The activity of the *Saccharomyces cerevisiae* pheromone signal transduction pathway is regulated by Cln1/2-Cdc28 cyclin-dependent kinase. High level expression of *CLN2* can repress activation of the pathway by mating factor or by deletion of the α-subunit of the heterotrimeric G-protein. We now show that *CLN2* overexpression can also repress *FUS1* induction if the signaling pathway is activated at the level of the β-subunit of the G-protein (*STE4*) but not when activated at the level of downstream kinases (*STE20* and *STE11*) or at the level of the transcription factor *STE12*. This epistatic analysis indicates that repression of pheromone signaling pathway by Cln2-Cdc28 kinase takes place at a level around *STE20*. In agreement with this, a marked reduction in the electrophoretic mobility of the Ste20 protein is observed at the time in the cell cycle of maximal expression of *CLN2*. This mobility change is constitutive in cells overexpressing *CLN2* and absent in cells lacking *CLN1* and *CLN2*. These changes in electrophoretic mobility correlate with repression of pheromone signaling and suggest Ste20 as a target for repression of signaling by G1 cyclins. Two morphogenic pathways for which Ste20 is essential, pseudohyphal differentiation and haploid-invasive growth, also require *CLN1* and *CLN2*. Together with the previous observation that Cln1 and Cln2 are required for the function of Ste20 in cytokinesis, this suggests that Cln1 and Cln2 regulate the biological activity of Ste20 by promoting morphogenic functions, while inhibiting the mating factor signal transduction function.

Binding of mating factor to a specific receptor in haploid *Saccharomyces cerevisiae* cells activates a signal transduction pathway that prepares for conjugation with cells of the opposite mating type. The transduction of the signal starts with binding of the peptide mating factor to a seven-transmembrane domain receptor (*Ste2* in *a* mating type cells and *Ste3* in *α*-cells), which then activates a heterotrimeric G-protein by releasing an active β-γ complex from the inhibitory α-subunit (*α*, β, and γ-subunits are encoded, respectively, by the *GPA1*, *STE4*, and *STE18* genes). The activated G-protein transmits the signal to a set of serine/threonine protein kinases that are activated in a sequential order. The first of these is Ste20, a member of the family of p21-activated kinases (PAKs), and then Ste11 (a MAP kinase kinase kinase), Ste7 (a MAP kinase kinase), and finally a MAP kinase (*Fus3* or in some cases *Kss1*) are activated. Activation of the MAP kinase (i) stimulates the transcription of many genes involved in the conjugation process through the transcription factor Ste12, (ii) results in arrest in G1-phase of the cell cycle through the Far1 protein, and (iii) leads to specific morphological changes that are required for efficient cell fusion. Several review articles (1–4) describe the mating factor signal transduction pathway and other MAP kinase-based pathways in more detail.

Some of the components of the mating factor signal transduction pathway are required for functions other than sexual differentiation. Agar-invasive growth of haploid cells (haploid-invasive growth) and pseudohyphal growth of diploid cells both require Ste20, Ste11, Ste7, and Ste12 (5–7). In addition, from the lethal phenotype of cells that are deleted for both Ste20 and Cla4, a related PAK family member, Ste20, appears to share a function in the budding/cytokinesis cycle with Cla4 (8). The overlap in function is only partial, as Cla4 has no known function in mating factor signal transduction. Therefore, Ste20 is not only critical for sexual differentiation in response to mating factor but can also play a role in morphogenesis during the vegetative cell cycle. The small G-protein Cdc42 can interact with a specific domain in the N terminus of Ste20 that is conserved among PAK family members (2). This interaction of Cdc42 with Ste20 is dispensable for *in vitro* kinase activity and the mating factor signal transduction functions of Ste20 but appears critical for the vegetative morphological roles of Ste20 (9–11). Similarly, full morphogenic function of Cla4 also requires interaction of Cla4 with Cdc42 (12).

Both the basal activity (in absence of ligand stimulation) and the mating factor-induced activity of the mating factor signal transduction pathway are cell cycle-regulated (13–15). In the absence of mating factor stimulation, fluctuations are observed for transcripts of many genes that are involved in the mating reaction and whose transcription involves Ste12 (13–16). A common pattern for transcription of such genes is that transcription is high in G1-phase and then declines as cells enter S-phase (15). The activity of the *Fus3* protein kinase shows a similar cell cycle pattern (17), and this regulation is likely to be required for the cell cycle regulation of basal transcription. The mating factor-induced signal transduction activity is also strictly regulated, with maximal activation during M/G1-phase, and reduced activation in S-phase (13, 14). This regulation of the induced signal transduction activity depends specifically on the G1 cyclins *CLN1* and *CLN2* (14). These cyclins are expressed in late G1-phase, and when Cln1 and Cln2 associate with the cyclin-dependent kinase (CDK) Cdc28, they help to promote the transition of cells from G1- to S-phase (18, 19). High level expression of *CLN2* strongly reduces induction of
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**TABLE I**

