Forkhead Genes in Transcriptional Silencing, Cell Morphology and the Cell Cycle: Overlapping and Distinct Functions for *FKH1* and *FKH2* in *Saccharomyces cerevisiae*

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ABSTRACT

The *SIR1* gene is one of four specialized genes in *Saccharomyces cerevisiae* required for repressing transcription at the silent mating-type cassettes, *HML*α and *HMR***a**, by a mechanism known as silencing. Silencing requires the assembly of a specialized chromatin structure analogous to heterochromatin. *FKH1* was isolated as a gene that, when expressed in multiple copies, could substitute for the function of *SIR1* in silencing *HMR***a**. *FKH1* (*F*ork*h*ead *H*omologue *One*) was named for its homology to the forkhead family of eukaryotic transcription factors classified on the basis of a conserved DNA binding domain. Deletion of *FKH1* caused a defect in silencing *HMR***a**, indicating that *FKH1* has a positive role in silencing. Significantly, deletion of both *FKH1* and its closest homologue in yeast, *FKH2*, caused a form of yeast pseudohyphal growth, indicating that the two genes have redundant functions in controlling yeast cell morphology. By several criteria, *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth was distinct from the nutritionally induced form of pseudohyphal growth observed in some strains of *S. cerevisiae*. Although *FKH2* is redundant with *FKH1* in controlling pseudohyphal growth, the two genes have different functions in silencing *HMR***a**. High-copy expression of *CLB2*, a G2/M-phase cyclin, prevented *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth and modulated some of the *fkh*Δ-induced silencing phenotypes. Interestingly, deletions in either *FKH1* or *FKH2* alone caused subtle but opposite effects on cell-cycle progression and *CLB2* mRNA expression, consistent with a role for each of these genes in modulating the cell cycle and having opposing effects on silencing. The differences between Fkh1p and Fkh2p *in vivo* were not attributable to differences in their DNA binding domains.

DIFFERENTIATION of eukaryotic cells into distinct cell types requires changes in both cellular transcription and cell-cycle progression. The single-celled *Saccharomyces cerevisiae* has served as a model organism for elucidating many of the fundamental transcription and cell-cycle mechanisms common to all eukaryotes (Murray and Hunt 1993; Carlson 1997) and has also provided insights into how the two processes may control cell differentiation. In *S. cerevisiae*, for example, cell shape, a significant component of cell differentiation, is in part dependent upon the relative lengths of different phases of the cell cycle (Lew and Reed 1993). Increased time in the G1/S phase of the cell cycle is associated with growth that promotes the spherical form of this yeast, whereas increased time in the G2/M phase is associated with growth that promotes an elongated form of this yeast. Pseudohyphal growth, a differentiated state characterized by elongated cells that remain attached to one another to form chains, or pseudohyphae, is associated with both an elongated G2/M phase and a number of changes in the yeast transcriptional program (Gimeno *et al.* 1992; Kron *et al.* 1994; Liu *et al.* 1996; Lo and Dranginis 1998; Rupp *et al.* 1999). Furthermore, perturbations in the cell cycle itself can cause significant changes in the transcription regulation of certain chromosomal regions. For example, transcriptional repression of the yeast silent mating-type cassette, *HMR***a**, which is required for the differentiation of a haploid yeast cell into a distinct mating type (Herskowitz *et al.* 1992), can be altered by perturbations in cell-cycle progression (Laman *et al.* 1995). The identification and characterization of genes in yeast required for both transcription and cell-cycle regulation should provide a foundation for elucidating the mechanisms that coordinate these two processes during eukaryotic cell differentiation.

Studies of the mechanisms that repress transcription of the silent mating-type cassettes, *HML* and *HMR*, have revealed several intriguing connections between this form of transcriptional regulation and cell-cycle progression (Loo and Rine 1995; Fox and Rine 1996). The silent mating-type cassettes are transcriptionally re-

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pressed by a mechanism known as silencing, which requires the assembly of a large domain of repressive chromatin that is analogous to heterochromatin in multicellular eukaryotes (Loo and Rine 1995). Efficient silencing of *HML* and *HMR* is required for the proper differentiation of haploid yeast cells into distinct mating types (Herskowitz *et al.* 1992). Mating type is regulated by the alleles present at a locus called *MAT*: the *MAT***a** allele confers the **a**-mating phenotype whereas the *MAT*α allele confers the α-mating phenotype. In normal yeast strains, a silenced copy of the *MAT***a** allele resides at *HMR* and a silenced copy of the *MAT*α allele resides at *HML*. Mutations that cause defects in silencing lead to the simultaneous expression of both **a**-mating-type and α-mating-type genes, which in turn causes a haploid cell to take on characteristics distinct to the diploid cell type, including the inability to mate. Silencing of *HMR* and *HML* requires the combined action of small DNA elements called silencers that flank these loci and several DNA binding proteins that bind to silencers directly (silencer-binding proteins; Shore 1994; Loo and Rine 1995). In addition, the four Sir (*S*ilent *I*nformation *R*egulator) proteins, silencing-specific proteins proposed to interact with the silencer-binding proteins and nucleate the assembly of silent chromatin, are essential for silencing (Shore 1994; Loo and Rine 1995; Grunstein 1997; Stone and Pillus 1998). The *de novo* assembly of silent chromatin requires passage through the S phase of the cell cycle (Miller and Nasmyth 1984; Fox *et al.* 1997). In addition, the two silencers that regulate silencing at *HMR***a**, *HMR*-E, and *HMR*-I function as chromosomal replication origins, providing another connection between an S-phase event, replication initiation, and silencing (Rivier and Rine 1992; Rivier *et al.* 1999). Significantly, the connection between cell-cycle progression and silencing extends beyond S phase; mutations in genes that perturb progression through the S, G2/M or G1/S phases of the cell cycle can also modulate silencing at *HMR***a** (Laman *et al.* 1995).

The effect of cell-cycle perturbations on the efficiency of transcriptional silencing at *HMR***a** can be observed in strains containing mutations in *SIR1* but not in strains containing mutations in any of the other three *SIR* genes (Laman *et al.* 1995), providing evidence that the role of *SIR1* in silencing is distinct from the roles of *SIR2*, *SIR3*, and *SIR4*. In addition, a classic genetic study indicates that *SIR1* is required for the establishment of silencing but not its maintenance (Pillus and Rine 1989). In contrast, the other three *SIR* genes encode proteins required for the maintenance of the silent state and have since been shown to encode structural components of silent chromatin (Hecht *et al.* 1995, 1996; Strahl-Bolsinger *et al.* 1997). In general, slowing progress through specific phases of the cell cycle, either by mutation or chemical interference, can partially bypass the requirement for *SIR1* in silencing (Laman *et al.* 1995). The mechanisms by which these cell-cycle perturbations substitute for *SIR1* function in silencing are unknown, but it is clear that simply slowing growth rate is not sufficient to enhance silencing (Laman *et al.* 1995). Regardless, the relationship between *SIR1* function and the cell cycle presents an opportunity to identify new genes that modulate both progress through the cell cycle and transcriptional silencing.

We identified *FKH1* (*F*or*k*head *H*omologue *One*) as a gene that could substitute for the function of *SIR1* in silencing when expressed from a high-copy plasmid. *FKH1* and its closest homologue in yeast, *FKH2*, are named for their similarity to an evolutionarily conserved family of transcription factors classified on the basis of their forkhead (winged-helix) DNA binding domains (Clark *et al.* 1993; Lai *et al.* 1993; Kaufmann and Knochel 1996). The name forkhead comes from the founding member of this family, a gene that, when mutated, causes patterning defects in the Drosophila embryo (Weigel *et al.* 1989). Transcription factors in the forkhead family have roles in early development, cell differentiation, and cell-cycle progression in a wide variety of multicellular eukaryotes and, significantly, represent a rare example of tissue-specific transcription factors with clear homologues in yeast (Kaufmann and Knochel 1996; Yang *et al.* 1997).

The data presented in this article provide evidence for roles for *FKH1* and *FKH2* in transcriptional silencing and pseudohyphal growth in yeast. Interestingly, although the two genes share a redundant function in preventing pseudohyphal growth, they exhibit different functions in silencing. The roles of *FKH1* and *FKH2* in pseudohyphal growth and silencing are related to their roles in cell-cycle progression, since both the silencing and pseudohyphal phenotypes caused by loss of *FKH* function could be modulated by high-copy expression of the G2/M-phase cyclin, *CLB2*. In addition, mutations in the *FKH* genes cause measurable changes in cell-cycle progression and levels of *CLB2* mRNA consistent with their opposing roles in silencing. The differences between Fkh1p and Fkh2p were not attributable to differences in their DNA binding domains.

MATERIALS AND METHODS

The genotypes of the yeast strains and the plasmids used in this study are listed in Tables 1 and 2. Yeast rich medium (YPD), minimal medium (YM), amino acid and base supplements, and standard yeast genetic methods were as described (Guthrie and Fink 1991). Recombinant DNA methods were as described (Sambrook *et al.* 1989).

Strain constructions: All strains were isogenic to W303-1A except as noted. All gene deletions described in this article were constructed as precise substitutions of the relevant gene's entire coding region with the indicated marker gene. DNA fragments for constructing gene deletions were prepared using the fusion polymerase chain reaction (PCR) method (Amberg *et al.* 1995). The amplified fragment was introduced into a diploid strain by one-step gene replacement, and haploid segregants containing the deletion of interest were ob-

