-$ cells, the MAP kinase homolog Ste7 and the MAP kinase homolog Fus3 are phosphorylated in response to pheromone (20, 47), and the level of Fus3 kinase activity seems to be similar to that in wild-type cells (16). The signal transduction pathway, at least up to the level of the Fus3 kinase, therefore seems to be fairly normal in *ste12*$^-$ cells. Given the strong effect of Ste12 depletion on *FAR1* transcript and protein levels, and given the fact that *far1*$^-$ cells do not arrest in response to pheromone, we wanted to test whether the failure of *ste12*$^-$ cells to arrest in response to pheromone could be due to a lack of *FAR1*. For that purpose we forced *ste12*$^-$ cells to express *FAR1* in G_1 phase by driving *FAR1* expression from the constitutive *GAL1* promoter, and we tested whether these cells would arrest in response to pheromone. The *GAL1*::*FAR1* construct does not result in strong overexpression of transcript and protein levels, compared with peak levels of normal expression (33; and data not shown). Cells expressing *FAR1* from the *GAL1* promoter

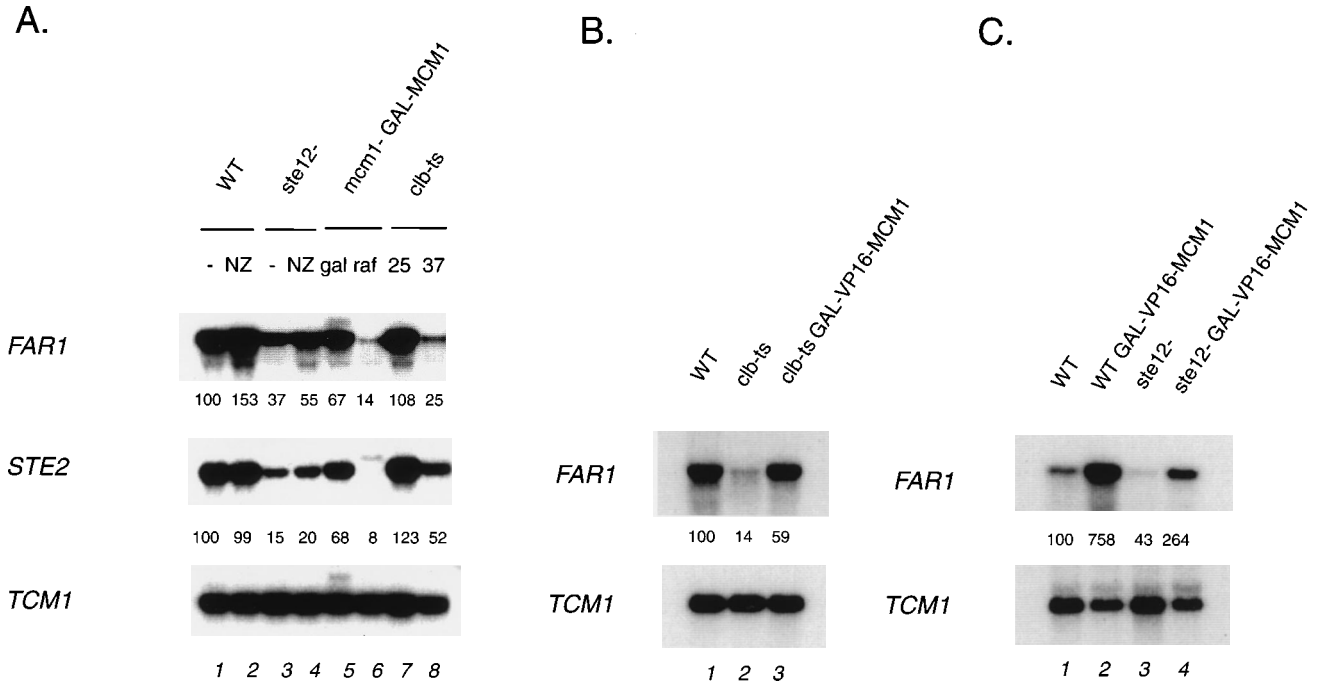

FIG. 4. G_2/M expression of *FAR1* is Mcm1 dependent. (A) Transcript levels in G_2/M-blocked cells. *MAT***a** cells of the indicated genotypes were grown under permissive conditions to early exponential phase in YEP-galactose medium, and a portion of the culture was then arrested in G_2/M by imposing restrictive conditions for 3 h. Wild-type and *ste12*$^-$ cells were treated with nocodazole (NZ; 15 μg/ml) (lanes 1 to 4). *mcm1*$^-$ *GAL1*::*MCM1* cells were shifted to YEP-raffinose medium (lanes 5 and 6). The *clb*-ts cells, which were grown at 25°C, were shifted to 37°C (lanes 7 and 8). Samples for Northern analysis were taken for permissive conditions (odd-numbered lanes) and for arrested cells (even-numbered lanes). The numbers below the *FAR1* lanes reflect quantitated RNA transcript levels, expressed in relation to the level in wild-type cells at 30°C (lane 1), which was arbitrarily chosen to represent 100 U. The strains used were W303-1a (wild type [WT]), W303-1a *ste12*::*LEU2* (*ste12*$^-$), GA231 (*mcm1-GAL1*::*MCM1*), and K3080 (*clb*-ts). (B) VP16-Mcm1 expression stimulates *FAR1* expression in Clb1- to Clb4-depleted cells. Wild type, *clb*-ts and *clb*-ts cells with a *GAL1*-driven *VP16-MCM1* fusion construct were grown to exponential phase in YEP-raffinose medium at 25°C and switched to 37°C. After 2 h, galactose was added to a final concentration of 3%, and samples