| Strains | Genotypes |
|---------|------------|
| 1255-5C | MATa bar1  |
| BOY391  | MATa bar1 his3 HIS2  |
| BOY1037 | MATa bar1 LEU2::GAL1::CLN2  |
| BOY539  | MATa bar1 his3 H2B TRP1::GAL1::CLN2  |
| BOY575  | MATa bar1 ste2::LEU2  |
| BOY1151 | MATa bar1 ste18::LEU2  |
| BOY527  | MATa bar1 ste4::LEU2  |
| BOY1149 | MATa bar1 ste5::LEU2  |
| BOY754  | MATa bar1 ste20::URA3  |
| BOY1370 | MATa bar1 ste20::LEU2  |
| BOY1277 | MATa bar1 ste11::URA3  |
| BOY1289 | MATa bar1 ste11::TRP1  |
| BOY783  | MATa bar1 ste7::LEU2  |
| BOY906  | MATa bar1 hs1::LEU2 fus3::TRP1  |
| BOY522  | MATa bar1 hs1::LEU2 fus3::URA3  |
| BOY529  | MATa bar1 ste12::LEU2  |
| BOY501a | MATa bar1 cdc15-2  |
| BOY443a | MATa  |
| BOY1427a | MATa LEU2::GAL1::CLN2  |
| BOY445a | MATa ste20::LEU2  |
| BOY743a | MATa bar1 cdc28-13  |
| BOY1143a | MATa bar1 cdc28-13 cln1 cln2 4×CLN3  |
| BOY836a | MATa bar1 cln1 cln2 3 pTRP1::GAL1::CLN1  |
| BOY188a | MATa bar1 cln1 cln2 cln3 LEU2::GAL1::CLN2  |
| BOY747a | MATa bar1 cln1 cln2 cln3 LEU2::GAL1::CLN3  |
| BOY798a | MATalpha bar1 cla::TRP1  |
| BOY1113a | MATa bar1 akr1::URA3 his3  |
| BOY1162a | MATalpha bar1 akr1::URA3  |
| BOY489a | MATalpha bar1 ste20::TRP1  |
| BOY1138a | MATa bar1 bem1::LEU2  |
| LS976a | MATa/MATa  |
| BOY1565a | MATa/MATa cln1::URA3 cln1::URA3 cln2::TRP1 cln2::LEU2  |
| 10560-4D | MATa  |
| BOY1452a | MATa cln1::URA3  |
| BOY1459a | MATa cln2::LEU2  |
| BOY1451a | MATa cln1::URA3 cln2::LEU2  |

- Strain isogenic to W303.
- Strain isogenic to BF264–15D (trp1-1a leu2-3, 112 ura3 ade1 his2).

mating specific genes by mating factor or by deletion of the α-subunit of the heterotrimeric G-protein (14). This suggests that regulation of mating factor signal transduction activity by Cln1/2-Clc28 takes place at a level downstream of the mating factor receptor and the α-subunit.

Here we present a more detailed analysis of the regulation of the mating factor signal transduction pathway by the G1 cyclins CLN1 and CLN2. A combination of genetic, biochemical, and cell biological observations suggests that Cln1/2-Clc28 regulate the function of the Ste20 protein kinase.

**MATERIALS AND METHODS**

**Yeast Strains and Plasmids**—The genotypes of the strains used in this study are given in Table I. Strains were isogenic to BP264-15D (trp1-1a leu2-3, 112 ura3 ade1 his2) except where indicated. Strains were constructed by standard techniques for crossing and gene replacement (20). Plasmids that provided fragments for the creation of disruption alleles are as follows: pAB506 (ste2::LEU2 (21)), pMS97 (ste18::URA3 (23)), pΔ121 (ste4::LEU2 (provided by V. MacKay, Seattle)), pSF32 (ste5::URA3 (provided by V. MacKay, Seattle)), pEL45 (ste20::URA3 (25)), pS11094 (ste11::URA3 (24)), pNC113 (ste7::LEU2 (25)), pB065 (hsu1::URA3 (26)), pYE99 (fus3::LEU2 (27)), pSUL16 (ste12::LEU2 (28)), pBB119 (cla::TRP1 (12)), pBB590 (ahr1::URA3 (29)), pKO2 (= pBP642, bem1::LEU2 (30)). Gene disruptions were made by one-step gene replacement with appropriately digested DNA. In some cases the original auxotrophic markers on disruption cassettes were altered using “marker swap” plasmids (31). Deletion alleles for CLN genes and CLN expression constructs were as described previously (14). Other plasmids used were as follows: pL19 (pURA3-GAL1::STE4 (32)), pURA3-GAL1::STE20AN (33), pVTU-STE20 (pURA3-ADH-STE20 (23)), pGA2003 (pTRP1-GAL1::STE5-myc (provided by G. Ammerer, Vienna)), pGU-STE11AN.