TABLE 1

Strains used in this study

Strain[a]	Genotype[b]	Reference[c]
JRY19	*MAT***a** *his4 leu2 trp1 ura3*	
CFY617	JRY19 *MAT*α	Gardner *et al.* (1999)
JRY2334	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (W303-1A)	Thomas and Rothstein (1989)
JRY3009	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (W303-1B)	
CFY35	JRY3009 *HMR-SS*Δ*I***a**	DeBeer and Fox (1999)
CFY145	JRY2334 *ADE2 lys2*Δ	Herman and Rine (1997)
CFY762	JRY3009 *HMR-SS***a** *sir1*Δ*::LEU2*[d]	Gardner *et al.* (1999)
CFY744	JRY3009 *HMR-SS***a** *sir1-101*	Gardner *et al.* (1999)
CFY720	JRY3009 *HMR-SS***a** *sir1-102*	Gardner *et al.* (1999)
CFY737	JRY3009 *hml***a**Δp *mat***a**Δp *HMR-SS*α *sir1-106*	Gardner *et al.* (1999)
CFY37	JRY3009 *HMR*Δ*I***a**	Fox *et al.* (1995)
CFY393	JRY3009 *HMR*Δ*I***a** *sir2*Δ*::LEU2*	DeBeer and Fox (1999)
CFY55	JRY2334 *HMR-SS*Δ*I***a** *fkh1*Δ*::HIS3*	
CFY62	JRY2334 *HMR-SS*Δ*I***a** *lys2*Δ *fkh1*Δ*::HIS3*	
CFY65	JRY2334 *HMR-SS*Δ*I***a** *fkh1*Δ*::TRP1 lys2*Δ	
CFY75	JRY2334 *HMR-SS*Δ*I***a** *fkh1*Δ*::TRP1*	
CFY94	JRY3009 *HMR-SS*Δ*I***a** *fkh2*Δ*::HIS3 lys2*Δ	
CFY95	JRY3009 *fkh2*Δ*::HIS3*	
CFY99	JRY2334 *fkh2*Δ*::HIS3*[d]	
CFY100	JRY2334 *HMR-SS*Δ*I***a** *lys2*Δ *fkh2*Δ*::HIS3*	
CFY103	JRY3009 *HMR-SS*Δ*I***a** *fkh2*Δ*::HIS3*	
CFY147	JRY3009 *HMR-SS*Δ*I***a** *lys2*Δ *fkh1*Δ*::TRP1 fkh2*Δ*::HIS3*	
CFY148	JRY3009 *HMR-SS*Δ*I***a** *lys2*Δ	
CFY149	JRY3009 *HMR-SS*Δ*I***a** *lys2*Δ *fkh1*Δ*::TRP1*	
CFY150	JRY3009 *HMR-SS*Δ*I***a** *lys2*Δ *fkh2*Δ*::HIS3*	
CFY158	JRY2334 *HMR-SS*Δ*I***a** *lys2*Δ	
CFY166	JRY2334 *HMR-SS*Δ*I***a** *lys2*Δ *fkh1*Δ*::TRP1 fkh2*Δ*::HIS3*	
CFY480	JRY2334 *ADE2 lys2*Δ *FKH1-3xHA*	
CFY854	JRY2334 *ADE2 lys2*Δ *FKH2-3xHA*	
CFY269	JRY2334 *HMR-SS*Δ*I***a**	
CFY270	JRY2334 *HMR-SS*Δ*I***a** *fkh1*Δ*::TRP1 fkh2*Δ*::HIS3*	
CG189	*MAT***a** *trp1 ura3* (Σ1279b)	Gimeno and Fink (1994)
CFY330	JRY2334 *fkh1*Δ*::TRP1 fkh2*Δ*::HIS3 flo11*Δ*::hisG URA3 hisG*	
CFY155	JRY2334 *fkh1*Δ*::TRP1 fkh2*Δ*::HIS3*	
CFY863[e]	JRY2334 *ADE2 lys2*Δ *FKH1/KFH2*$_{DBD}$	
CFY902[e]	JRY2334 *KFH1/FKH2*$_{DBD}$ *fkh2*Δ*::HIS3*	

[a] All strains except JRY19, CFY617, and CG189 are isogenic derivatives of W303.

[b] All gene deletions described in this article are substitutions of the entire open reading frame for the relevant gene with the marker gene indicated.

[c] Unless noted, strains were from the laboratory collection or constructed during the course of this work.

[d] The *HMR***a** status of this strain is unknown. It is either *HMR-SSI***a** or *HMR***a**. The *HMR* genotype was not relevant to the *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth described in this article.

[e] *FKH1/FKH2*$_{DBD}$ is a hybrid gene in which the coding region for the DNA binding domain of *FKH1* has been precisely replaced with the coding region for the DNA binding domain of *FKH2*. This hybrid gene replaces *FKH1* at its normal chromosomal position in this strain.

tained by sporulation and dissection of the diploid. To construct isogenic strains carrying gene deletions in combination with the desired *HMR* and *MAT* genotypes, standard genetic crosses were performed and the *HMR* and *MAT* genotypes were determined by mating assays and/or analysis of genomic DNA using PCR or DNA blot hybridization.

To construct an isogenic set of *MAT*α *HMR-SS*Δ*I***a** strains that differed only in their *FKH* genotype [CFY147 (*fkh1*Δ*::TRP1 fkh2*Δ*::HIS3*), CFY148 (*FKH1 FKH2*), CFY149 (*fkh1*Δ*::TRP1 FKH2*), and CFY150 (*FKH1 fkh2*Δ*::HIS3*)], a *MAT***a** *HMR-SS*Δ*I***a** *fkh1*Δ*::TRP1* strain (CFY65) was crossed to a *MAT*α *HMR-SS*Δ*I***a** *fkh2*Δ*::HIS3* strain (CFY94). The results from this cross were typical of the results from similar crosses in terms of spore viability and the appearance of segregants with a ruffled colony morphology. Specifically, out of 19 tetrads analyzed from this cross, only 8 contained 4 viable spores, indicating a relatively low spore viability for this strain background (W303-1A). The *FKH* genotype for only 6 out of the 13 dead spores could be accurately deduced from analysis of the remaining live segregants from the tetrad: 2 were *fkh1*Δ*::TRP1*, 2 were *fkh1*Δ*::TRP1 fkh2*Δ*::HIS3*, and 2 were wild type. Thus a clear correlation between individual spore viability and *FKH* genotype was not evident. However, the remaining 62 viable spores indicated an association between the *FKH* genotype and the ruffled colony morphology. Specifically, 13 of the viable 62 segregants were Trp^+ His^+ prototrophs and each of

TABLE 2

Plasmids used in this study

Plasmid	Description	Reference[a]
Yep24	2-μm plasmid	Carlson and Botstein (1982)
pCF345[b]	*SIR1* genomic clone in Yep24	
pCF337	*FKH1* genomic clone in Yep24	
pCF341	*FKH1* genomic clone in Yep24	
pSEY8	2-μm plasmid	Emr *et al.* (1986)
pCF351	*SIR4* genomic clone in Yep24	
pCF290[b]	*SIR4* in pRS416	
pCF293[b]	*SIR3* genomic clone in Yep24	
pCF462[b]	*SIR2* genomic clone in pSEY8	
pCF399	*FKH2* in Yep24	
pCF561	*FKH2* genomic clone in Yep24	
pCF547	*FKH1-3xHA* in pRS426	
pCF665	*FKH2-3xHA* in pRS426	
pCF633	*CLB2* in pRS426	
pCF587	*FKH1-GFP* in pRS426	
pCF480	*FKH1* in pRS426	
pCF569	$FKH1_{DBD\Delta}$ in pRS426	
pCF589	$FKH1_{DBD\Delta}$-*3xHA* in pRS426	
pCF574	$FKH1_{FKH2DBD}$ in pRS426	
pCF662	$FKH1_{FKH2DBD}$ in pRS406	
pRS416		Sikorski and Hieter (1989)
pRS426		Sikorski and Hieter (1989)

[a] Unless noted, plasmids were from the laboratory collection or constructed during the course of this work.
[b] These plasmids were gifts from the laboratory of Jasper Rine.

these segregants exhibited the ruffled colony morphology. No other segregants exhibited this morphology. In addition this ruffled morphology could be suppressed by transforming these segregants with a plasmid containing either *FKH1* or *FKH2* (C. A. Fox, unpublished results).

To examine the levels of Fkh1p and Fkh2p expressed from chromosomal copies of *FKH1* and *FKH2*, respectively, three copies of the hemagglutinin epitope (3xHA) were inserted in frame and just upstream of the codon for the C-terminal amino acid for each gene in a *MAT***a** strain (CFY145) using the PCR epitope tagging method for *S. cerevisiae* (Schneider *et al.* 1995). Both *FKH1-3xHA* and *FKH2-3xHA* fusion genes provided wild-type *FKH* function based upon their ability to prevent pseudohyphal growth when supplied as the sole source of *FKH* in yeast (P. C. Hollenhorst, unpublished results).

To test whether the *FKH1* and *FKH2* DNA binding domains were equivalent *in vivo*, a *FKH1* hybrid gene in which the *FKH1* DNA binding domain was precisely replaced with the *FKH2* DNA binding domain ($FKH1_{FKH2DBD}$) was introduced at the *FKH1* locus in a *MAT***a** strain (CFY863). Specifically, an integrating plasmid containing the $FKH1_{FKH2DBD}$ hybrid gene (pCF662) was cleaved at the unique *Msc*I site within the *FKH1* gene and the hybrid gene was introduced into a *MAT***a** strain (CFY145) by two-step gene replacement. Integrants containing $FKH1_{FKH2DBD}$ were determined by analytical PCR and diagnostic restriction enzyme digests. To construct a strain in which the $FKH1_{FKH2DBD}$ was the only form of *FKH*, the *MAT***a** $FKH1_{FKH2DBD}$ *FKH2* strain (CFY863) was crossed to a *MAT*α *fkh2*Δ*::HIS3* strain (CFY95) and the $FKH1_{FKH2DBD}$ genotype of several His^+ segregants was determined.

Identification of *FKH1* as a high-copy suppressor of a *SIR1* defect: Two identical plasmids that contained *FKH1* were identified in the screen discussed in this article. One isolate (pCF337) from the Yep24 library (Carlson and Botstein 1982) was characterized further and contained a 7-kb *Sau*3AI genomic fragment that included the 5′ portion of the YIL130W gene, the entire YIL131C (*FKH1*), YIL132C, YIL133C, and YIL134W genes, and the 5′ portion of the YIL135C gene. To determine which of the several genes present on this plasmid (pCF337) was responsible for the silencing phenotype, two subclones were constructed. (1) A 2-kb *Sph*I fragment containing the YIL130W gene and the 5′ half of *FKH1* was released from the original plasmid isolate (pCF337), creating a new plasmid (pCF343) that contained the YIL132C, YIL133C, and YIL134W genes and the 5′ portion of YIL135C. This plasmid failed to enhance silencing in a *sir1* mutant strain, indicating that these genes did not contribute to silencing (C. A. Fox, unpublished results). (2) A 5-kb *Nhe*I fragment was released from the original plasmid isolate (pCF337), creating a new plasmid (pCF341) that contained only the 5′ portion of YIL130C and the entire *FKH1* gene. This plasmid enhanced silencing in a *sir1* mutant strain as efficiently as the original isolate. As an additional test of whether *FKH1* alone was responsible for the enhanced silencing phenotype, a plasmid containing only *FKH1* was constructed (pCF480) by high-fidelity PCR amplification of *FKH1*. This plasmid (pCF480) enhanced silencing as effectively as the original isolate (pCF337).

Isolation of *SIR1*, *SIR4*, and *FKH2* genomic clones from the Yep24 library: In the course of the experiments described in this article, Yep24 genomic clones containing *SIR4* (pCF351) and *SIR1* were isolated. The *SIR4* plasmid was used for experiments described in Figure 2 and behaved identically to previously characterized *SIR4* plasmids.

In the course of investigating *fkh1*Δ *fkh2*Δ-induced yeast pseudohyphal growth, a genomic clone containing *FKH2* (pCF561) was isolated from the same Yep24 library used for the silencing screen. This clone behaved identically to a PCR-amplified clone that contained only the *FKH2* gene (pCF399), indicating that the *FKH2* phenotypes associated with our engi-

neered *FKH2* clones were accurate representations of *FKH2* function (P. C. Hollenhorst, unpublished results).

Plasmid constructions: To measure the level of expression of Fkh1 and Fkh2 proteins, two clones were constructed that contained *FKH1-3xHA* (pCF547) and *FKH2-3xHA* (pCF665), respectively, in pRS426. To construct the high-copy plasmid containing *FKH1-3xHA* (pCF547), a fragment containing the 3′ region of *FKH1-3xHA* was amplified by high-fidelity PCR from total genomic DNA prepared from a yeast strain harboring a chromosomal copy of *FKH1-3xHA* (CFY480) and cloned into the *Bcl*I/*Nhe*I sites of the *FKH1* plasmid (pCF480), creating *FKH1-3xHA* in pRS426 (pCF547). To construct the high-copy plasmid containing *FKH2-3xHA* (pCF665), a fragment containing the entire *FKH2-3xHA* locus was amplified by high-fidelity PCR from total genomic DNA prepared from a yeast strain harboring a chromosomal copy of *FKH2-3xHA* (CFY-854). The amplified fragment was cloned into the *Sma*I site of pRS426 to create *FKH2-3xHA* in pRS426 (pCF665). The *FKH1-3xHA* and *FKH2-3xHA* plasmids each provided wild-type *FKH* function (P. C. Hollenhorst, unpublished results).