for Northern analysis were taken 3 h after the addition of galactose. RNA transcript levels were quantitated and expressed in relation to the level in wild-type cells (lane 1), which was arbitrarily chosen to represent 100 U. The strains used were W303-1a (WT), K3080 (*clb*-ts) and K3080 VP16-MCM1 (*clb*-ts *GAL-VP16-MCM1*). (C) VP16-Mcm1 expression stimulates *FAR1* expression in wild-type and *ste12*$^-$ cells. Wild-type and *ste12*$^-$ cells with and without a *GAL1*-driven *VP16-MCM1* fusion construct were grown to exponential phase in YEP-galactose medium, and samples for Northern analysis were taken. The numbers below the *FAR1* lanes reflect quantitated RNA transcript levels, expressed in relation to the level in wild-type cells (lane 1), which was arbitrarily chosen to represent 100 U. The strains used were 1255-5C (wild type [WT]), 1255-5C VP16-MCM1 (wild type *GAL-VP16-MCM1*), 1255-5C *ste12*$^-$ (*ste12*$^-$), and 1255-5C *ste12*$^-$ VP16-MCM1 (*ste12*$^-$ *GAL-VP16-MCM1*).

are large, and the population contains a higher percentage of unbudded cells than in cells that do not express *GAL1*::*FAR1* (Fig. 5A) (33). Unlike normal *ste12*$^-$ cells, *ste12*$^-$ *GAL1*::*FAR1* cells arrested in response to pheromone as large unbudded cells (Fig. 5A), with a 1 N content of DNA (Fig. 5B). It is noteworthy that the cells arrested without the formation of the typical mating projections, called shmoos, suggesting that this response requires the Ste12-dependent induction of other genes. As shown in Fig. 6A, the arrest caused by pheromone in the *ste12*$^-$ *GAL1*::*FAR1* cells was not permanent, indicating that prolonged arrest also requires transcriptional induction of other Ste12-dependent factors. Our observation that expression of *FAR1* from the *GAL1* promoter could not restore the ability to mate to *ste12*$^-$ cells (tested by a qualitative patch assay [42] [data not shown]) is consistent with the idea that this Ste12-dependent transcriptional induction is essential for successful mating (25). Reduced *FAR1* transcript levels were found not only in *ste12*$^-$ cells but also in cells lacking a functional *STE4* gene or lacking both MAP kinase homologs *KSS1* and *FUS3* (data not shown). We found that *ste4*$^-$ *GAL1*::*FAR1* and *kss1*$^-$ *fus3*$^-$ *GAL1*::*FAR1* cells did not show G_1 arrest in response to pheromone (data not shown). These results indicate that both a functional signal transduction pathway and the G_1 expression of *FAR1* are required for this response.