**RESULTS**

Repression of Pheromone Signal Transduction Takes Place Around the Level of Ste20—We have previously shown that constitutive expression of CLN2 from the strong GAL1 promoter can effectively block the response to mating factor (14). Activation of the mating factor pathway by deletion of the α-subunit of the heterotrimeric G-protein can also be repressed by overexpression of CLN2 (14). This latter observation suggests that inhibition of the mating factor pathway by CLN2 takes place at a level downstream of the mating factor receptor and the α-subunit of the G-protein. We wanted to determine the site of action of Cln2 on the mating factor pathway more precisely. For this epistatic analysis, we used expression constructs of particular genes, whose high level expression has been shown to induce the mating factor response pathway. Among these are STE4 (the β-subunit of the G-protein) (32), activated alleles of the STE20 and STE11 kinases (24, 33, 34), the “scaffolding” protein STE5 (36, 37), and the transcription factor STE12 (35). We studied the effect of simultaneous expression of CLN2 and these activators of the mating factor response pathway (Fig. 1). In all cases the signal transduction activity of wild type cells and GAL1::CLN2 cells without the expression construct served as a control. As shown in Fig. 1A, overexpression of CLN2 from the GAL1 promoter can prevent the induction of FUS1 transcription caused by overexpression of STE4. Even when STE4-overexpressing cells were treated with mating factor, simultaneous overexpression of CLN2 could prevent the induction of FUS1. In contrast, overexpression of a truncated allele of STE20 (STE20AN, Fig. 1B), a truncated allele of STE11 (STE11AN, Fig. 1D) and STE12 (Fig. 1E), resulted in elevated levels of FUS1 transcription in the presence of high levels of CLN2. In all these cases, expression of CLN2 from the GAL1 promoter also failed to prevent the additional elevation of FUS1 transcript levels by addition of mating factor (Fig. 1, B, D, and E). (It should be noted that, in a previous publication (14), we have referred to preliminary results that appeared to show enhanced expression of GAL1::STE4 to GAL1::CLN2. These data now turn out to be incorrect and were probably due to a mix-up of plasmids. We wish to apologize for any problem that this may have caused.)

We found that high level CLN2 expression not only failed to down-regulate elevated FUS1 transcription induced by GAL1::STE11AN (Fig. 1D) but also SST2 transcription (the
transcript is regulated similarly to FUS1 (38) induced by activated STE11 alleles (STE11-1 and STE11-4 (24)) expressed from their own promoter (Fig. 2D). In fact, high level CLN2 expression may somewhat enhance the effect of the STE11-4 allele on SST2 transcription. This finding is in contrast to results that were recently reported by others (17). Even using the same strains that were used in that study (17), we have been unable to reproduce the result that high level CLN2 expression represses the elevation of FUS1 transcription caused by the activated alleles STE11-1 and STE11-4 (data not shown). The results shown in Fig. 2D cannot be explained by ineffective expression of CLN2, as mating factor-induced transcription of SST2 is effectively blocked by high level CLN2 expression in these cells (Fig. 2E). We have no explanation at present for this discrepancy.

The GAL1::STE20ΔN construct fails to complement the mating defect of ste4, ste5, ste11, ste7, and ste12 cells (33). To provide additional epistatic information, we determined the effects of overexpression of GAL1::STE20ΔN on FUS1 transcription in strains that were deleted for various components of the mating factor signal transduction pathway (Fig. 2A). Induction of FUS1 by GAL1::STE20ΔN was observed in cells lacking the mating factor receptor (STE2), components of the heterotrimeric G-protein (STE4 and STE18), and STE5. However, other components of the signal transduction pathway were required for GAL1::STE20ΔN-induced FUS1 transcription. These data are consistent with the established epistatic position of STE20 downstream of the G-protein and upstream of the STE11-STE7-MAP kinase cassette (23) and thus demonstrate the usefulness of the GAL1::STE20ΔN construct for epistatic analysis.

The epistatic position of STE5 in the mating factor response pathway is complicated, possibly because of the many proteins with which Ste5 interacts (39). A GAL1::STE5 construct has been used previously in epistatic experiments, but when plating efficiency of cells containing a GAL1::STE5 plasmid was monitored, this yielded rather complex results (36). We tested the FUS1 induction by the GAL1::STE5 construct in strains deleted for various components of the signal transduction pathway (Fig. 2B). FUS1 induction by overexpression of STE5 required, with the exception of STE2, the presence of all the tested components of the mating factor signal transduction pathway. Activated alleles of STE5 were previously shown to partially complement the mating defect of strains deleted for the mating factor receptor, components of the heterotrimeric G-protein or Ste20, but not deletion of components of the STE11-STE7-MAP kinase cassette (23, 37). Our data confirm the findings of Hasson et al. (37) and extend previous analyses (37, 40) by showing that induction of FUS1 by high level expression of STE5 also requires STE20. The failure of induction of FUS1 by GAL1::STE5 in ste20 (36) suggests that slow growth of such cells that was observed by Akada et al. (36) is not due to increased transcriptional activity of the mating factor response pathway. On the whole, the data obtained with

