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, HMLα and HMRa, 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 HMRa. FKH1 (Forkhead Homologue 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 HMRa, 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, fkh1Δ fkh2Δ-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 HMRa. High-copy expression of CLB2, a G2/M-phase cyclin, prevented fkh1Δ fkh2Δ-induced pseudohyphal growth and modulated some of the fkhΔ-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, HMRa, 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 (Lo and Rine 1995; Fox and Rine 1996). The silent mating-type cassettes are transcriptionally re-
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 MATα allele confers the α-mating phenotype whereas the MATα allele confers the α-mating phenotype. In normal yeast strains, a silenced copy of the MATα allele resides at HMR and a silenced copy of the MATα allele resides at HML. Mutations that cause defects in silencing lead to the simultaneous expression of both α-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 (Silent Information Regulator) 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 HMRα, 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 HMRα (Laman et al. 1995).

The effect of cell-cycle perturbations on the efficiency of transcriptional silencing at HMRα 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 (Forkhead Homologue 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 Knockel 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 Knockel 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

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<tr>
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<th>Reference</th>
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<td>MATa his4 leu2 trp1 ura3</td>
<td>Gardner et al. (1999)</td>
</tr>
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<td>CFY617</td>
<td>JRY19 MATα</td>
<td>Thomas and Rothstein (1989)</td>
</tr>
<tr>
<td>CFY2334</td>
<td>MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 (W303-1A)</td>
<td>Gardner et al. (1999)</td>
</tr>
<tr>
<td>JRY3009</td>
<td>MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 (W303-1B)</td>
<td>Gardner et al. (1999)</td>
</tr>
<tr>
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</tr>
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<td>Gardner et al. (1999)</td>
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<tr>
<td>CFY902</td>
<td>JRY2334 FKH1/KFH2Δ</td>
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1. All strains except JRY19, CFY617, and CG189 are isogenic derivatives of W303.
2. All gene deletions described in this article are substitutions of the entire open reading frame for the relevant gene with the marker gene indicated.
3. Unless noted, strains were from the laboratory collection or constructed during the course of this work.
4. The HMRα status of this strain is unknown. It is either HMR-SSα or HMRα. The HMR genotype was not relevant to the fkh1Δ fkh2Δ-induced pseudohyphal growth described in this article.
5. FKH1/KFH2Δ 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.

The table lists various strains used in the study, along with their genotypes and references. The strains are generally constructed by mating assays and/or analysis of genomic DNA using PCR or DNA blot hybridization. The study focuses on the role of FKH1 and FKH2 in yeast pseudohyphal growth, and the table provides specific information about the strains used for this purpose.
FKH1 DNA binding domain (FKH1 DNA binding domain was precisely replaced with the isolate. As an additional test of whether FKH1 S. cerevisiae the PCR epitope tagging method for in vivo source of FKH provided wild-type et al. (1995). Both FKH1 FKH2 DBD FKH2 provided wild-type 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 Sau3AI 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 SphI 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 NheI 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 fkh1Δ fkh2Δ-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-

### TABLE 2

<table>
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<tr>
<th>Plasmid</th>
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<td>FKH1 genomic clone in Yep24</td>
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* Unless noted, plasmids were from the laboratory collection or constructed during the course of this work.
* These plasmids were gifts from the laboratory of Jasper Rine.
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 (CFY854) and cloned into the BsiI/NheI 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 (CFY854). The amplified fragment was cloned into the Smal 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 Smal 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 Smal 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 Smal sites flanking the coding region for the FKH1 DNA binding domain. Each Smal site introduced a codon for a single glycine residue into the recombiant 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 Fkh1p functioned identically to wild-type FKH1 (M. Mielke, unpublished results). To construct the FKH1-GFP 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 Smal and cloned into the Smal 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 Smal site in place of the coding region for the FKH1 DNA binding domain (FKH1<sup>ΔDBD</sup>; pCF569). To determine whether the Fkh1<sup>ΔDBD</sup> encoded a stable mutant protein, the coding region for the 3xHA C-terminal epitope was introduced into the FKH1<sup>ΔDBD</sup> clone (pCF569) to generate FKH1<sup>ΔDBD</sup>-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<sup>ΔDBD</sup> 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 Smal site of FKH1<sup>ΔDBD</sup> (pCF569) to create a FKH1<sup>ΔDBD</sup> hybrid gene in pRS426 (pCF574). To construct an integrating version of this hybrid gene, a fragment containing the FKH1<sup>ΔDBD</sup> 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 α1, 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 HMRa locus under the control of the synthetic HMR-E silencer (HMR-SSa; McNally and Rine 1991). The sir1-101 allele is defective for silencing, but is not as defective as a sir1Δ allele, and thus contributed to a sensitized genetic background. The synthetic HMR-E silencer (HMR-SSa), a simplified version of the HMR-E silencer that provides full silencing to HMRa in combination with the HMR-E silencer, requires SIR1 for efficient silencing. Thus a MATα HMR-SSa sir1-101 strain is unable to mate because the simultaneous expression of both α and SIR genes causes the nonmating phenotype of a diploid (Her skowitz et al. 1992). This strain was transformed with a high-copy-number yeast genomic library (Carlson and Boetstein 1982). If a transformant expressed a gene that could restore silencing to HMR-SSa, then it would mate efficiently with an α-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 MATα HMR-SSa sir1Δ::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 HMRa.
FKH1 (pCF341). Mating assays were performed as described in Figure 1A. 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 sIR1Δ mutant strain.