We tested pheromone sensitivity by incubating cells with different doses of α-factor for 3 h and scored the percentage of unbudded cells. The sensitivity of *ste12*$^-$ *GAL1*::*FAR1* cells for pheromone was similar to that of *STE12*$^+$ cells with or without *GAL1*::*FAR1* (Fig. 6B). Taken together, these data indicate that the Ste12-mediated transcription of *FAR1* in the G_1 phase of the cell cycle and the resulting accumulation of Far1 protein are critical for cell cycle arrest in response to pheromone.

DISCUSSION

Ste12-dependent G_1 expression of genes involved in mating. The transcription factor Ste12 plays a key role in the basal and pheromone-induced transcription of many genes involved in mating. This notion is based on the observations that several mating genes are very poorly expressed and cannot be induced by mating pheromone in cells lacking Ste12 activity (13, 18, 22, 31, 41). In this report we show that the basal transcripts of various genes involved in mating are cell cycle regulated, with peak levels of expression in G_1 phase and reduced expression as cells enter S phase. Given the fact that basal expression of several of these genes (e.g., *FUS1* and *AGA1*) is strongly Ste12 dependent, this finding suggests that the activity of Ste12 to support basal transcription is cell cycle regulated. Our finding that loss of Ste12 has the strongest effect on *FAR1* transcription specifically in the G_1 phase is fully consistent with this