A high-copy plasmid containing *CLB2* was constructed by synthesizing the *CLB2* gene by high-fidelity PCR amplification of total yeast genomic DNA prepared from W303-1A and cloning it into pRS426 (pCF633).

To construct the plasmids used to examine the cellular localization of Fkh1p (pCF587) and the role of the Fkh1p DNA binding domain in *FKH1* function (pCF569, pCF574, pCF589, and pCF662), three parent plasmids were constructed (pCF543, pCF555, and pCF557). High-fidelity PCR was used to generate *FKH1* fragments that were combined using standard recombinant techniques to generate the following two parent *FKH1* clones: (1) An *FKH1* clone in pRS426 identical to pCF480 except for a *Sma*I site engineered at the 5′ end of the *FKH1* DNA binding domain (pCF543) and (2) an *FKH1* clone in pRS426 identical to pCF480 except for a *Sma*I site engineered at the 3′ end of the *FKH1* DNA binding domain (pCF555). Fragments from pCF543 and pCF555 were combined to generate a third parent *FKH1* clone in pRS426 (pCF557) identical to pCF480 except that it contained two *Sma*I sites flanking the coding region for the *FKH1* DNA binding domain. Each *Sma*I site introduced a codon for a single glycine residue into the recombinant *FKH1* such that this engineered Fkh1p contained one glycine inserted after the proline at position 291 and one after the proline at position 420. This engineered *FKH1* functioned identically to wild-type *FKH1* (M. Mielke, unpublished results). To construct the *FKH1-Green Fluorescent Protein* (*FKH1-GFP*) fusion gene, the entire coding region for *GFP* was amplified by high-fidelity PCR from pSP65T (Hampton *et al.* 1996). The amplified product was cleaved with *Sma*I and cloned into the *Sma*I site of pCF555 to generate *FKH1-GFP*, which provided wild-type *FKH1* function (M. Mielke, unpublished results). To determine whether the DNA binding domain was required for Fkh1p function, a *FKH1* clone was generated that was identical to *FKH1* in pRS426 (pCF480) except that it contained a single in-frame *Sma*I site in place of the coding region for the *FKH1* DNA binding domain ($fkh1_{DBD\Delta}$; pCF569). To determine whether the $fkh1_{DBD\Delta}$ encoded a stable mutant protein, the coding region for the *3xHA* C-terminal epitope was introduced into the $FKH1_{DBD\Delta}$ clone (pCF569) to generate $FKH1_{DBD\Delta}$-*3xHA* in pRS426 (pCF589). To determine whether a Fkh1p containing the Fkh2p DNA binding domain in place of its own possessed *FKH1* function, a $FKH1_{FKH2DBD}$ hybrid gene in pRS426 was generated (pCF574). Specifically, the coding region for the *FKH2* DNA binding domain was amplified by high-fidelity PCR and cloned into the *Sma*I site of $FKH1_{DBD\Delta}$ (pCF569) to create a $FKH1_{FKH2DBD}$ hybrid gene in pRS426 (pCF574). To construct an integrating version of this hybrid gene, a fragment containing the $FKH1_{FKH2DBD}$ hybrid gene from pCF574 was cloned into pRS406 (pCF662).

Immunoblot analysis of chromosomal and overexpressed versions of Fkh1p-3xHA and Fkh2p-3xHA: The level of Fkh1p-3xHA or Fkh2p-3xHA in crude yeast extracts was determined as described previously (Gardner *et al.* 1999) except that 0.15 OD cell equivalents of crude yeast extracts were examined for the appropriate fusion protein and the primary antibody in immunoblot analysis was a mouse monoclonal antibody raised against the hemagglutinin epitope (Berkeley Antibody Company).

RNA blot analysis: Total yeast RNA was prepared and RNA blot hybridization was performed with probes for **a1**, *SIR4*, *CLB2*, or *SCR1*, as indicated and as described previously (Fox *et al.* 1995, 1997).

RESULTS

To identify new genes that could provide insights into *SIR1* function and the relationship between silencing and cell-cycle progression, we performed a genetic screen to isolate genes that, when expressed at a high copy number, enhanced silencing in a *sir1-101* strain. We exploited this recessive hypomorphic allele of *SIR1* (Gardner *et al.* 1999) together with an *HMR***a** locus under the control of the synthetic *HMR*-E silencer (*HMR-SS***a**; McNally and Rine 1991). The *sir1-101* allele is defective for silencing, but is not as defective as a *sir1*Δ allele, and thus contributed to a sensitized genetic background. The synthetic *HMR*-E silencer (*HMR-SS***a**), a simplified version of the *HMR*-E silencer that provides full silencing to *HMR***a** in combination with the *HMR*-I silencer, requires *SIR1* for efficient silencing. Thus a *MAT*α *HMR-SS***a** *sir1-101* strain is unable to mate because the simultaneous expression of both **a** and α genes causes the nonmating phenotype of a diploid (Herskowitz *et al.* 1992). This strain was transformed with a high-copy-number yeast genomic library (Carlson and Botstein 1982). If a transformant expressed a gene that could restore silencing to *HMR-SS***a**, then it would mate efficiently with an **a**-mating-type strain. From ~10,000 transformants, we identified 6 transformants that mated efficiently in a plasmid-dependent manner. Recovery and sequencing of the relevant plasmids revealed that two of the plasmids were identical and contained *SIR1*, one contained *SIR4*, one contained an intact uncharacterized yeast open reading frame, and two contained identical plasmids that contained the *FKH1* gene. In this article, we present characterization of *FKH1* and its yeast homologue, *FKH2*, in silencing and yeast biology.

Multicopy expression of *FKH1* enhanced silencing in strains containing defects in *SIR1*: To test whether multicopy expression of *FKH1* enhanced silencing in a strain containing a complete deletion of *SIR1*, a *MAT*α *HMR-SS***a** *sir1*Δ*::LEU2* strain was transformed with a high-copy plasmid containing *FKH1*. Multiple copies of either *SIR1* or *FKH1* conferred the α-mating phenotype to this strain, consistent with restored silencing at *HMR***a**

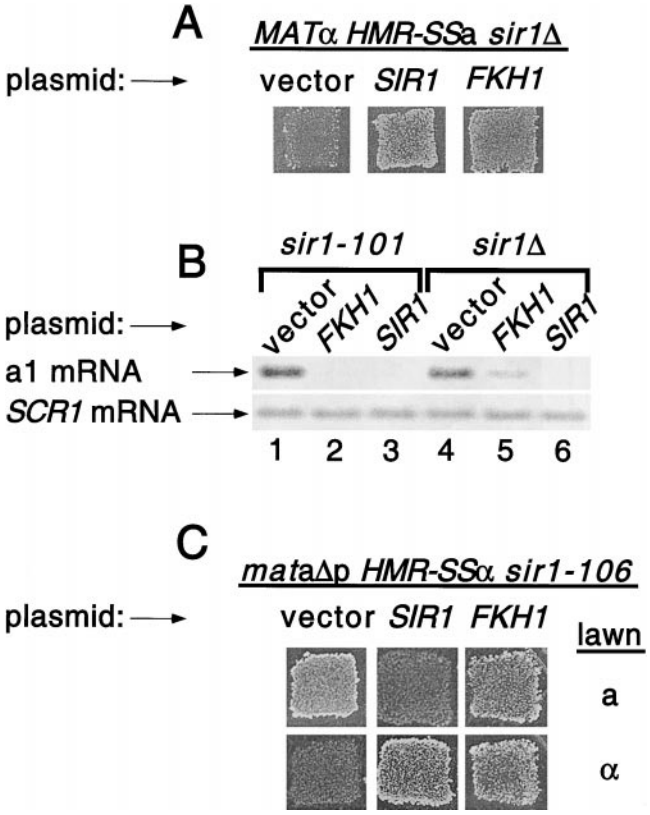

Figure 1.—Multicopy expression of *FKH1* substituted for the function of *SIR1* in silencing *HMR***a**. (A) Mating phenotypes observed in a *MAT*α *HMR-SS***a** *sir1*Δ*::LEU2* strain (CFY762) harboring either a 2-μm plasmid (vector; Yep24), or 2-μm *SIR1* (pCF345) or *FKH1* (pCF341). The transformants were grown as patches for 18 hr at 30° on medium lacking uracil, replica-plated to a *MAT***a** lawn (JRY19) on selective medium, and incubated at 30° for 2 days to select for the formation of diploids. (B) The steady-state levels of **a1** mRNA and *SCR1* mRNA were measured by RNA blot hybridization of 25 μg of total RNA from isogenic *MAT*α *HMR-SS***a** strains that were *sir1-101* (*sir1-101*; CFY744) or *sir1*Δ*::LEU2* (*sir1*Δ; CFY762), each transformed with a 2-μm plasmid (lanes 1 and 4, vector; Yep24), or 2-μm *FKH1* (lanes 2 and 5; pCF341) or *SIR1* (lanes 3 and 6; pCF345). (C) Mating phenotypes observed in a *mat***a**Δ**p** *HMR-SS*α *sir1-106* strain (CFY737) transformed with either a 2-μm plasmid (vector; Yep24), or 2-μm *SIR1* (pCF345) or *FKH1* (pCF341). Mating assays were performed as described in Figure 1A.

(Figure 1A, compare *SIR1* and *FKH1* to vector). Further analysis indicated that *FKH1* was responsible for the enhanced mating efficiency in this mutant yeast strain; a plasmid containing only *FKH1* enhanced the mating efficiency of this mutant strain to the same degree as the plasmid isolated from the genomic library. Thus *FKH1* restored the ability to mate to this *sir1*Δ mutant strain.

As a second measure of the ability of *FKH1* to restore silencing to *HMR***a**, the levels of **a1** mRNA were analyzed directly by RNA blot hybridization in a *MAT*α *HMR-SS***a** strain harboring either a *sir1-101* or a *sir1*Δ allele (Figure 1B). In the absence of silencing at *HMR***a**, **a1** mRNA is expressed (Herskowitz *et al.* 1992). The levels of **a1** mRNA were similar in the *MAT*α strains containing either *sir1-101* or *sir1*Δ, indicating that by this criterion *sir1-101* behaved similarly to a *sir1*Δ allele (Figure 1B, lanes 1 and 4). Multicopy expression of wild-type *SIR1* in either *sir1* mutant strain restored full silencing to *HMR***a** as indicated by the disappearance of **a1** mRNA (Figure 1B, lanes 3 and 6). Multicopy expression of *FKH1* restored some silencing to *HMR***a** in both *sir1* mutant strains as indicated by a reduction in the level of **a1** mRNA (Figure 1B, lanes 2 and 5). However, *FKH1* reduced the levels of **a1** mRNA more efficiently in the strain harboring *sir1-101* than in the strain harboring *sir1*Δ*::LEU2* (Figure 1B, lanes 2 and 5). Thus, multicopy expression of *FKH1* could substitute only partially for *SIR1* function in silencing. These data also provide evidence that the *sir1-101* allele provided some residual *SIR1* function, consistent with the previously published characterization of this allele (Gardner *et al.* 1999).