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**Fig. 1. Epistatic analysis of repression of mating factor signal transduction by high level expression of CLN2.** Cells were grown to early exponential phase in synthetic dropout medium with raffinose as a carbon source. Then galactose was added to all cultures for several hours to induce the various constructs driven from the GAL1 promoter. Samples were taken for Northern analysis before and after 15 min mating factor stimulation. Blots were probed for FUS1 transcript to monitor activity of the signal transduction pathway and for TCMI transcript to control for loading in all lanes. Transcript levels were quantitated using a STORM PhosphorImager system, and the data shown are FUS1 levels corrected for loading. Samples before mating factor stimulation are shown by gray bars and mating factor-stimulated samples by black bars. For each panel, the mating factor-induced FUS1 level in wild-type cells was arbitrarily chosen as 100 units, and other transcript levels in that panel are given in relation to this value. A, strains 12255-C (wt) or BOY1035 (GAL1::CLN2) were transformed with pl19 (pURA3-GAL1::STE4) or vector controls. Induction in galactose was for 3 h. B, same strains as in A transformed with pURA3-GAL1::STE20ΔN or vector controls. Induction in galactose was for 14 h. C, same strains as in A transformed with pGA2013 (pTRP1-GAL1::STE5) or vector controls. Induction in galactose was for 5 h. D, strains BOY391 (wt) or BOY389 (GAL1::CLN2) were transformed with pG-U1E11ΔN (pURA3-GAL1::STE11ΔN) or vector controls. Induction in galactose was for 5 h. E, same strains as in D were transformed with pGRK10 (pURA3-GAL1::STE12) or vector controls. Induction in galactose was for 5 h.
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GAL1::STE5 constructs yield rather complex results that are difficult to place in an epistatic series and therefore are of limited use in establishing the position of the negative effect of Cln2-Cdc28 on the mating factor signal transduction pathway. Because of this, the observation that GAL1::CLN2 expression represses the signal generated by GAL1::STE5 only in the absence, and not in the presence of mating factor (Fig. 1C), is also difficult to interpret.

The epistatic position of Ste11 in relation to most other components of the mating factor signal transduction pathway is fairly well established by transcriptional-induction and mating-complementation assays using STE11-1 and STE11-4 alleles (24) or the GAL1::STE11AN construct (34). The observation that STE11-1 and STE11-4 also induce significant levels of SST2 in ste20 cells (Fig. 2C) is consistent with the general notion that Ste11 acts downstream of Ste20.

Taken together, the data presented in Figs. 1 and 2 show that GAL1::CLN2 represses the mating factor pathway at a level which is at or downstream of Ste4 and at or upstream of Ste20. The observations that mating factor-induced hyper-phosphorylation of Ste7 (41), tyrosine phosphorylation of Fus3 (42) (assayed with anti-phosphotyrosine antibodies in immunoprecipitates of Fus3), and Fus3 kinase activation can be prevented by high level expression of CLN2 (data not shown (15)) are consistent with this epistatic placement of the negative effect of Cln2-Cdc28 on the mating factor response pathway. Since the Ste20 protein is one of the potential targets suggested by epistatic analysis and since studies of the Ste20 homolog Cla4 suggest a potential genetic interaction between Ste20 and the G1 cyclins CLN1 and CLN2 (8, 12), we focused on Ste20 as a potential site for repression of mating factor signal transduction.

The Electrophoretic Mobility of Ste20 Changes during the Cell Cycle—We first looked at the abundance of the Ste20 protein at various cell cycle stages. For this purpose, temperature-sensitive cdc15-2 cells were arrested in late M-phase at restrictive temperature, and synchronous cell cycle progression was then initiated by lowering the temperature. Ste20 appears to be present at all cell cycle positions, but there are marked changes in the mobility of the protein when cells progress through the cell cycle (Fig. 3). The mobility of the protein on SDS-PAGE gels was relatively fast in late M- and early G1-phase, but Ste20 in late G1 cells and S-phase migrated markedly slower (Fig. 3). This “upshift” in mobility of Ste20 followed peak levels of CLN2 transcription and roughly coincided with the previously identified period of repression of the mating factor pathway by Cln1/2-Cdc28 (Fig. 3 (14)). Similar observations were made on the native STE20 driven from its own promoter, showing that the continued presence of Ste20 through the cell cycle is not some artifact of overexpression of the protein (data not shown). Since Ste20 protein levels of the STE20 gene expressed from its own promoter were markedly lower than if STE20 is expressed from either the ADH promoter or the GAL1 promoter (data not shown), further analysis.
of the Ste20 protein was performed using these ectopic expression constructs.

Changes in the Mobility of Ste20 Are Cln1/2-dependent—Combining the data from the epistatic analysis (Fig. 1) with the data on the cell cycle-regulated mating factor signal transduction (14) and cell cycle-regulated mobility changes of Ste20 (Fig. 3), we wanted to test whether the change in mobility of Ste20 was important for repression of the mating factor pathway by Cln1/2-Cdc28 kinase. If the upshift in mobility was important for repression, it could be expected that the upshift, like repression, is (i) dependent on the presence of CLN1/2 and (ii) constitutive in cells that overexpress CLN2. To test this potential connection, we first followed the mobility of Ste20 in cln1 cln2 CLN3 cells compared with cells that did contain the CLN1 and CLN2 cyclins. The same strains and synchronization protocols were used for this analysis that were used previously to show that repression of the mating factor response pathway depends on the presence of CLN1 and CLN2 (14). Also with this different method of synchronization, the CLN wild type cells showed a change in the mobility of Ste20 at about the time when CLN1/2 are normally maximally expressed and cells enter S-phase (Fig. 4A). The cell cycle pattern of the electrophoretic mobility changes of Ste20 in CLN1 CLN2 cln3 cells was very similar to that in wild type cells (data not shown). In contrast, the upshift in mobility of Ste20 was not observed in cells lacking CLN1 and CLN2 (Fig. 4B). The absence of an upshift of Ste20 in cln1 cln2 CLN3 cells correlates with the previously shown absence of repression of the mating factor signal transduction in these cells (14).