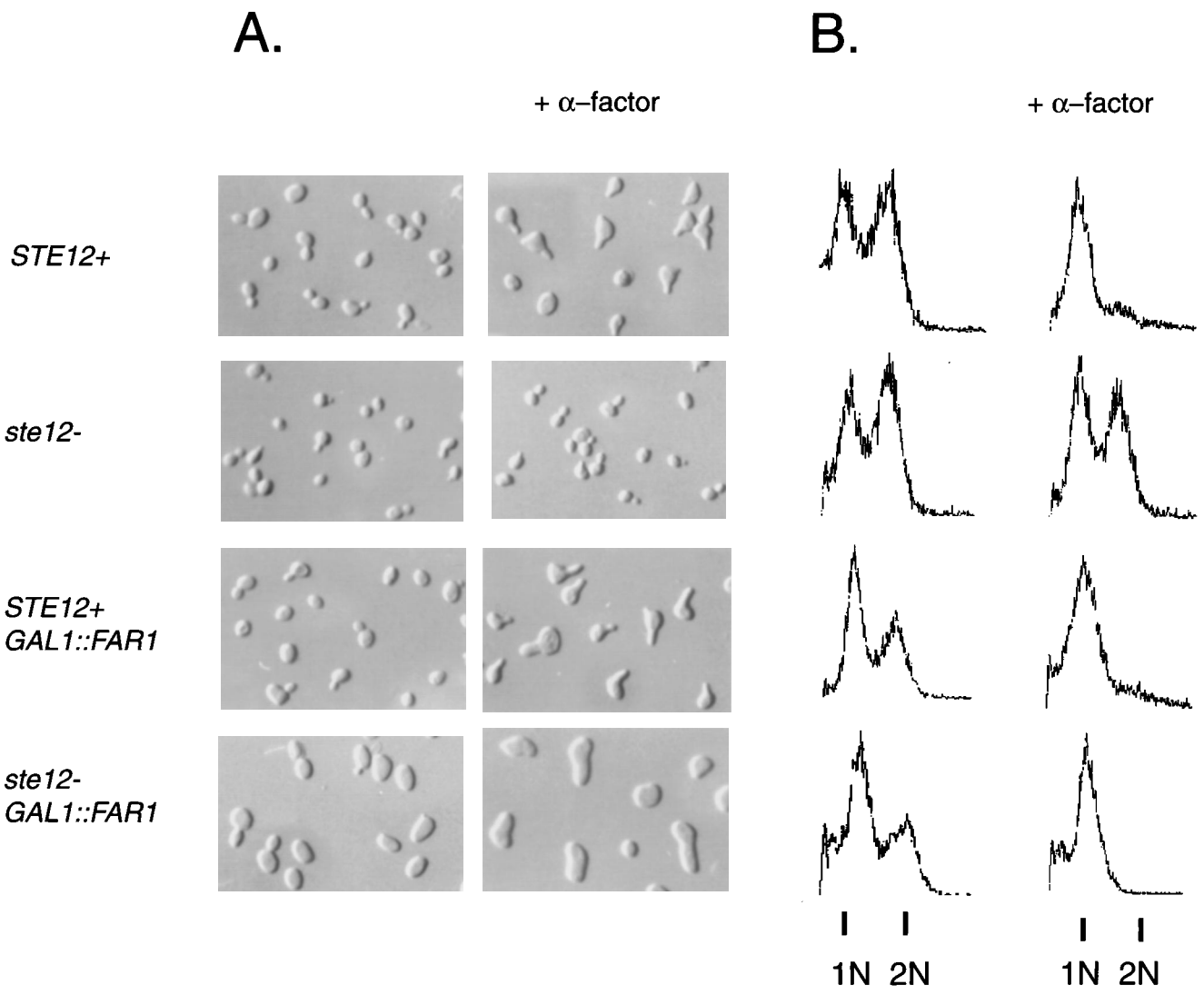

FIG. 5. Ectopic expression of *FAR1* restores pheromone-induced cell cycle arrest to *ste12*$^-$ cells. Cells of the indicated genotypes were grown to early exponential phase in YEP-galactose medium and then treated with 0.1 μM α-factor for 3 h. (A) Cell morphology was examined by differential interference contrast microscopy. The morphologies for untreated cells (left panels) and α-factor-treated cells (right panels) are shown. All photomicrographs are shown at the same magnification. (B) Analysis of the cellular DNA content as determined by flow cytometry for untreated cells (left) and pheromone-treated cells (right). The strains used were *STE12*$^+$ (1255-5C), *ste12*$^-$ (1255-5C ste12::LEU2), *STE12*$^+$ *GAL1*::*FAR1* (1255-5C GALFAR1), and *ste12*$^-$ *GAL1*::*FAR1* (1255-5C ste12::L>U GALFAR1).

idea. It may be relevant that the level of this Ste12-mediated transcription activity is high during the G_1 interval of the cell cycle, the only phase in which cells are mating competent (39). Cell cycle-regulated transcription has been observed for a variety of genes, and coordinated transcription is thought to be important for several cell cycle transitions (reviewed in reference 26). Ste12 could be a key component in a novel pattern of cell cycle-regulated transcription, which might promote the transition from a pheromone-sensitive and conjugation-competent phase in early G_1 phase to a pheromone-resistant and mating-incompetent stage as cells execute START and enter S phase.

Transcriptional regulation and *FAR1* function. Cell cycle arrest caused by pheromone is remarkably specific and occurs only in G_1-phase cells. It has been shown that Far1 is stable only in the G_1 phase of the cell cycle and unstable at other cell cycle positions (33). It is likely that this cell cycle-regulated instability of Far1 helps to ensure G_1-specific Far1 accumulation and that this contributes to confine pheromone-induced cell cycle arrest to the G_1 phase. Our observation here, that a reduction in Ste12-dependent G_1-specific transcription of *FAR1* results in strongly reduced Far1 levels, indicates that transcriptional regulation also contributes to the control of *FAR1* function.