The data presented above indicate that multicopy expression of *FKH1* reduced the levels of **a1** mRNA expressed from *HMR***a** in strains containing defects in *SIR1*, consistent with a role for *FKH1* in silencing. Two additional experiments provided evidence that *FKH1* was mediating its effects on **a1** mRNA levels through a *bona fide* silencing mechanism. First, multicopy expression of *FKH1* failed to enhance silencing by *HMR-SS***a** in *sir2*Δ, *sir3*Δ, or *sir4*Δ strains (M. Mielke, unpublished results). The *SIR2*, *SIR3*, and *SIR4* genes encode structural components of silenced chromatin and a requirement for these genes is a hallmark of silencing. Second, we determined whether multicopy expression of *FKH1* could silence a gene other than **a1** at *HMR*, since another hallmark of silencing is that it is not gene specific (Loo and Rine 1995). Specifically, we measured silencing in a haploid strain that harbored a deletion for the promoter of the **a** genes at the *MAT* locus, the *sir1-106* allele, and the α-mating-type genes at an *HMR* locus controlled by the synthetic silencer (*mat***a**Δ**p** *HMR*-SSα *sir1-106*; Figure 1C). The α-mating-type genes, controlled by a different promoter than the **a1** genes (Herskowitz *et al.* 1992), provided an independent measure of silencing at *HMR*. *sir1-106*, a weak *SIR1* allele (Gardner *et al.* 1999), provided an additional level of sensitivity required by this experiment. The *mat***a**Δ**p** *HMR*-SSα *sir1-106* strain had an **a**-mating-type and mated with the α-mating-type lawn when it expressed multicopy *SIR1* because *SIR1* silenced *HMR*α, and **a**-mating is the default mating pathway (Herskowitz *et al.* 1992; Figure 1C, *SIR1*). However, in the absence of plasmid-expressed *SIR1*, this strain mated primarily with an α-mating-type because the α genes at *HMR* were not silenced (Figure 1C, vector). In contrast, when this strain contained multicopy *FKH1*, silencing was restored; the strain expressing *FKH1* mated with the α-mating-type lawn, indicating that the α genes present at *HMR* were silenced in a significant fraction of the

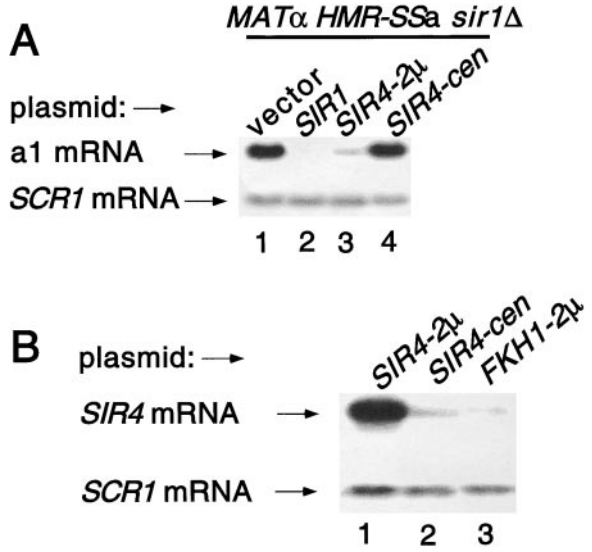

Figure 2.—Multicopy expression of *FKH1* did not increase the steady-state levels of *SIR4* mRNA. (A) Expression of *SIR4* from a 2-μm plasmid substituted for the function of *SIR1* in silencing. The steady-state levels of **a1** mRNA and *SCR1* mRNA were measured as described in Figure 1B from a *MAT*α *HMR-SS***a** *sir1*Δ*::LEU2* strain (CFY762), transformed with a 2-μm plasmid (lane 1, vector; Yep24), 2-μm *SIR1* (lane 2; pCF345), 2-μm *SIR4* (lane 3, *SIR4*-2μ; pCF351), or a low-copy centromere vector containing *SIR4* (lane 4, *SIR4*-cen; pCF290). (B) Multicopy expression of *FKH1* did not increase the level of *SIR4* mRNA sufficiently to enhance silencing. The steady-state levels of *SIR4* mRNA and *SCR1* mRNA were measured from a *MAT*α *HMR-SS***a** *sir1*Δ*::LEU2* strain (CFY762) transformed with 2-μm *SIR4* (lane 1, *SIR4*-2μ; pCF351), a centromere vector containing *SIR4* (lane 2, *SIR4*-cen; pCF290), or 2-μm *FKH1* (lane 3, *FKH1*-2μ; pCF341).

cell population (Figure 1C, *FKH1*). The bimating phenotype indicated that the plasmid containing *FKH1* did not silence the α genes at *HMR* as efficiently as the plasmid containing *SIR1*, consistent with the data obtained from RNA blot hybridization of *HMR***a** (Figure 1B). Taken together, these data indicate that multicopy expression of the *FKH1* partially substituted for the function of *SIR1* in silencing *HMR*.

Multicopy expression of *FKH1* did not increase levels of *SIR4* mRNA: Previous studies indicate that increasing the dosage of *SIR4* enhances silencing at *HMR* in a strain that lacks *SIR1* (Laman *et al.* 1995). Consistent with this observation, we isolated a plasmid containing *SIR4* that enhanced mating in the *MAT*α *HMR*-SS**a** *sir1-101* strain. RNA blot hybridization indicated that multicopy *SIR4* expression silenced *HMR***a** in a *MAT*α *HMR-SS***a** *sir1*Δ strain (Figure 2A, compare lanes 1–3). However, low-copy expression of *SIR4* failed to enhance silencing in this strain (Figure 2A, compare lanes 1 and 4). In contrast to *SIR4*, multicopy expression of *SIR2* or *SIR3* failed to silence *HMR-SS***a** in this strain (M. Mielke, unpublished results).

One possible role for *FKH1* in silencing was that it functioned in transcription of *SIR4*, consistent with the proposed role of the Fkh1p as a transcription factor. Therefore we measured *SIR4* mRNA levels in a strain transformed with either a multicopy plasmid encoding *SIR4* or *FKH1* or a low-copy plasmid encoding *SIR4*. The level of *SIR4* mRNA in the strain expressing high-copy *FKH1* was below the level of *SIR4* mRNA required for silencing *HMR***a** in this *sir1*Δ mutant strain (Figure 2B), indicating that multicopy expression of *FKH1* did not enhance silencing by increasing the level of *SIR4* mRNA.

FKH1* and *FKH2* have different functions in silencing:** The data presented above indicate that multicopy expression of *FKH1* could enhance silencing. If these data reflect a natural role for *FKH1* in silencing, then one prediction is that a deletion of *FKH1* would cause a defect in silencing. Therefore one copy of *FKH1* was deleted from a diploid strain in which one *HMRa** locus was controlled by the synthetic version of the *HMR*-E silencer and lacked the *HMR*-I element (*MAT***a**/*MAT*α *fkh1*Δ*::HIS3*/*FKH1 HMR-SS*Δ*I***a**/*HMR***a**), and the segregants that resulted from sporulation and dissection of this strain were analyzed. From over 20 tetrads analyzed, every spore was viable, and the growth and morphology of individual segregants were indistinguishable, indicating that the *FKH1* gene was not essential. Qualitative analysis of the mating properties of *MAT*α *HMR-SS*Δ*I***a** *fkh1*Δ*::HIS3* segregants indicated that silencing was not affected dramatically (C. A. Fox, unpublished results). However, the sensitivity of *HMR-SS*Δ*I***a** permits the detection of small changes in silencing at the level of **a1** mRNA expression (Fox *et al.* 1995). Importantly, deletion of *FKH1* caused a reproducible defect in silencing at the sensitized *HMR-SS*Δ*I***a** locus as demonstrated by the small increase in levels of **a1** mRNA compared to an isogenic wild-type strain (Figure 3A, compare lanes 5 and 6). These data are consistent with a positive role for *FKH1* in silencing.

One explanation for the small role of *FKH1* in silencing at *HMR***a** and its nonessential role in haploid yeast growth was that *FKH1* has overlapping functions with another gene(s). In fact, a query of the yeast genome database revealed a second gene, *FKH2*, with a high degree of similarity to *FKH1*. The two genes are 44% identical over the length of *FKH1* and 75% identical within their conserved DNA binding domains. This sequence similarity raised the possibility that the two genes might share overlapping functions that could complicate analysis of the role of *FKH1*. Therefore, to analyze *FKH2* and its possible overlapping function with *FKH1*, one copy of *FKH2* was deleted from the diploid strain described above. Analysis of the segregants from over 20 tetrads obtained from sporulation and dissection of this strain (*MAT***a**/*MAT*α *fkh2*Δ*::HIS3*/*FKH2 HMR-SS*Δ*I***a**/*HMR***a**) indicated that *FKH2* was not required for haploid yeast growth. Unexpectedly, based on the strong sequence similarity between *FKH1* and *FKH2*, a deletion of *FKH2* reduced the levels of **a1** mRNA expressed by *HMR-SS***a**, consistent with a negative role for

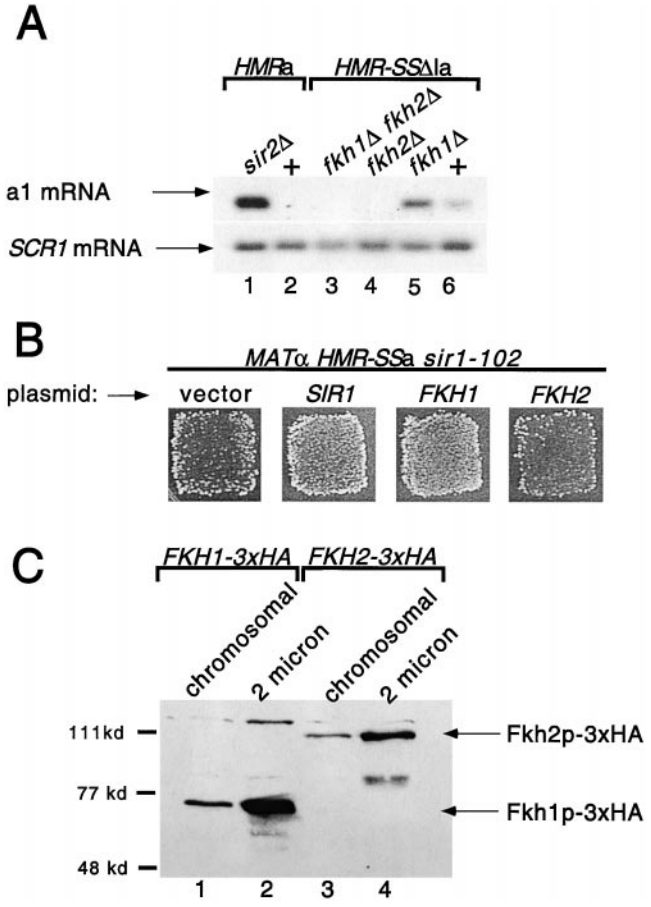

Figure 3.—The *FKH1* and *FKH2* genes have different functions in silencing *HMR***a**. (A) The steady-state levels of **a1** mRNA and *SCR1* mRNA were measured for isogenic *MAT*α *HMR***a** strains that were either *sir2*Δ (CFY393) or wild type (+; CFY37; lanes 1 and 2). These mRNAs were also measured from an isogenic set of *MAT*α *HMR-SS*Δ*I***a** strains differing by their *FKH* genotype as indicated [lanes 3–6; *fkh1*Δ *fkh2*Δ, CFY147; *fkh2*Δ, CFY150; *fkh1*Δ, CFY149; and wild type (+), CFY148]. (B) Multicopy expression of *FKH2* did not enhance silencing at *HMR***a** in a strain containing a defect in *SIR1*. Mating phenotypes observed for a *MAT*α *HMR-SS***a** *sir1-102* strain (CFY720) harboring either a 2-μm plasmid (vector; Yep24), or 2-μm *SIR1* (pCF345), *FKH1* (pCF341), or *FKH2* (pCF399) were determined as described in Figure 1. (C) The steady-state levels of either Fkh1p-3xHA or Fkh2p-3xHA were determined for an isogenic set of strains containing a chromosomal copy of either *FKH1-3xHA* (lane 1; CFY480) or *FKH2-3xHA* (lane 3; CFY854) or a 2-μm plasmid containing either *FKH1-3xHA* (lane 2; CFY762 containing pCF547) or *FKH2-3xHA* (lane 4; CFY145 containing pCF665). The steady-state levels of Fkh1p-3xHA and Fkh2p-3xHA expressed from a 2-μm plasmid were ~10- and 7-fold higher, respectively, than the levels expressed from chromosomal copies of each tagged gene (P. C. Hollenhorst, unpublished results).