To test the second prediction that overexpression of CLN2 might strongly affect the mobility of Ste20, we monitored the mobility of Ste20 in cln– cells that were synchronized by conditional expression of either CLN1, CLN2, or CLN3 from the GAL1 promoter. These cells were grown on galactose medium and then shifted to raffinose, which results in a quantitative arrest at START due to G1 cyclin deprivation. Re-addition of galactose to these cultures then started synchronous cell cycle progression. We previously showed that repression of the mating factor pathway is strong in cells with GAL1::CLN2 and is absent in GAL1::CLN3 cells (14). It was found that the upshift in Ste20 mobility as cells enter S-phase was only observed in GAL1::CLN1 and GAL1::CLN2 cells (Fig. 5, A and B) but not in GAL1::CLN3 cells (Fig. 5C). After turning on the GAL1 promoter, the upshift in the GAL1::CLN2 cells was almost quantitative at all cell cycle positions (Fig. 5B). This strong alteration of electrophoretic mobility of Ste20 therefore correlates well with the strong negative effect of this CLN2 construct on the pheromone signaling pathway (14) (Fig. 1). There are various indications that Cln1 and Cln2 have distinct biological activities from Cln3 (e.g. Ref. 43). It has been shown that epitope-tagged versions of CLN3 expressed at high level, from high copy number plasmids or from the GAL1 promoter, have at least equal in vitro H1 kinase activity as CLN2 expressed from its own promoter (43). Also, CLN3 expressed from either its own or from the GAL1 promoter provides sufficient biological activity in vivo to efficiently promote START. The absence of an alteration in Ste20 mobility in the GAL1::CLN3-synchronized cells is therefore unlikely due to a general reduction in START-promoting Cln-Cdc28 kinase activity but is much more likely to be another example where Cln1 and Cln2 differ in their biological activities from Cln3. Treatment of purified fast and slow migrating forms of Ste20 with phosphatase shows that the Cln1/2-Cdc28-mediated changes in electrophoretic mobility are probably mostly due to phosphorylation (data not shown).2

Taken together these data show a strong correlation between retardation of Ste20 mobility in SDS-PAGE gels and repression of the mating factor response pathway as follows: both the upshift and repression (i) occur at about the time of maximal activity of the Cln1/2-Cdc28 kinase, (ii) depend on CLN1/2, and (iii) are constitutive in cells expressing CLN2 from the GAL1 promoter.

One specific model that could explain the tight correlation between upshift and repression is that Cln1/2-Cdc28 inacti-

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2 C. L. Wu, E. Leberer, and M. Whiteway, personal communication.
nized to identify potential factors that are required for the cytokinesis function of Ste20, we tested the synthetic phenotype of CLA4 deletion in combination with deletion of other proteins that are known to interact directly or indirectly with Ste20. In both genetic and biochemical assays, Ste4 has been shown to interact with Ste20 (4, 45). Tetrad analysis revealed that ste::LEU2 cla::TRP1 cells were viable (Table II, entry A), which is consistent with the notion that an intact mating factor signal transduction pathway is not required for the essential morphogenic role of Ste20. Ste4 has been shown to physically interact with Akr1, a protein with a role in mating factor signal transduction and cell morphology (29, 46). Tetrad analysis of spores from a cross of MATa akr::URA3 to MATalpha cla::TRP1 cells revealed that cla::TRP1 akr::URA3 cells were inviable (Table II, entry B). The Akr1 requirement for viability is specific to cla− cells, since ste20− akr1− cells are fully viable (46) (Table II, entry C). This suggests that Akr1 is required for the essential vegetative morphogenic role of Ste20 in the absence of Cla4. Ste20 has also been shown to be part of a complex of proteins that includes actin, Bem1, and Ste5 (47). Since Bem1 is important for proper morphogenesis in response to mating factor (29, 48, 49), we tested whether it could also be important for the vegetative morphogenic role of Ste20. We crossed MATa bemi::LEU2 to MATalpha cla::TRP1 cells and found no viable cla::TRP1 bemi::LEU2 double mutants upon sporulation and tetrad analysis (Table II, entry D). This synthetic lethality of cla4− and bemi1− is consistent with the idea that the interaction between Bem1 and Ste20 is important for the vegetative morphogenic role of Ste20.