There are some interesting similarities in the transcriptional regulation of *STE2* and *FAR1*. Both can be induced by the mating factor (8, 22), and both are transcribed in G_2/M- and G_1-phase cells. The transcription factor Mcm1 can bind to the *STE2* and *FAR1* promoters (2, 6, 10, 24), and it has been shown that mutations in the *STE2* Mcm1 binding site eliminate Mcm1 binding and reduce transcription (24). We describe here that high G_2/M transcription of both *FAR1* and *STE2* requires Mcm1. It is most likely that the G_2/M expression of these genes is mediated by Mcm1. *FAR1* and *STE2* may thus resemble *SWI5* (29), whose transcription is stimulated by Mcm1 at this cell cycle stage. This set of genes also includes *CLB1* and *CLB2*

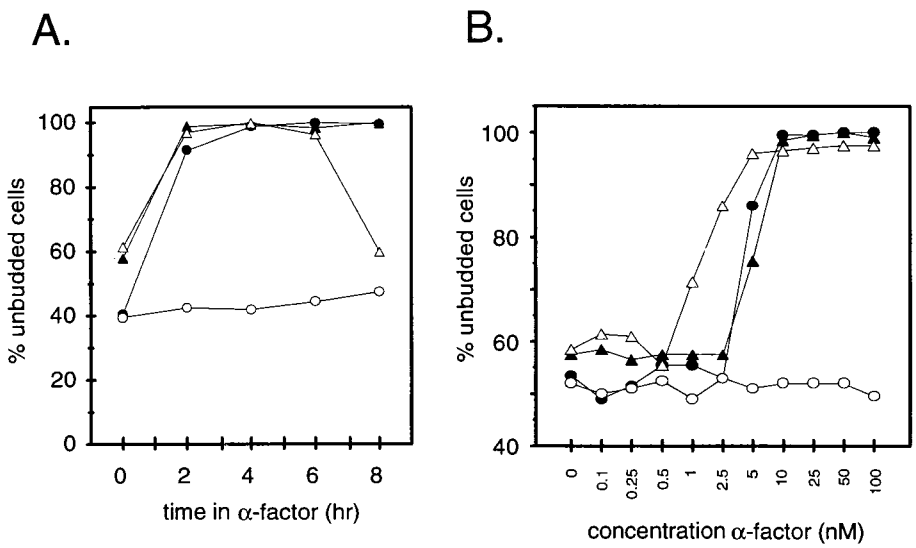

FIG. 6. Arrest kinetics and dose response for α-factor-induced cell cycle arrest. Wild-type and *ste12*$^-$ cells with and without *GAL1*::*FAR1* were grown on YEP-galactose medium to early exponential phase. (A) The percentage of unbudded cells was monitored in cultures treated with 0.1 μM α-factor at time zero. (B) Cells were incubated at different concentrations of α-factor for 3 h, and the percentage of unbudded cells was then scored. Closed circles, wild type (strain 1255-5C); open circles, *ste12*$^-$ (1255-5C ste12::LEU2); closed triangles, *STE12*$^+$ *GAL1*::*FAR1* (1255-5C GALFAR1); open triangles, *ste12*$^-$ *GAL1*::*FAR1* (1255-5C ste12::L>U GALFAR1).

(1, 21, 30, 44), which are thought to stimulate their own transcription through a positive feedback loop (3) that could involve Mcm1. The reduction of *FAR1* and *STE2* transcripts in cells lacking Clb1- and Clb2-Cdc28 kinase activities might therefore be the result of a failure of the Clb-Cdc28 kinase to fully activate Mcm1.