FKH2 in silencing *HMR***a** (Figure 3A, compare lanes 4 and 6; a very faint band corresponding to **a1** mRNA could be detected in the original autoradiogram; also see Figure 7, below). Moreover, deletion of both *FKH1* and *FKH2* caused an even further reduction in **a1** mRNA levels (Figure 3A, lane 3, and see Figure 7, below). Thus, the silencing phenotypes associated with loss of *FKH1* and *FKH2* were not predicted from their sequence similarities. In particular, rather than having overlapping functions in silencing, these data indicated that *FKH1* and *FKH2* had opposing functions in silencing.

One simple prediction based on the data described above was that multicopy expression of *FKH2* would fail to enhance silencing at *HMR***a**. Significantly, in contrast to multicopy expression of either *SIR1* or *FKH1*, multicopy expression of *FKH2* failed to enhance silencing in a *MAT*α *HMR-SS***a** *sir1-102* strain (Figure 3B). Thus, multicopy expression of *FKH2* failed to substitute for *SIR1* function in silencing *HMR***a**, consistent with the view that *FKH2* behaved differently from *FKH1*.

One explanation for the inability for multicopy expression of *FKH2* to enhance silencing was that some mechanism prevented the overexpression of the Fkh2 protein. Therefore, we compared the levels of Fkh1p and Fkh2p in a population of yeast cells expressing *FKH1* or *FKH2* fused to the coding region of three tandem copies of the hemagglutinin epitope (*3xHA*; Figure 3C). Both *FKH1-3xHA* and *FKH2-3xHA* provided for wild-type *FKH* function (P. C. Hollenhorst, unpublished results; see materials and methods). The levels of both Fkh1p-3xHA and Fkh2p-3xHA were elevated relative to their normal wild-type levels when either fusion gene was expressed from a high-copy-number plasmid (Figure 3C, compare "chromosomal" to "2 micron"). The levels of Fkh2p-3xHA appeared to be lower than the levels of Fkh1p-3xHA in these experiments, but the larger size of Fkh2p compared to Fkh1p could have contributed to a reduced transfer efficiency of Fkh2p. Regardless, the Fkh2p-3xHA levels could be substantially increased over wild-type levels when *FKH2-3xHA* was expressed from a high-copy plasmid, suggesting that the inability for *FKH2* to enhance silencing was not due to an inability to generate a higher level of Fkh2p. Taken together, these data indicate that *FKH1* and *FKH2* behaved differently in silencing *HMR***a**.

FKH1* and *FKH2* had redundant functions in preventing pseudohyphal growth:** The silencing data indicate that, despite their strong sequence similarity, *FKH1* and *FKH2* had opposite effects on silencing *HMRa**. Significantly, a cross between a strain containing a deletion of *FKH1* (*MAT***a** *fkh1*Δ*::TRP1 HMR-SS*Δ*I***a**) and a strain containing a deletion of *FKH2* (*MAT*α *fkh2*Δ*::HIS3 HMR-SS*Δ*I***a**) indicated that the two *FKH* genes did indeed share overlapping functions in controlling another form of yeast cell differentiation. Specifically, segregants containing deletions in both *FKH1* and *FKH2* (*fkh1*Δ*::TRP1 fkh2*Δ*::HIS3*) gave rise to colonies with ruffled edges and a chalky appearance and texture. Furthermore, diploids that were homozygous for deletions in both *FKH1* and *FKH2* also exhibited this colony phenotype (C. A. Fox, unpublished results). Therefore, *FKH1* and *FKH2* have redundant functions in controlling yeast colony morphology.

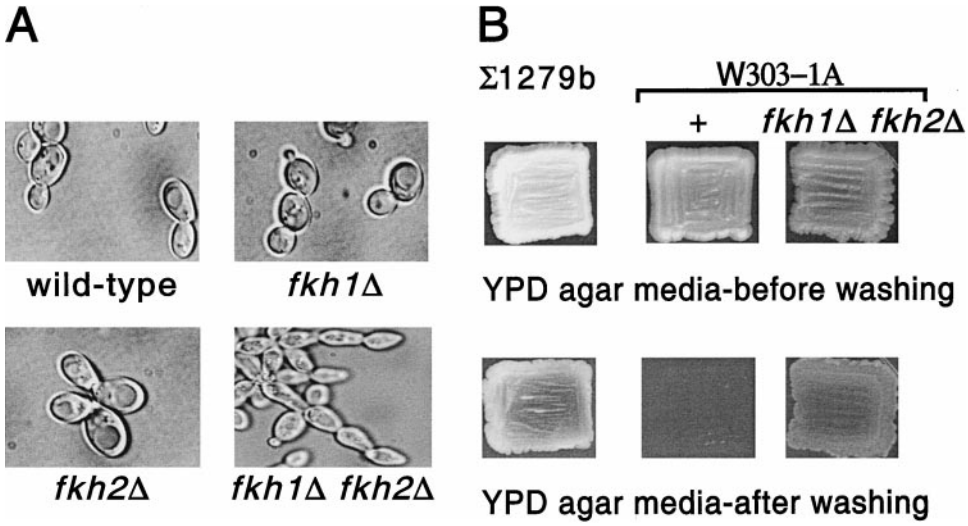

Figure 4.—*FKH1* and *FKH2* have redundant functions in pseudohyphal growth. (A) DIC optics were used to image a set of four isogenic haploid strains: wild type (CFY269), *fkh1*Δ (CFY55), *fkh2*Δ (CFY99), and *fkh1*Δ *fkh2*Δ (CFY-270). (B) *fkh1*Δ *fkh2*Δ cells penetrated solid agar medium. Haploid strains were gently patched to YPD media and grown for 3 days (top). The plate was then washed with a gentle stream of water (bottom). The haploid strains were Σ1279b (CG189), W303-1A wild type (+; CFY269) and *fkh1*Δ *fkh2*Δ (CFY270). W303-1A was *ade2* and thus produced a darker scar than Σ1279b (*ADE2*).

The ruffled colony phenotype observed in yeast strains containing deletions in both *FKH1* and *FKH2* suggested that the individual cell morphology in these strains might be different from wild-type strains. In liquid culture, yeast strains harboring deletions in both *FKH1* and *FKH2* exhibited a clumpy, flocculent phenotype characteristic of yeast strains that grow pseudohyphally (C. A. Fox, unpublished results; Liu *et al.* 1996). To test whether individual cells from a *fkh1*Δ *fkh2*Δ strain grew similarly to pseudohyphal yeast cells, cells were viewed under light microscopy (Figure 4A). Cells containing a deletion of both *FKH1* and *FKH2* had an elongated morphology relative to wild-type cells or cells containing a deletion of either *FKH1* or *FKH2* alone. Furthermore, the elongated cells grew in chains in a manner similar to characterized pseudohyphal growth in some strains of *S. cerevisiae* (Gimeno *et al.* 1992), suggesting that *FKH1* and *FKH2* were redundant negative regulators of yeast pseudohyphal growth.

One documented characteristic of pseudohyphal yeast cells is that many of the cells within a colony penetrate or invade solid agar media. This agar penetration causes a "scar" of imbedded cells to be left on the medium after the surface cells are washed off (Roberts and Fink 1994). To determine whether strains harboring deletions in both *FKH1* and *FKH2* also exhibited this characteristic of pseudohyphal growth, we compared agar-scarring of the characterized pseudohyphal strain of *S. cerevisiae*, Σ1279B, which in its haploid form exhibits pseudohyphal growth under glucose starvation (Roberts and Fink 1994), to a wild-type W303-1A strain and an isogenic *fkh1*Δ *fkh2*Δ strain (Figure 4B). Significantly, the strain containing deletions in both *FKH1* and *FKH2* (W303-1A, *fkh1*Δ *fkh2*Δ) caused agar scarring to a degree similar to that caused by strain Σ1279B, whereas the wild-type strain used in these studies caused no agar scarring (W303-1A, wild type). A strain containing a deletion of either *FKH1* or *FKH2* alone exhibited no agar scarring in an analogous experiment (C. A. Fox, unpublished results). Analysis of the plates after washing indicated that the scarring was due to a large number of cells that had penetrated beneath the agar surface. Thus by the second criterion of agar penetration, *FKH1* and *FKH2* have redundant functions in preventing yeast pseudohyphal growth.

***fkh1*Δ *fkh2*Δ-induced pseudohyphal growth is distinct from nutritionally induced pseudohyphal growth:** Pseudohyphal growth exhibited under nutritional starvation in yeast strain Σ1279b requires several genes, including *FLO11*. In particular, Flo11p, a cell-surface flocculin, is a critical terminal gene product required for the pseudohyphal cell morphology and agar scarring exhibited by strain Σ1279b (Lo and Dranginis 1998; Rupp *et al.* 1999). One hypothesis was that *FKH1* and *FKH2* normally repressed *FLO11* expression and that the *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth observed in W303-1A also required *FLO11*. Therefore, we constructed a strain that harbored complete deletions of *FLO11*, *FKH1*, and *FKH2* in W303-1A and determined whether this strain formed pseudohyphae and penetrated solid agar media (Figure 5). Significantly, a strain lacking *FKH1*, *FKH2*, and *FLO11* (*fkh1*Δ *fkh2*Δ *flo11*Δ) formed pseudohyphae and penetrated solid agar as efficiently as a strain lacking *FKH1* and *FKH2* but containing wild-type *FLO11* (*fkh1*Δ *fkh2*Δ *FLO11*), indicating that *FLO11* was not required for the pseudohyphal growth associated with deletion of the *FKH* genes. In a separate set of experiments, we also demonstrated that *STE12*, another gene required for pseudohyphal growth in strain Σ1279b (Roberts and Fink 1994), was not required for the *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth or agar penetration in strain W303-1A (C. A. Fox, unpublished results). Thus, although the pseudohyphal growth caused by deletion of both *FKH1* and *FKH2* was morphologically similar to the pseudohyphal growth described for strain Σ1279B, it was distinct by at least two genetic criteria.