**TABLE II**

| Cross Progeny genotype | Number of spores |
|------------------------|------------------|
|                         | Viable | Inviable |
| A. 2219 STE4 CLA4      | 12     | 0       |
| A. 2219 ste4::LEU2 CLA4 | 6      | 0       |
| A. 2219 ste4::LEU2 cla4::TRP1 | 12 | 0 |
| B. 2224 AKR1 CLA4      | 10     | 0       |
| B. 2224 ste::LEU2 AKR1 | 14     | 0       |
| B. 2224 ste::LEU2 cla4::TRP1 | 14 | 0 |
| B. 2224 ste::LEU2 cla4::TRP1 akr1::URA3 | 13 | 1 |
| B. 2224 ste::LEU2 cla4::TRP1 akr1::URA3 | 0 | 10 |
| C. 2232 AKR1 STE20     | 20     | 0       |
| C. 2232 AKR1 ste::LEU2 | 14     | 0       |
| C. 2232 AKR1 ste::LEU2 cla4::TRP1 | 14 | 0 |
| C. 2232 AKR1 ste::LEU2 cla4::TRP1 akr1::URA3 | 20 | 0 |
| C. 2232 AKR1 ste::LEU2 cla4::TRP1 akr1::URA3 | 0 | 10 |
| D. 2226 BEM1 CLA4      | 11     | 0       |
| D. 2226 BEM1 cla4::TRP1 | 13     | 0       |
| D. 2226 BEM1 cla4::TRP1 bemi::LEU2 | 13 | 0 |
| D. 2226 BEM1 cla4::TRP1 bemi::LEU2 | 0 | 11 |

**FIG. 5. Ste20 Mobility in GAL1::CLN Synchronized Cells.** cln− cells, containing plasmid VTU::STE20 (pURA3-ADH-STE20), with CLN1, CLN2, or CLN3 expressed from the GAL1 promoter were grown to early exponential phase in galactose and then arrested at START by shift to raffinose medium for 2.5 h. Synchronous cell cycle progression was initiated by addition of galactose. Samples were taken every 12 min for Western analysis and morphological examination. The percentage of unbudded cells in each sample was determined by scoring 200 cells. Ste20 protein levels were determined in total denaturing extracts upon SDS-PAGE on 6% gels. A, samples taken from synchronized cln−::GAL1::CLN1 cells (strain BOY536). B, samples taken from synchronized cln−::GAL1::CLN2 cells (strain BOY183). C, samples taken from synchronized cln−::GAL1::CLN3 cells (strain BOY747).

vates Ste20 kinase. However, no effect was observed of CLN overexpression or CLN1/2 deletion on overall in vitro Ste20 kinase activity (9). Since there did not seem to be an alteration in the chemical activity of the Ste20 kinase, we considered the possibility that CLN1/2 affects the biological activity of Ste20 and that the fast migrating and slower migrating forms of Ste20 represent species of Ste20 with distinct biological activities.

**Genetic Interactions Suggest Bem1 and Akr1 as Components of a Pathway for Ste20-dependent Regulation of Cytokinesis—** Cells lacking either Ste20 or Cla4 are viable, whereas cells lacking both Ste20 and Cla4 are inviable (8). Based on the morphology of ste20 cla4− cells, it was suggested that these two PAK family members share an essential morphogenic function at cytokinesis (8). Interestingly, cln1 cla2 cla4− cells are also inviable, with an arrest phenotype that resembles that of ste20− cla4− cells (8, 12). This suggests that Cln1/2 and Ste20 function in a similar pathway and that maybe the modification of Ste20 by Cln1/2 is required for its cytokinesis function. The mechanism by which Ste20 and Cla4 promote cytokinesis is unknown, but since ste11 cla4− and ste5 cla4− cells are viable, an intact mating factor or haploid-invasive signal transduction route is not essential (8). In an attempt to identify potential factors that are required for the cytokinesis function of Ste20, we studied the synthetic phenotype of CLA4 deletion in combination with deletion of other proteins that are known to interact directly or indirectly with Ste20. In both genetic and biochemical assays, Ste4 has been shown to interact with Ste20 (4, 45). Tetrad analysis revealed that ste::LEU2 cla::TRP1 cells were viable (Table II, entry A), which is consistent with the notion that an intact mating factor signal transduction pathway is not required for the essential morphogenic role of Ste20. Ste4 has been shown to physically interact with Akr1, a protein with a role in mating factor signal transduction and cell morphology (29, 46). Tetrad analysis of spores from a cross of MATa akr1::URA3 to MATalpha cla4::TRP1 cells revealed that cla4::TRP1 akr1::URA3 cells were inviable (Table II, entry B). The Akr1 requirement for viability is specific to cla4− cells, since ste20− akr1− cells are fully viable (46) (Table II, entry C). This suggests that Akr1 is required for the essential vegetative morphogenic role of Ste20 in the absence of Cla4. Ste20 has also been shown to be part of a complex of proteins that includes actin, Bem1, and Ste5 (47). Since Bem1 is important for proper morphogenesis in response to mating factor (29, 48, 49), we tested whether it could also be important for the vegetative morphogenic role of Ste20. We crossed MATa bemi1::LEU2 to MATalpha cla4::TRP1 cells and found no viable cla4::TRP1 bemi1::LEU2 double mutants upon sporulation and tetrad analysis (Table II, entry D). This synthetic lethality of cla4− and bemi1− is consistent with the idea that the interaction between Bem1 and Ste20 is important for the vegetative morphogenic role of Ste20.