Mcm1 is known to interact with various transcription factors. In combination with α1, it activates α-cell-type-specific transcription; with α2, it represses **a**-cell-type-specific expression in α cells; with Ste12, it controls the expression of **a**-specific genes; with SFF, it controls the cell cycle regulation of *SWI5* transcription and possibly *CLB1* and *CLB2* (1, 7). The data presented here suggest that interactions between Mcm1 and Ste12 may also play a role in the proper cell cycle regulation of *FAR1* and other genes involved in mating. Further study of the cell cycle regulation of *FAR1* transcription may give more insight into the exact nature of these interactions and into how two transcription factors combine to control a G_2/M/G_1 pattern of transcription like that of *FAR1*.

***FAR1*-mediated cell cycle arrest of *ste12*$^-$ cells.** Mutants in *STE12* were originally identified by their failure to arrest, mate, and induce agglutinin synthesis in response to mating pheromone (23). Pheromone-induced transcription is dependent on the presence of functional Ste12 and has been found to be a prerequisite for mating (25). However, both Ste7 and Fus3 are phosphorylated in response to pheromone in cells lacking Ste12 (20, 47), and pheromone-treated *ste12*$^-$ cells seem to have a level of Fus3 kinase activity and a spectrum of endogenous substrates similar to those of wild-type cells (16). Cells lacking Ste12 therefore appear to have a fairly normal level of signal transduction activity, at least up to the level of the Fus3 kinase. It has been unclear, then, why cells lacking Ste12 do not arrest in response to pheromone. Our findings indicate that the failure of *ste12*$^-$ cells to accumulate Far1 may largely account for their inability to arrest in response to pheromone. Mutants in *FAR1* and *FUS3* that fail to arrest in response to pheromone but show normal transcriptional induction have been isolated (8, 15). This finding was taken as an indication that transcription and cell cycle arrest are separate responses to pheromone. Here we present a case in which the reverse is observed: *ste12*$^-$ *GAL*::*FAR1* cells are able to arrest in response to pheromone, without being able to induce transcript levels of mating genes, thus supporting the notion that cell cycle arrest and transcriptional induction (with the possible exception of *FAR1* induction) are separate phenomena. The observation that these cells are deficient in mating, shmoo formation, and prolonged arrest in response to pheromone is in agreement with the idea that these responses require transcriptional induction by pheromone (25). Our data indicate that the cell cycle-regulated transcription of *FAR1* is under combinatorial control by the transcription factors Ste12 and Mcm1 and that the Ste12-mediated transcription of *FAR1* in the G_1 phase of the cell cycle is essential for the accumulation of sufficient Far1 protein to allow the mating factor-induced cell cycle arrest at START.

ACKNOWLEDGMENTS

We thank J. Hirsch, P. Lipke, S. Michaelis, K. Nasmyth, and especially G. Ammerer for providing plasmids and yeast strains. We thank G. Ammerer and S. Dalton for communicating results prior to publication, B. Benton and L. Vallen for critical reading of the manuscript, and J. Liu for excellent technical assistance.

This study was supported by the Human Frontiers Science Program Organization, the Norman and Rosita Winston Foundation, the National Science Foundation, and Public Health Service grant GM49716.

REFERENCES

1. **Althoefer, H., A. Schleiffer, K. Wassmann, A. Nordheim, and G. Ammerer.** 1995. Mcm1 is required to coordinate G_2-specific transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**5917–5928.
2. **Ammerer, G.** 1990. Identification, purification, and cloning of a polypeptide (PRTF/GRM) that binds to mating-specific promoter elements in yeast. Genes Dev. **4:**299–312.
3. **Amon, A., M. Tyers, B. Futcher, and K. Nasmyth.** 1993. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. Cell **74:**993–1007.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. Wiley Interscience, New York.
5. **Bardwell, L., J. G. Cook, C. J. Inouye, and J. Thorner.** 1994. Signal propagation and regulation in the mating pheromone response pathway of the yeast Saccharomyces cerevisiae. Dev. Biol. **166:**363–379.