Multicopy expression of *CLB2* prevented *fkh1*Δ *fkh2*Δ-

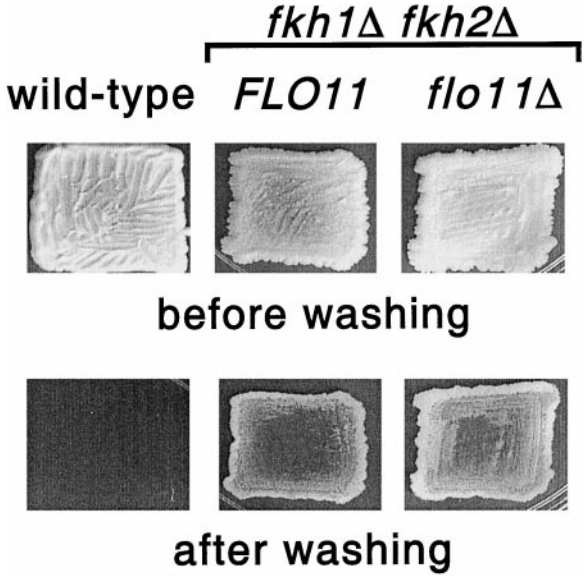

Figure 5.—*FLO11* is not required for *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth. Agar penetration was assessed as described in Figure 4. The isogenic strains were wild type (CFY269), *fkh1*Δ *fkh2*Δ (CFY270), or *fkh1*Δ *fkh2*Δ *flo11*Δ (CFY330).

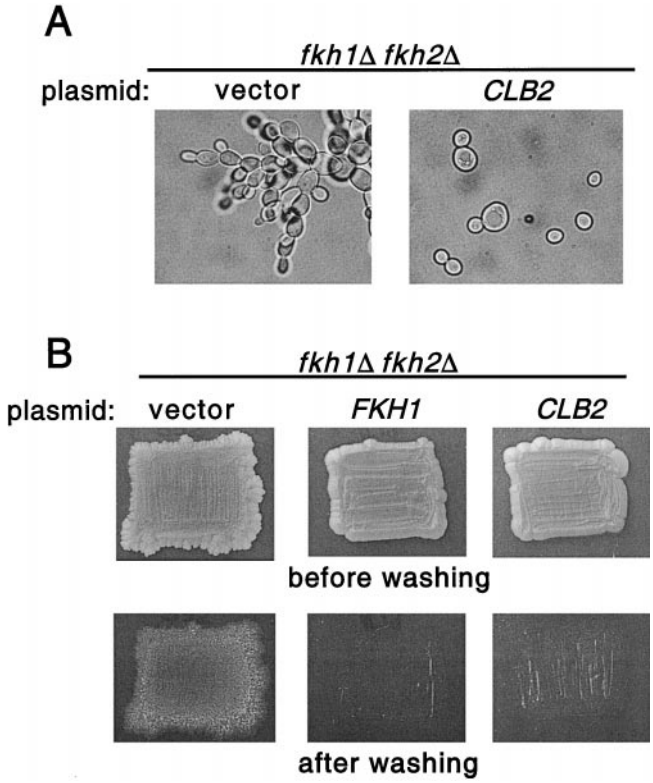

Figure 6.—Multicopy expression of *CLB2* prevented *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth. (A) An *fkh1*Δ *fkh2*Δ strain (CFY147) transformed with a 2-μm vector (pRS426) or 2-μm *CLB2* (pCF633) was imaged with DIC optics. (B) The *fkh1*Δ *fkh2*Δ strain (CFY147) transformed with a 2-μm vector (pRS426), or 2-μm *FKH1* (pCF480) or *CLB2* (pCF633) was assessed for agar penetration as described in Figure 4.

induced pseudohyphal growth: Pseudohyphal differentiation in yeast is characterized by growth during the G2/M phase of the cell cycle (Kron *et al.* 1994; Kron and Gow 1995). Furthermore, an elongated cell morphology, a component of pseudohyphal differentiation, is promoted by mutations in the G2/M-phase cyclin *CLB2* (Lew and Reed 1993) and abrogated by overexpression of *CLB2* (Kron *et al.* 1994; Ahn *et al.* 1999). Therefore, a reasonable hypothesis was that *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth could be abrogated by multicopy expression of *CLB2*. Significantly, multicopy expression of *CLB2* completely suppressed the formation of elongated cells and pseudohyphae associated with the loss of *FKH* function (Figure 6A, compare vector to *CLB2*). In addition, *fkh1*Δ *fkh2*Δ-induced agar penetration was also abolished (Figure 6B). Thus, multicopy expression *CLB2* abolished *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth, providing evidence that the pseudohyphal phenotype associated with loss of the *FKH* genes was related to yeast cell-cycle progression.

Multicopy expression of *CLB2* prevented *fkh1*Δ *fkh2*Δ-enhanced silencing: Previous studies indicated that mutations in *CLB2* enhance silencing at *HMR***a** (Laman *et al.* 1995). Since multicopy expression of *CLB2* could abrogate *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth, we postulated that the enhanced level of silencing observed in a *fkh1*Δ *fkh2*Δ strain might be abrogated by multicopy expression of *CLB2*. Therefore we measured the **a1** mRNA levels in an isogenic set of *MAT*α *HMR-SS*Δ*I***a** strains (*HMR-SS*Δ*I***a**) that differed only by their *FKH1* or *FKH2* genotypes and the plasmid that they contained (Figure 7). Specifically, the same set of strains was transformed with either a 2-μm plasmid (vector) or a 2-μm plasmid containing the *CLB2* gene (*CLB2*). As a control in these experiments, the **a1** mRNA levels from two isogenic *MAT*α strains containing wild-type *HMR***a** (*HMR***a**) and differing only in their *SIR2* genotype were also measured.

As discussed above, deletion of *FKH1* (*fkh1*Δ) reduced silencing, whereas deletion of *FKH2* (*fkh2*Δ) enhanced silencing as measured by a reduction in **a1** mRNA levels (Figure 7, compare lanes 2, 3, and 4). Deletion of both *FKH1* and *FKH2* (*fkh1*Δ *fkh2*Δ) enhanced silencing further than deletion of *FKH2* alone (*fkh2*Δ); **a1** mRNA was undetectable even after a long exposure of the RNA blot in a *fkh1*Δ *fkh2*Δ strain (Figure 7, compare lanes 1 and 2). Thus the selective growth conditions used to retain the plasmid in these experiments yielded results similar to those observed under rich growth conditions.

If multicopy expression of *CLB2* could abrogate the silencing phenotypes caused by deletion of *FKH2* (*fkh2*Δ) or deletion of both *FKH1* and *FKH2* (*fkh1*Δ *fkh2*Δ), then the *fkh2*Δ and *fkh1*Δ *fkh2*Δ strains harboring a *CLB2* plasmid should express more **a1** mRNA than these same strains harboring vector alone. Significantly, the level of **a1** mRNA expressed from these strains was

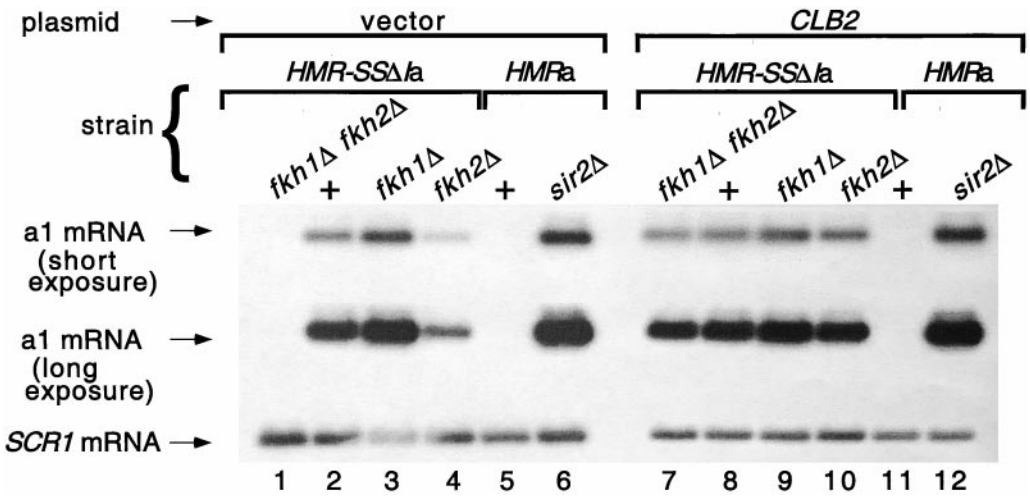

Figure 7.—Multicopy expression of *CLB2* suppressed the enhanced silencing phenotypes of *fkh2*Δ and *fkh1*Δ *fkh2*Δ strains. The steady-state levels of **a1** mRNA and *SCR1* mRNA were measured as described in Figure 1B from isogenic *MAT*α *HMR-SSΔI***a** strains that were *fkh1*Δ *fkh2*Δ (CFY147; lanes 1 and 7), wild type (+; CFY148; lanes 2 and 8), *fkh1*Δ (CFY149; lanes 3 and 9), or *fkh2*Δ (CFY150; lanes 4 and 10) and from *MAT*α *HMR***a** strains that were wild type (+; CFY37; lanes 5 and 11) or *sir2*Δ (CFY393; lanes 6 and 12). Each strain contained either a 2-μm vector (pRS426; lanes 1–6) or 2-μm *CLB2* (lanes 7–12; pCF633).

markedly increased in the presence of multicopy *CLB2* (Figure 7, compare lanes 7, 8, and 10 to lanes 1, 2, and 4). Thus, multicopy *CLB2* expression abrogated the pseudohyphal growth and silencing phenotypes caused by the simultaneous deletions of *FKH1* and *FKH2* and the silencing phenotype caused by deletion of *FKH2* alone. Multicopy expression of *CLB2* did not significantly affect silencing in either the wild-type or *fkh1*Δ strains (Figure 7, compare lanes 2 and 3 to lanes 8 and 9), supporting the observation that *FKH1* and *FKH2* functioned differently in silencing. Moreover, these data raise the possibility that the silencing and pseudohyphal growth phenotypes caused by simultaneous deletion of both *FKH1* and *FKH2* were associated with similar changes in the cell cycle.

Deletion of the *FKH* genes affected cell-cycle progression and *CLB2* mRNA expression: The data discussed above indicate that multicopy expression of *CLB2* suppressed the pseudohyphal growth and some of the silencing phenotypes caused by deletion of the *FKH* genes, raising the possibility that deletion of the *FKH* genes caused defects in cell-cycle progression and *CLB2* expression. To test these possibilities, cell-cycle progression and *CLB2* mRNA levels were measured in an isogenic set of *MAT***a** strains that differed only in their *FKH* genotype (Figure 8). A growing liquid culture was synchronized in G1 phase by α-factor arrest, released from arrest into fresh medium, and at 15-min intervals cell-cycle progression was monitored by counting the number of cells in the G1 (no buds), S (small buds), and G2/M (large buds) phases of the cell cycle (Figure 8A). *CLB2* mRNA levels were also measured at each interval by RNA blot hybridization (Figure 8B).