**CLN1/2 Are Required for Pseudohyphal and Haploid Invasive Growth**—Besides roles in mating factor signal transduction and the budding/cytokinesis cycle, Ste20 is essential for agar invasive growth in haploid cells (7) and pseudohyphal growth in diploids (5). Activation of these signal transduction pathways leads, among other phenotypes, to a more polarized cell morphology. Since CLN1/2 has been implicated in polarized growth during the vegetative cell cycle (50), and since the several lines of experimentation shown above suggest a connection between Ste20 and these G1 cyclins, we tested whether pseudohyphal and haploid invasive growth depend on CLN1/2. For these assays we used strains of the Σ1278b background (6)
since strains from our usual laboratory background (BF264-15D) did not display pseudohyphal or haploid invasive growth (data not shown). On plates with low ammonium sulfate concentration, wild type Σ1278b diploid cells display filament formation which is clearly visible at the fringe of colonies (Ref. 5 and Fig. 6A). In contrast, diploid cells without CLN1 and CLN2 were strongly defective in the formation of these filaments (Fig. 6A). As a result of inversion of the agar in rich medium, haploid Σ1278b wild type cells (7) and cells deleted for either CLN1 or CLN2 remain attached to the agar plates upon rinsing with water (Fig. 6B). However, isogenic cells deleted for both CLN1 and CLN2 were strongly defective in haploid invasive growth (Fig. 6B). These data show that CLN1 and CLN2 are required for both pseudohyphal and haploid invasive growth.

**DISCUSSION**

**Ste20 as the Site of Repression of Mating Factor Signal Transduction by Cln1/2-Cdc28 Kinase**—Based on epistatic analysis by several laboratories, the most likely linear sequence of activation of mating factor pathway components is Ste2 → Gpa1 → Ste4/Ste18 → Ste20 → Ste5 → Ste11 → Ste7 → Fus3/Kss1 → Ste12 (1). It was previously shown that repression of mating factor signal transduction by Cln1/2-Cdc28 kinase takes place at a level in the pathway downstream of Ste2 and Gpa1 (14). The effect of high level CLN2 expression in strains in which the pathway is activated at the level of Ste4, Ste5, Ste20, Ste11, or Ste12 (Fig. 1) is most readily explained by assuming that repression acts at a level or downstream of Ste4 and at or upstream of Ste5, i.e. around the site of action of Ste20. There is some indication, however, that the mating factor signaling pathway may not be a linear pathway, especially in the region around Ste20 (36). This makes interpretation of the epistatic data presented in Fig. 1 somewhat difficult. It is clear that the effect is upstream of the Ste11-Ste7-MAP kinase cassette, based on observations that high level CLN2 expression prevents mating factor-induced hyperphosphorylation of Ste7 and activation of Fus3 (data not shown and Ref. 17), and, most importantly, on the observation that high level CLN2 expression does not reduce SST2 transcription induced by hyperactive alleles of Ste11 (Figs. 1 and 2). On this latter point our experimental results differ from those obtained by Wassmann and Ammerer (17), although the conclusion that repression takes place upstream of Ste11 is not necessarily inconsistent with the data from Wassmann and Ammerer (see Ref. 17). We do not know the reason for the difference in some experimental results, but strain differences are unlikely, since in our hands the same strains used by Wassmann and Ammerer show no signs of signaling repression (data not shown).

If repression acted at the level of Ste11, as has been suggested (17), high level CLN2 expression would be expected to repress any signal resulting from activation of the signal transduction pathway upstream of Ste11. However, even though FUS1 induction by high level expression of an active allele of Ste20 requires Ste11 (confirming that Ste20 acts upstream of Ste11), high level CLN2 expression cannot repress the signal generated at the level of Ste20. This observation suggests that repression of the mating factor signal transduction pathway takes place at a level upstream of Ste11. Most of the epistatic experiments involved high level expression of signaling components in combination with high levels of Cln2. In the interpretation of these experiments, it should be kept in mind that the precise stoichiometry between cyclin-CDK and mating factor signaling component could be an important factor in the transcriptional induction of the mating factor pathway. However, stoichiometric effects are unlikely to affect the overall conclusions from our epistatic analysis, since experiments with high and low level expression of activated alleles of Ste11 gave essentially the same epistatic results. It is therefore reasonable to suppose that the negative effect of Cln1/2-Cdc28 on the mating factor signal transduction pathway takes place upstream of Ste11.