6. **Bender, A., and G. F. Sprague, Jr.** 1987. MAT alpha 1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific genes. Cell **50:**681–691.
7. **Bruhn, L., and G. F. Sprague, Jr.** 1994. MCM1 point mutants deficient in expression of α-specific genes: residues important for interaction with α1. Mol. Cell. Biol. **14:**2534–2544.
8. **Chang, F., and I. Herskowitz.** 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: *FAR1* is an inhibitor of a G1 cyclin, *CLN2*. Cell **63:**999–1011.
9. **Cross, F. R., and A. H. Tinkelenberg.** 1991. A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. Cell **65:**875–883.
10. **Dalton, S.** 1996. Personal communication.
11. **De Koning, W., K. Groeneveld, L. J. W. M. Oehlen, J. A. Berden, and K. Van Dam.** 1991. Changes in the activities of key enzymes of glycolysis during the cell cycle in yeast: a rectification. J. Gen. Microbiol. **137:**971–976.
12. **Dietzel, C., and J. Kurjan.** 1987. Pheromonal regulation and sequence of the *Saccharomyces cerevisiae SST2* gene: a model for desensitization to pheromone. Mol. Cell. Biol. **7:**4169–4177.
13. **Dolan, J. W., and S. Fields.** 1990. Overproduction of the yeast STE12 protein leads to constitutive transcriptional induction. Genes Dev. **4:**492–502.
14. **Dolan, J. W., C. Kirkman, and S. Fields.** 1989. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proc. Natl. Acad. Sci. USA **86:**5703–5707.
15. **Elion, E. A., P. L. Grisafi, and G. R. Fink.** 1990. *FUS3* encodes a $cdc2^{+}$/CDC28-related kinase required for the transition from mitosis into conjugation. Cell **60:**649–664.
16. **Elion, E. A., B. Satterberg, and J. E. Kranz.** 1993. FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. Mol. Biol. Cell **4:**495–510.
17. **Errede, B., and G. Ammerer.** 1989. STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. Genes Dev. **3:**1349–1361.
18. **Fields, S., D. T. Chaleff, and G. F. Sprague, Jr.** 1988. Yeast *STE7*, *STE11*, and *STE12* genes are required for expression of cell-type-specific genes. Mol. Cell. Biol. **8:**551–556.
19. **Fields, S., and I. Herskowitz.** 1987. Regulation by the yeast mating-type locus of STE12, a gene required for cell-type-specific expression. Mol. Cell. Biol. **7:**3818–3821.
20. **Gartner, A., K. Nasmyth, and G. Ammerer.** 1992. Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Genes Dev. **6:**1280–1292.
21. **Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I. Reed.** 1991. A cyclin B homolog in S. cerevisiae: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. Cell **65:**163–174.
22. **Hartig, A., J. Holly, G. Saari, and V. L. MacKay.** 1986. Multiple regulation of STE2, a mating-type-specific gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**2106–2114.
23. **Hartwell, L. H.** 1980. Mutants of Saccharomyces cerevisiae unresponsive to cell division control by polypeptide mating hormone. J. Cell Biol. **85:**811–822.
24. **Hwang-Shum, J.-J., D. C. Hagen, E. E. Jarvis, C. A. Westby, and G. F. Sprague, Jr.** 1991. Relative contributions of MCM1 and STE12 to transcriptional activation of a- and α-specific genes from *Saccharomyces cerevisiae*. Mol. Gen. Genet. **227:**197–204.
25. **Kirkman-Correia, C., I. L. Stroke, and S. Fields.** 1993. Functional domains of the yeast STE12 protein, a pheromone-responsive transcriptional activator. Mol. Cell. Biol. **13:**3765–3772.
26. **Koch, C., and K. Nasmyth.** 1994. Cell cycle regulated transcription in yeast. Curr. Opin. Cell Biol. **6:**451–459.
27. **Kronstad, J. W., J. A. Holly, and V. L. MacKay.** 1987. A yeast operator overlaps an upstream activation site. Cell **50:**369–377.