Deletion of either *FKH1* (*fkh1*Δ *FKH2*) or *FKH2* (*FKH1 fkh2*Δ) caused subtle but measurable changes in cell-cycle progression compared to an isogenic wild-type strain (*FKH1 FKH2*). Specifically, deletion of *FKH1* caused a slight increase in progression through the S and G2/M phases of the cell cycle such that the peak of cells in the second G1 phase occurred slightly earlier than the corresponding peak in the wild-type strain (Figure 8A). In addition, the *fkh1*Δ *FKH2* strain progressed more rapidly and synchronously through S phase and into G2/M phase than did the wild-type strain. In contrast, deletion of *FKH2* (*FKH1 fkh2*Δ) reduced the rate of progress through the cell cycle relative to the wild-type and *fkh1*Δ *FKH2* strains (Figure 8A, *FKH1 fkh2*Δ). The filamentous morphology of the isogenic *fkh1*Δ *fkh2*Δ strain prevented a similar analysis of this strain. However, vigorous sonication of an asynchronously growing *fkh1*Δ *fkh2*Δ strain indicated that the majority of cells released from filaments had a large two-budded morphology. In contrast, after exposure to α-factor, a large number of cells released from filaments after sonication had a single-budded morphology, suggesting that these cells had responded to α-factor and arrested in the G1 phase (C. A. Fox, unpublished results). These observations are consistent with the majority of individual cells in an asynchronously growing *fkh1*Δ *fkh2*Δ culture exisiting in the G2/M phase of the cell cycle. Taken together, these data indicated that reductions in *FKH* gene function altered cell-cycle progression. Moreover, deletion of either *FKH1* or *FKH2* alone caused detectable and opposite effects on cell-cycle progression.

Analysis of *CLB2* mRNA levels during cell-cycle progression revealed that deletion of the *FKH* genes also altered *CLB2* expression (Figure 8B). In the G1 phase, all four strains expressed very low levels of *CLB2* mRNA, as expected (Fitch *et al.* 1992; Figure 8B, time 0). However, after release from α-factor, each strain exhibited

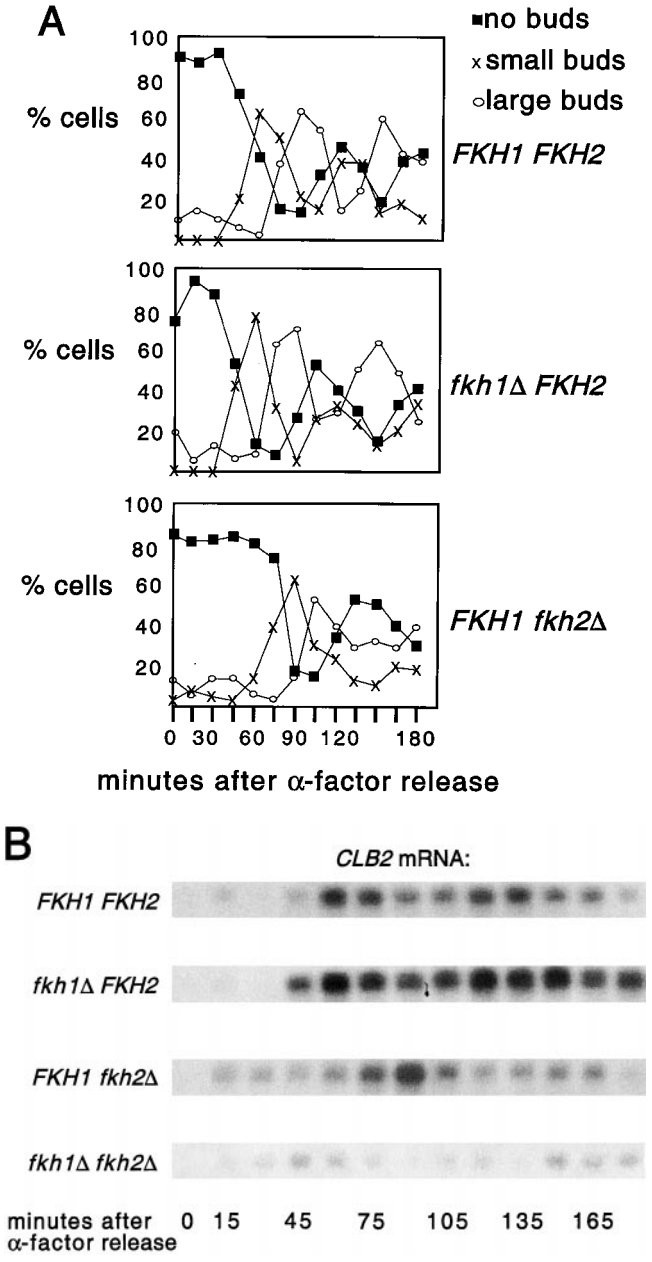

Figure 8.—Deletion of the *FKH* genes affected cell-cycle progression and *CLB2* mRNA expression. Isogenic *MAT***a** cells that were wild-type (*FKH1 FKH2*; CFY158), *fkh1*Δ (*fkh1*Δ FKH2; CFY62), or *fkh2*Δ (*FKH1 fkh2*Δ; CFY100) or *fkh1*Δ *fkh2*Δ (CFY166) were grown into log phase in rich media, arrested in G_1 with α-factor, and then released into fresh media. Every 15 min, an aliquot from each culture was harvested for (A) analysis of individual cell morphology and (B) levels of *CLB2* mRNA as described in Figure 1. The same level of total RNA (10 μg) was analyzed in each lane of each blot as determined by A_{260} units and the level of *SCR1* RNA. Total RNA from the wild-type strain was included in each blot, and the exposures for wild type on each blot were adjusted so that they were identical to the exposure shown for wild type (*FKH1FKH2*, top). Thus the levels of *CLB2* mRNA for each strain shown can be compared directly.

a different expression pattern for *CLB2* mRNA. Deletion of *FKH1* (*fkh1*Δ *FKH2*) elevated the levels of *CLB2* mRNA at each time interval relative to wild type, although cycling of *CLB2* mRNA was similar. Significantly, the *CLB2* mRNA levels in the *fkh1*Δ strain did not return to their low G1-phase levels as they did in the wild-type strain during the course of this experiment, although the *fkh1*Δ cells continued to cycle similarly to the wild-type strain (Figure 8A). In contrast, deletion of *FKH2* (*FKH1 fkh2*Δ) reduced the levels of *CLB2* mRNA at most time intervals. Interestingly, *CLB2* mRNA was detected early after release from α-factor, but this level remained constant until *CLB2* mRNA levels peaked sharply and much later at 90 min. Deletion of both *FKH1* and *FKH2* (*fkh1*Δ *fkh2*Δ) dramatically reduced the levels of *CLB2* mRNA. A shallow cycling of *CLB2* mRNA was still observable in this strain, although compared to the other strains in this experiment, cycling of *CLB2* mRNA was less evident. Thus reductions in *FKH* gene function altered *CLB2* mRNA expression. Moreover, deletion of either *FKH1* or *FKH2* alone caused opposite effects on the levels of *CLB2* mRNA expressed at most intervals during cell-cycle progression.

The Fkh1p was nuclear and required its DNA binding domain for function: To test whether Fkh1p functioned through its DNA binding domain, we determined whether Fkh1p was nuclear by constructing a fusion gene in which the coding region for the GFP was fused immediately downstream of the coding region for the Fkh1p DNA binding domain. This *FKH1-GFP* fusion functioned as wild-type *FKH1* (M. Mielke, unpublished results). Fluorescence microscopy indicated that the fusion protein localized to the nucleus, suggesting that Fkh1p was a nuclear protein (Figure 9A). To test whether the Fkh1p DNA binding domain was required for Fkh1p function, we constructed a *FKH1* gene that contained a precise deletion of the coding region for the *FKH1* DNA binding domain ($fkh1_{DBD\Delta}$). This $fkh1_{DBD\Delta}$ failed to provide *FKH1* function in either silencing or pseudohyphal growth (Figure 9, B and C). Importantly, deletion of the DNA binding domain did not reduce the steady-state levels of the mutant protein significantly (Figure 9D). Thus, the Fkh1p was a nuclear protein that required its DNA binding domain for its functions in silencing and pseudohyphal growth.

The DNA binding domains of Fkh1p and Fkh2p were interchangeable: One explanation for differences between *FKH1* and *FKH2* was that the two proteins had different DNA binding specificities *in vivo* and thus regulated different sets of target genes. In this view, pseudohyphal growth would require that expression of both the Fkh1p and Fkh2p gene targets be disrupted, whereas the silencing phenotypes would be affected differently depending on whether Fkh1p or Fkh2p gene targets were affected. Although the DNA binding domains of Fkh1p and Fkh2p are 75% identical, several of the amino acids that do differ between the domains are

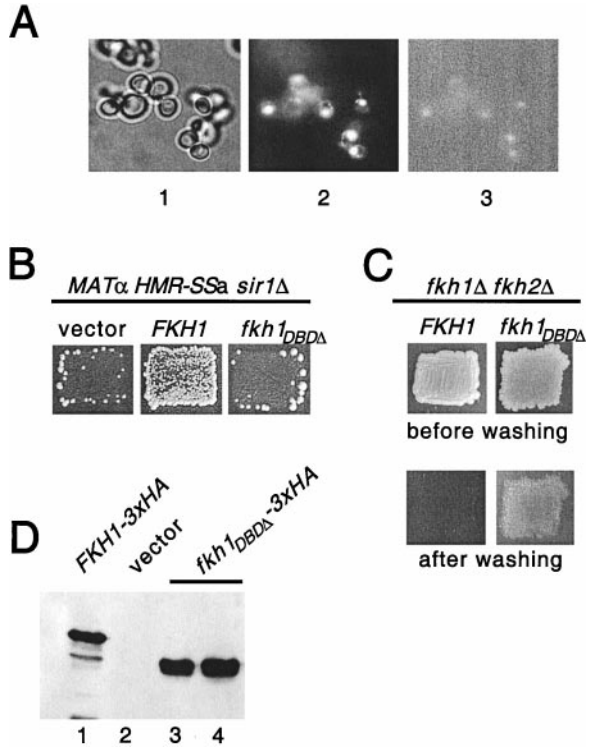

Figure 9.—Fkh1p was nuclear and required its DNA binding domain. (A) Nuclear localization of a *FKH1-GFP* fusion protein (pCF587) expressed in a *MAT***a** *fkh1*Δ*::HIS3 fkh2*Δ*::TRP1* strain (CFY155) was determined by staining cells with 4′,6-diamidino-2-phenylindole (DAPI) and imaging with (1) DIC optics, (2) a UV filter, and (3) a GFP filter. The DAPI stain was not detectable with the GFP filter. (B) Fkh1p requires its DNA binding domain for silencing function. Mating phenotypes observed in a *MAT*α *HMR-SS***a** *sir1*Δ*::LEU2* strain (CFY762) harboring a 2-μm vector (pRS426), 2-μm *FKH1* (pCF480), or 2-μm $fkh1_{DBD\Delta}$ (pCF569). Mating assays were performed as described in Figure 1. (C) Fkh1p requires its DNA binding domain for agar penetration. The assay described in Figure 4B was used to measure the agar penetration of a *MAT***a** *fkh1*Δ *fkh2*Δ strain (CFY270) transformed with 2-μm *FKH1* (pCF480) or $fkh1_{DBD\Delta}$ (pCF569). (D) Fkh1p lacking its DNA binding domain was expressed at levels similar to wild-type Fkh1p. Anti-HA antibody detected the steady-state level of Fkh1p-3xHA or $Fkh1_{DBD\Delta}$p-3xHA in CFY762 transformed with a 2-μm vector (lane 2; pRS426), 2-μm *FKH1-3xHA* (lane 1; pCF547) or $fkh1_{DBD\Delta}$*-3xHA* [lanes 3 and 4 (two separate transformants); pCF589].