Independent from the epistatic analysis, our study of the mobility of Ste20 on SDS-PAGE gels provides evidence for a connection between Cln1/2-Cdc28 and Ste20. We find that the mobility of Ste20 changes during the cell cycle, most likely as a
Mechanism of Repression—It is interesting to note that even though Ste20 is present at all cell cycle positions, localization of the protein to a specific site is only observed at emergent buds (9, 10), which is by approximation the period when the slower migrating form of Ste20 is observed. Even though CLN1/2 is required for observation of the slow-migrating form of Ste20, there is no indication that Cln1/2-Cdc28 is required for localization of Ste20: GFP-Ste20 is properly localized to buds even in the absence of CLN1 and CLN2. Proper localization of Ste20 has been shown to require interaction with the small G-protein Cdc42 (9, 10). It does not appear that the Ste20-Cdc42 interaction is affected by Cln1/2-Cdc28, first because Ste20 localization is not affected in cln1 cln2 cells, and second because we did not see effects of high level expression of CLN2 on the association between Ste20 and Cdc42 in a two-hybrid analysis (Ref. 8 and data not shown). Also, since Ste20 proteins with defective Cdc42 interaction are proficient in mating factor signal transduction (9, 10), elimination of the interaction between Cdc42 and Ste20 could not by itself explain the signaling defect of cells with high level expression of CLN2.

Both the fast and slow migrating forms of Ste20 are phosphoproteins (Ref. 51 and data not shown). The slower migrating form is most likely phosphorylated on additional residues but does not differ in in vitro kinase activity from the fast migrating form. There are numerous TP and SP residues (potential Cdc28 phosphorylation sites) in Ste20 (23), some of which have been shown to be phosphorylated (51). Alteration of those residues that are specifically phosphorylated as a result of Cln1/2-Cdc28 activity is likely to improve the understanding of the precise role of these modifications in mating factor signaling repression by Cln1/2-Cdc28. Whether Ste20 is a direct in vivo substrate for the Cln1/2-Cdc28 kinase remains to be established. The fact that Ste20 can serve as an in vitro substrate for Cln2-Cdc28 is consistent with that possibility. Our data leave open the possibility that other proteins that act at the level Ste20 in the signal transduction pathway (and that possibly interact with Ste20) are the direct targets for the Cln1/2-Cdc28 kinase. The results from the epistatic experiments with high level expression of Ste5 could indicate the existence of an effector of the Cln1/2-Cdc28 kinase besides Ste20. One candidate, Akr1 (29, 46), does not appear to be involved in repression of mating factor signal transduction, as high level CLN2 expression effectively down-regulates mating factor signal transduction in akr1− cells.

Can Cln1 and Cln2 Alter Ste20 Function?—In addition to a role in mating factor signal transduction, Ste20 can function in pseudohyphal growth (5), agar invasive growth (7), and in the budding/cytokinesis cycle (8). It was shown previously that cln1 cln2 cla4 cells are inviable, with a phenotype similar to that ste20 cla4 cells (8). This suggests that Cln1/2-Cdc28 and Ste20 function in a similar pathway to rescue the inviability of cla4 cells. Based on the lethal phenotype of akr1 cla4 and bem1 cla4 cells, this pathway may also include Akr1 and Bem1. Here we show that the two other pathways that are known to require Ste20 also require Cln1 and Cln2 (Fig. 6). Therefore, Cln1 and Cln2 appear to be required for all the vegetative morphogenic functions in which Ste20 has been implicated. In contrast, Cln1 and Cln2 are not required for morphogenesis or the signal transduction functions of Ste20 in response to mating factor. In fact, those sexual functions are inhibited by Cln1/2-Cdc28 (14). Therefore, the effect of Cln1/2-Cdc28 on Ste20 may be to promote the vegetative morphogenic functions, while inhibiting the sexual functions (Figs. 7, A and B). Thus Cln1/2-Cdc28 may contribute to make a switch at Start from a mating differentiation-competent state in G1−phase to a mating differentiation-incompetent and budding and morphogenesis-competent state in post-Start cells. In haploid cells, various components like Ste20, Ste11, Ste7, and Ste12 can be used for both mating factor signal transduction and haploid invasive growth (7). This has raised the issue of signaling specificity. How does the signal from different environmental triggers travel through the same pathway to produce different biological outputs? It has been proposed that part of the mechanism for achieving signal transduction specificity may be to use different MAP kinases (Fus3 for mating and Kss1 for haploid invasive growth) for different outputs (44, 52). The regulation of Ste20 function by Cln1/2-Cdc28 might be another way to achieve signaling specificity by precluding the simultaneous activation of different morphogenic programs which require similar components for signal transduction. In this view, cells would be responsive to mating factor signals in G1−phase, and once Ste20 is modified by Cln1/2-Cdc28 at Start, cells will be unresponsive to mating factor and able to respond to other environmental signals. This mechanism of ensuring signaling specificity at a level upstream of the MAP kinase cassette would rely on evolutionary conserved classes of proteins like p21-activated-kinases (PAKs) and cyclin-dependent kinases (CDKs). The degree of conservation is quite good and extends beyond simple sequence similarities, as various PAKs can complement functions of Ste20 (2) and some CDKs can complement the yeast counterpart. It is possible that regulation of PAKs by CDKs is conserved in evolution and provides a general mechanism for achieving signaling specificity.

3 K. Huang et al., unpublished observations.
4 L. Oehlen, unpublished observations.
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