As a second measure of the ability of FKH1 to restore silencing to HMRa, the levels of al mRNA were analyzed directly by RNA blot hybridization in a MATα MAT-SSα strain harboring either asir1-101 or asir1Δ allele (Figure 1B). In the absence of silencing at HMRa, al mRNA is expressed (Hershkowicz et al. 1992). The levels of al mRNA were similar in the MATα strains containing either sir1-101 or sir1Δ, indicating that by this criterion sir1-101 behaved similarly to a sir1Δ allele (Figure 1B, lanes 1 and 4). Multicopy expression of wild-type FKH1 in either sir1 mutant strain restored full silencing to HMRa as indicated by the disappearance of al mRNA (Figure 1B, lanes 3 and 6). Multicopy expression of FKH1 restored some silencing to HMRa in both sir1 mutant strains as indicated by a reduction in the level of al mRNA (Figure 1B, lanes 2 and 5). However, FKH1 reduced the levels of al mRNA more efficiently in the strain harboring sir1-101 than in the strain harboring sir1Δ::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 al mRNA expressed from HMRa 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 al mRNA levels through a b0na fide silencing mechanism. First, multicopy expression of FKH1 failed to enhance silencing by HMR-SSa in sir2Δ, sir3Δ, or sir4Δ 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 al 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 a1 genes (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 sIR1Δ mutant strain.

Figure 1.—Multicopy expression of FKH1 substituted for the function of SIR1 in silencing HMRa. (A) Mating phenotypes observed in a MATα HMR-SSα sir1Δ::LEU2 strain (CFY762) harboring either a 2μm plasmid (vector; Yep24), or 2μm SIR1 (pCF341) or FKH1 (pCF341). The transformants were grown as patches for 18 hr at 30° on medium lacking uracil, replica-plated to a MATα 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 al mRNA and SCR1 mRNA were measured by RNA blot hybridization of 25 μg of total RNA from isogenic MATα HMR-SSα strains that were sir1-101 (sir1::LEU2 or sir1Δ; CFY774) or sir1Δ::LEU2 (sir1Δ; CFY762), each transformed with a 2μm plasmid (lanes 1 and 4, vector; Yep24), or 2μm FKH1 (lanes 2 and 5; pCF341) or SIR1 (lanes 3 and 6; pCF345). (C) Mating phenotypes observed in a mataΔp HMR-SSa sir1-106 strain (CFY737) transformed with either a 2μm plasmid (vector; Yep24), or 2μm SIR1 (pCF345) or FKH1 (pCF341). Mating assays were performed as described in Figure 1A.
Figure 2.—Multicopy expression of FKH1 did not increase the steady-state levels of SIR4 mRNA. (A) Expression of SIR4 from a 2μm 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 MATα HMR-SSa sir1Δ::LEU2 strain (CFY762), transformed with a 2μm plasmid (lane 1, vector; Yep24), 2μm SIR1 (lane 2; pCF345), 2μm 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 MATα HMR-SSa sir1Δ::LEU2 strain (CFY762) transformed with 2μm SIR4 (lane 1, SIR4-2μ; pCF351), a centromere vector containing SIR4 (lane 2, SIR4-cen; pCF290), or 2μm 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 HMRa (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 MATα HMR-SSa sir1Δ-101 strain. RNA blot hybridization indicated that multicopy SIR4 expression silenced HMRα in a MATα HMR-SSa sir1Δ 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-SSa 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 HMRα in this sir1Δ 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 (MATα/MATα fkh1Δ::HIS3 FKH1 HMR-SSΔα/HMRa), 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 MATα HMR-SSΔα fkh1Δ::HIS3 segregants indicated that silencing was not affected dramatically (C. A. Fox, unpublished results). However, the sensitivity of HMR-SSΔα 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-SSΔα 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 HMRa 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 (MATα/MATα fkh2Δ::HIS3/FKH2 HMR-SSΔα/HMRa) 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-SSα, consistent with a negative role for...
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. Significantly, in contrast to multicopy expression of either SIR1 or FKH1, multicopy expression of FKH2 failed to enhance silencing in a MATα HMR-SSa strain (Figure 3B). Thus, multicopy expression of FKH2 failed to substitute for SIR1 function in silencing HMR.