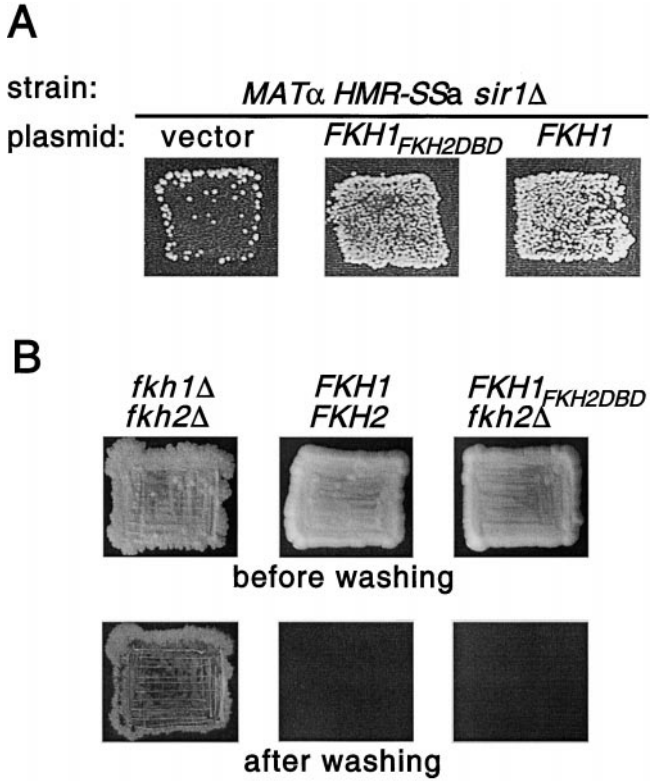

Figure 10.—The DNA binding domains of Fkh1p and Fkh2p are equivalent in silencing and pseudohyphal growth. (A) A multicopy *FKH1* hybrid gene containing the coding region for the *FKH2* DNA binding domain ($FKH1_{FKH2DBD}$) substitutes for *SIR1* function in silencing. Mating phenotypes observed in a *MAT*α *HMR-SS***a** *sir1*Δ*::LEU2* strain (CFY762) harboring a 2-μm vector (pRS426), 2-μm *FKH1* (pCF480), or 2-μm $FKH1_{FKH2DBD}$ (pCF574) were determined as described in Figure 1. (B) A chromosomal copy of $FKH1_{FKH2DBD}$ is sufficient to prevent pseudohyphal growth. Agar penetration was compared between three isogenic *MAT***a** haploid strains that were *fkh1*Δ *fkh2*Δ (CFY155), wild-type (*FKH1 FKH2*; JRY2334), or $FKH1_{FKH2DBD}$ fkh2Δ (CFY902) as described in Figure 4.

proposed to regulate DNA binding specificity and affinity in other Fkh proteins (Overdier *et al.* 1994; Marsden *et al.* 1997). Therefore, a fusion gene was constructed that contained a precise substitution of the *FKH1* DNA binding domain with the *FKH2* DNA binding domain ($FKH1_{FKH2DBD}$). Multicopy expression of the $FKH1_{FKH2DBD}$ enhanced silencing in a *MAT*α *HMR-SS***a** *sir1*Δ strain to a level similar to that caused by multicopy expression of wild-type *FKH1* (Figure 10A). Furthermore, substitution of the *FKH1* gene with the $FKH1_{FKH2DBD}$ hybrid gene at the normal *FKH1* chromosomal position provided a level of *FKH1* function sufficient to prevent pseudohyphal growth in a strain containing a deletion of *FKH2* (Figure 10B). These data suggest that the Fkh1 and Fkh2 proteins bound at least a subset of the same gene targets *in vivo* that were sufficient to modify both phenotypes associated with these genes. Thus, any differences between *FKH1* and *FKH2* could not be explained simply by differences in the DNA binding domains of Fkh1p and Fkh2p.

DISCUSSION

The work presented here was based on the prediction that the function of *SIR1* in silencing *HMR***a** could be enhanced or bypassed by the overexpression of a particular gene(s). Since changes in cell-cycle progression can enhance silencing at *HMR***a** in strains containing mutations in *SIR1* (Laman *et al.* 1995), in principle such a gene(s) could have a role(s) in regulating cell-cycle progression. In this article, we identify *FKH1* as a gene that, when expressed at high copy, could partially substi-

tute for the function of *SIR1* in silencing *HMR***a**. The data presented here provide evidence that *FKH1* and its homologue *FKH2* are genes with redundant functions in yeast cell morphology and opposing functions in silencing. Moreover, both the cell morphology and silencing phenotypes associated with loss of *FKH* function are associated with perturbations in cell-cycle progression. Thus, genetic studies of silencing have revealed the identity of two redundant regulators of cell-cycle progression and cell differentiation in *S. cerevisiae*.

Redundant functions for *FKH1* and *FKH2* revealed by their effects on yeast cell morphology: In the absence of both *FKH1* and *FKH2*, yeast cells grew with an elongated morphology and in filaments that failed to separate except under vigorous sonication (M. E. Bose, unpublished results), were flocculent when grown in liquid culture (C. A. Fox, unpublished results), and penetrated solid agar medium. These observations indicate that *FKH1* and *FKH2* have overlapping functions in preventing yeast pseudohyphal growth. These overlapping functions are not entirely surprising given the sequence conservation between the two genes; *FKH2* arose from a duplication of a multigene chromosomal region that includes *FKH1* (Pohlmann and Philippsen 1996) and the most obvious difference between the two genes is a coding region for an additional 300 C-terminal amino acids in *FKH2*. Thus, the overlapping structural features of Fkh1p and Fkh2p give rise to overlapping functions in controlling yeast cell morphology.

Significantly, although *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth appeared similar to the nutritionally induced pseudohyphal growth observed in some strains of *S. cerevisiae*, it also differed in two important ways from this relatively well-characterized form of pseudohyphal growth. First, the nutritionally induced form of pseudohyphal growth occurs in response to different nutritional signals depending on whether the yeast cells are in the haploid or diploid form (Roberts and Fink 1994). Specifically, diploids form pseudohyphae in response to nitrogen starvation (Gimeno *et al.* 1992), whereas haploids form pseudohyphae in response to glucose starvation (Roberts and Fink 1994). Furthermore, the nutritionally induced form of pseudohyphal growth is significantly more extensive in the diploid form of yeast (Roberts and Fink 1994). In contrast, the *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth is constitutive; both haploids and diploids form equivalent pseudohyphae and both do so on rich media. Second, the nutritionally induced form of pseudohyphal growth requires a number of genes, including *STE12* and *FLO11*. However, *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth require neither *STE12* nor *FLO11*, indicating that morphologically similar forms of yeast differentiation can occur by different genetic mechanisms. It will be interesting to learn whether *FKH1* or *FKH2* plays any regulatory role in the formation of nutritionally induced pseudohyphae in *S. cerevisiae* or the regulated transitions to filamentous growth in infectious yeast such as *Candida albicans*.

Interestingly, *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth was completely suppressed by overexpression of *CLB2*, a gene that encodes a G2/M-phase cyclin. Nutritionally induced pseudohyphal growth is associated with an elongated G2/M phase and can be enhanced by reductions in *CLB2* and abrogated by overexpression of *CLB2* (Kron *et al.* 1994). However, reductions in *CLB2* are not sufficient to induce the formation of pseudohyphae (Lew and Reed 1993). Regardless, these data provided genetic evidence that an elongated G2/M phase was a possible component of the *fkh1*Δ *fkh2*Δ-induced pseudohyphal growth, as did morphological analysis of individual cells in a *fkh1*Δ *fkh2*Δ strain, suggesting that, at the level of cell-cycle progression, *fkh1*Δ *fkh2*Δ-induced and nutritionally induced pseudohyphal growth were similar.

Distinct functions for *FKH1* and *FKH2* revealed by their effects on silencing and the cell-cycle: The silencing phenotypes associated with loss of *FKH2* or both *FKH1* and *FKH2* were also modulated by multicopy expression of *CLB2*, suggesting that the roles of the *FKH* genes in silencing are related to their roles in cell-cycle progression. However, in contrast to the redundant relationship between Fkh1p and Fkh2p in pseudohyphal growth, the silencing phenotypes associated with *FKH* function revealed a more complex relationship. Specifically, *FKH1* has a positive role in silencing whereas *FKH2* has a negative role. If the two genes have overlapping functions in controlling cell morphology, why would *FKH1* behave differently from *FKH2* in silencing? The analysis of cell-cycle progression and *CLB2* mRNA levels in strains lacking *FKH1*, *FKH2*, or both *FKH1* and *FKH2* provided a clue. In particular, *FKH1* and *FKH2* have opposite effects on cell-cycle progression and *CLB2* mRNA levels; deletion of *FKH1* enhanced progression through the S/G2/M phases of the cell-cycle and elevated *CLB2* mRNA levels relative to wild type, whereas deletion of *FKH2* reduced both cell-cycle progression and *CLB2* mRNA levels. Furthermore, deletion of both *FKH1* and *FKH2* caused a *CLB2* mRNA expression pattern more similar to deletion of *FKH2* than deletion of *FKH1*. These effects are consistent with the effects deletion of the *FKH* genes have on silencing (Laman *et al.* 1995). Thus, although each gene can provide for the function of the other in controlling cell morphology, under normal circumstances each gene has distinct functions in cell-cycle progression that could explain its distinct role in silencing.

The changes in *CLB2* mRNA levels caused by deletion of the *FKH* genes raise the possibility that Fkh1p and Fkh2p normally function as cell-cycle transcriptional regulators of *CLB2*. Indeed, Fkh1p and Fkh2p are proposed to be components of the *S*wi *F*ive *F*actor (SFF) that binds near Mcm1p binding sites in the regulatory regions of genes within the *CLB2* gene cluster (Maher *et al.* 1995; Spellman *et al.* 1998; T. N. Davis and A. B.

Futcher, unpublished results). Thus, the phenotypes associated with the *FKH* genes may be explained by their effects on the expression of genes within the *CLB2* cluster, including *CLB2* itself. However, since reductions in cell-cycle progression are not sufficient to induce pseudohyphal growth, elucidation of the precise mechanisms by which high-copy expression of *CLB2* is sufficient to prevent *fkh1*Δ *fkh2*Δ-induced pseudohyphal differentiation should provide insights into the relationships between cell differentiation, cell-cycle progression, and the transcriptional control of specific genes.

Significantly, the differences in *FKH1* and *FKH2* function are not attributable to differences in the functions of the Fkh1p and Fkh2p DNA binding domains, suggesting that the two proteins could regulate at least some gene targets in a similar manner. However, the differences in *FKH1* and *FKH2* function argue that, for at least some target genes, the functions of Fkh1p and Fkh2p are not interchangeable under normal conditions. The differences in Fkh1p and Fkh2p could be due to differences in the structure of the two proteins, such as the long C-terminal domain of Fkh2p missing from Fkh1p, that could affect interactions with other gene regulatory proteins. Regardless, it is difficult to explain the different functions of *FKH1* and *FKH2* without invoking target genes that, under normal conditions, are regulated uniquely by either Fkh1p or Fkh2p. Thus, the Fkh1p and Fkh2p DNA binding domains may not be the exclusive determinants of the sites at which each protein functions *in vivo*.

Continued studies that exploit phenotypes that reveal nonoverlapping functions for *FKH1* and *FKH2* should provide insights into the unique functions of each gene. Moreover, given the evolutionary conservation of *FKH* genes in eukaryotic organisms, continued studies in yeast should provide insights into how this important class of transcription factors links changes in gene expression to cell-cycle progression and cell differentiation.

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