28. **Kuo, M. H., and E. Grayhack.** 1994. A library of yeast genomic MCM1 binding sites contains genes involved in cell cycle control, cell wall and membrane structure, and metabolism. Mol. Cell. Biol. **14:**348–359.
29. **Lydall, D., G. Ammerer, and K. Nasmyth.** 1991. A new role for MCM1 in yeast: cell cycle regulation of *SWI5* transcription. Genes Dev. **5:**2405–2419.
30. **Maher, M., F. Cong, D. Kindelberger, K. Nasmyth, and S. Dalton.** 1995. Cell cycle-regulated transcription of the CLB2 gene is dependent on Mcm1 and a ternary complex factor. Mol. Cell. Biol. **15:**3129–3137.
31. **McCaffrey, G., F. J. Clay, K. Kelsay, and G. F. Sprague, Jr.** 1987. Identification and regulation of a gene required for cell fusion during mating of the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**2680–2690.
32. **McKinney, J. D., F. Chang, N. Heintz, and F. R. Cross.** 1993. Negative regulation of *FAR1* at the start of the yeast cell cycle. Genes Dev. **7:**833–843.
33. **McKinney, J. D., and F. R. Cross.** 1995. *FAR1* and the G1 phase specificity of cell cycle arrest by yeast mating factor. Mol. Biol. Cell **15:**2509–2516.
34. **Michaelis, S., and I. Herskowitz.** 1988. The **a**-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. Mol. Cell. Biol. **8:**1309–1318.
35. **Oehlen, L. J. W. M., and F. R. Cross.** 1994. G1 cyclins *CLN1* and *CLN2* repress the mating factor response pathway at START in the yeast cell cycle. Genes Dev. **8:**1058–1070.
36. **Osley, M. A., J. Gould, S. Kim, M. Y. Kane, and L. Hereford.** 1986. Identification of sequences in a yeast histone promoter involved in periodic transcription. Cell **45:**537–544.
37. **Peter, M., A. Gartner, J. Horecka, G. Ammerer, and I. Herskowitz.** 1993. FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. Cell **73:**747–760.
38. **Peter, M., and I. Herskowitz.** 1994. Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. Science **265:**1228–1231.
39. **Reid, B. J., and L. H. Hartwell.** 1977. Regulation of mating in the cell cycle of *Saccharomyces cerevisiae*. J. Cell Biol. **75:**355–365.
40. **Roy, A., C. F. Lu, D. L. Marykwas, P. N. Lipke, and J. Kurjan.** 1991. The *AGA1* product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein **a**-agglutinin. Mol. Cell. Biol. **11:**4196–4206.
41. **Song, O., J. W. Dolan, Y. O. Yuan, and S. Fields.** 1991. Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation. Genes Dev. **5:**741–750.
42. **Sprague, G. F.** 1990. Assay of yeast mating reaction. Methods Enzymol. **194:**77–93.
43. **Sprague, G. F., and J. W. Thorner.** 1992. Pheromone response and signal transduction during the mating process of Saccharomyces cerevisiae, p. 657–744. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular and cellular biology of the yeast Saccharomyces: gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth.** 1991. The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell **65:**145–161.
45. **Tyers, M., and B. Futcher.** 1993. Far1 and Fus3 link the mating pheromone signal transduction pathway to three G_1-phase Cdc28 kinase complexes. Mol. Cell. Biol. **13:**5659–5669.
46. **Zanolari, B., and H. Riezman.** 1991. Quantitation of α-factor internalization and response during the *Saccharomyces cerevisiae* cell cycle. Mol. Cell. Biol. **11:**5251–5258.
47. **Zhou, Z., A. Gartner, R. Cade, G. Ammerer, and B. Errede.** 1993. Pheromone-induced signal transduction in *Saccharomyces cerevisiae* requires the sequential function of three protein kinases. Mol. Cell. Biol. **13:**2069–2080.