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 hamagglutinin epitope (3xHA; Figure 3C). Both FKH1-3xHA and FKH2-3xHA were 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.

**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 HMR. Significantly, a cross between a strain containing a deletion of FKH1 (MATα fkh1Δ::TRP1 HMR-SSa) and a strain containing a deletion of FKH2 (MATα fkh2Δ::HIS3 HMR-SSa) 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 (fkh1Δ::TRP1 fkh2Δ::HIS3) gave rise to colonies with ruffled edges and a chalky appearance. Furthermore, diploids that were heterozygous 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.
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 fkh1Δ fkh2Δ 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 fkh1Δ fkh2Δ strain (Figure 4B). Significantly, the strain containing deletions in both FKH1 and FKH2 (W303-1A, fkh1Δ fkh2Δ) 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.

*fhk1Δ fkh2Δ*-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 fkh1Δ fkh2Δ-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 (fkh1Δ fkh2Δ flo11Δ) formed pseudohyphae and penetrated solid agar as efficiently as a strain lacking FKH1 and FKH2 but containing wild-type FLO11 (fkh1Δ fkh2Δ 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 fkh1Δ fkh2Δ-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 *fhk1Δ fkh2Δ*-
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 fkh1Δ fkh2Δ-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, fkh1Δ fkh2Δ-induced agar penetration was also abolished (Figure 6B). Thus, multicopy expression of CLB2 abolished fkh1Δ fkh2Δ-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 fkh1Δ fkh2Δ-enhanced silencing: Previous studies indicated that mutations in CLB2 enhance silencing at HMRα (Laman et al. 1995). Since multicopy expression of CLB2 could abrogate fkh1Δ fkh2Δ-induced pseudohyphal growth, we postulated that the enhanced level of silencing observed in a fkh1Δ fkh2Δ strain might be abrogated by multicopy expression of CLB2. Therefore we measured the α1 mRNA levels in an isogenic set of MATα HMR-SSα strains (HMR-SSαα) 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 α1 mRNA levels from two isogenic MATα strains containing wild-type HMRα (HMRαα) and differing only in their SIR2 genotype were also measured.

As discussed above, deletion of FKH1 (fkh1Δ) reduced silencing, whereas deletion of FKH2 (fkh2Δ) enhanced silencing as measured by a reduction in α1 mRNA levels (Figure 7, compare lanes 2, 3, and 4). Deletion of both FKH1 and FKH2 (fkh1Δ fkh2Δ) enhanced silencing further than deletion of FKH2 alone (fkh2Δ); α1 mRNA was undetectable even after a long exposure of the RNA blot in a fkh1Δ fkh2Δ 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 (fkh2Δ) or deletion of both FKH1 and FKH2 (fkh1Δ fkh2Δ), then the fkh2Δ and fkh1Δ fkh2Δ strains harboring a CLB2 plasmid should express more α1 mRNA than these same strains harboring vector alone. Significantly, the level of α1 mRNA expressed from these strains was...
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 fkh1Δ 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 MATα 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 (fkh1Δ FKH2) or FKH2 (FKH1 fkh2Δ) 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 fkh1Δ 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 fkh2Δ) reduced the rate of progress through the cell cycle relative to the wild-type and fkh1Δ FKH2 strains (Figure 8A, FKH1 fkh2Δ). The filamentous morphology of the isogenic fkh1Δ fkh2Δ strain prevented a similar analysis of this strain. However, vigorous sonication of an asynchronously growing fkh1Δ fkh2Δ 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 fkh1Δ fkh2Δ culture existing 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
a different expression pattern for CLB2 mRNA. Deletion of FKH1 (fkh1Δ 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 fkh1Δ 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 fkh1Δ cells continued to cycle similarly to the wild-type strain (Figure 8A). In contrast, deletion of FKH2 (FKH1 fkh2Δ) 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 (fkh1Δ fkh2Δ) 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 (fkh1DBDΔ). This fkh1DBDΔ 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 MATα fkh1Δ::His3 fkh2Δ::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 MATα HMR-SSα sir1Δ::LEU2 strain (CFY762) harboring a 2-μm vector (pRS426), 2-μm FKH1 (pCF480), or 2-μm fkh1DBD (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 MATα fkh1Δ fkh2Δ strain (CFY270) transformed with 2-μm FKH1 (pCF480) or fkh1DBD (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 Fkh1DBD-3xHA in CFY762 transformed with a 2-μm vector (lane 2; pRS426), 2-μm FKH1-3xHA (lane 1; 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.

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 MATα HMR-SSα sir1Δ::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 MATα haploid strains that were fkh1Δ fkh2Δ (CFY155), wild-type (FKH1 FKH2; JRY2334), or FKH1_FKH2DBD fkh2Δ (CFY902) as described in Figure 4.

DISCUSSION

The work presented here was based on the prediction that the function of SIR1 in silencing HMRa could be enhanced or bypassed by the overexpression of a particular gene(s). Since changes in cell-cycle progression can enhance silencing at HMRa 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 HMRa. 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 silenc-
ing. 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 pro-
gression 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 elon-
gated morphology and in filaments that failed to sepa-
rate except under vigorous sonication (M. E. Bose, un-
published results), were flocculent when grown in liquid
culture (C. A. Fox, unpublished results), and pene-
trated solid agar medium. These observations indicate
that FKH1 and FKH2 have overlapping functions in pre-
venting 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 Philippen 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 fkh1Δ fkh2Δ-induced pseu-
dohyphal growth appeared similar to the nutritionally
induced pseudohyphal growth observed in some strains
of S. cerevisiae, it also differed in two important ways.
First, the nutritionally induced form of pseu-
dohyphal growth occurs in response to different nutri-
tional signals depending on whether the yeast cells are
in the haploid or diploid form (Roberts and Fink 1994).
Specifically, diploids form pseudohyphae in re-

response to nitrogen starvation (Gimeno et al. 1992),
whereas haploids form pseudohyphae in response to

glucose starvation (Roberts and Fink 1994). Further-
more, the nutritionally induced form of pseudohyphal
growth is significantly more extensive in the diploid
form of yeast (Roberts and Fink 1994). In contrast,
the fkh1Δ fkh2Δ-induced pseudohyphal growth is con-
stitutive; both haploids and diploids form equivalent pseudohyphae and both do so on rich media. Second, the
nutritionally induced form of pseudohyphal growth re-
quires a number of genes, including STE12 and FLO11.
However, fkh1Δ fkh2Δ-induced pseudohyphal growth re-
quire neither STE12 nor FLO11, indicating that morpho-

logically 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 pseudohy-
phae in S. cerevisiae or the regulated transitions to fila-

mentous growth in infectious yeast such as Candida albic-
ans.

Interestingly, fkh1Δ fkh2Δ-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 pseu-
dohyphae (Lew and Reed 1993). Regardless, these data
provided genetic evidence that an elongated G2/M
phase was a possible component of the fkh1Δ fkh2Δ-
induced pseudohyphal growth, as did morphological
analysis of individual cells in a fkh1Δ fkh2Δ strain, sug-
gest that, at the level of cell-cycle progression, fkh1Δ
fkh2Δ-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 silen-
encing phenotypes associated with loss of FKH2 or both
FKH1 and FKH2 were also modulated by multicopy ex-
pression 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 rela-
tionship between Fkh1p and Fkh2p in pseudohyphal
growth, the silencing phenotypes associated with FKH
function revealed a more complex relationship. Speci-

fically, FKH1 has a positive role in silencing whereas
FKH2 has a negative role. If the two genes have over-
lapping 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 ele-
vated 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 pat-
tern 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 pro-
posed to be components of the Swi Five Factor (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 fkh1Δ fkh2Δ-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 euukaryotic 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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