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Asymmetric Accumulation of Ash1p in Postanaphase Nuclei Depends on a Myosin and Restricts Yeast Mating-Type Switching to Mother Cells

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Summary

Cell division in haploid yeast gives rise to a "mother" cell capable of mating-type switching and a "daughter" cell that is not. Switching is initiated by the *HO* endonuclease, whose gene is only transcribed in cells that have previously given birth to a bud (mother cells). *HO* expression depends on a minimyosin, She1p/Myo4p, which accumulates preferentially in growing buds. We describe a gene, *ASH1*, that is necessary to repress *HO* in daughters. *ASH1* encodes a zinc finger protein whose preferential accumulation in daughter cell nuclei at the end of anaphase depends on She1p/Myo4p. The greater abundance of Ash1p in daughter cells is responsible for restricting *HO* expression to mother cells.

Introduction

The ability of cells to give birth to progeny that adopt different patterns of gene expression in the absence of any differences in their surroundings is an important aspect of multicellular development. A good example of this phenomenon occurs during the diploidization of homothallic spores of the budding yeast Saccharomyces cerevisiae (reviewed by Nasmyth, 1993). Upon germination, haploid spores divide to produce a mother cell that replaces the mating-type-specific sequences at its *MAT* locus by those of an opposite type (from **a** to α or vice versa) and a daughter cell that does not. Both mother and daughter cells proceed with another division, and each thereby produces a pair of cells, which, owing to differences at their mating-type loci, secrete different pheromones and express different receptors on their cell surfaces. The complementary nature of these two pheromone/receptor pairs enables the progeny descended from mother and daughter cells to conjugate with each other. Homothallic haploid spores thus regain the diploid state without having to encounter progeny of spores born with an opposite mating type. Switching ceases when both types of mating-type loci are expressed in the same cell, i.e., in zygotes and their diploid progeny.

The ability to switch mating type is confined to cells that have previously given rise to a bud or "daughter" cell for as long as cells remain haploid. Using micromanipulation, it is possible to separate mothers from their daughters after each division and show that, in the absence of any difference in their environment, mother cells switch at 70% of their divisions, whereas daughter cells never do so (Strathern and Herskowitz, 1979). There does not seem to be any great difference in the ability of old or new mothers to switch; that is, giving birth to a single daughter cell is sufficient qualification to switch.

Mating-type switching is initiated by a double-stranded break made at the *MAT* locus by an endonuclease encoded by the *HO* gene (Strathern et al., 1982). The differential ability of mothers and daughters to switch is due to differences in *HO* transcription. *HO* is transcribed transiently during a narrow window at the G1 to S phase of the mother cell cycle, shortly before budding, but it is not transcribed at any point during the cell cycle of a daughter (Nasmyth, 1983).

The isolation of mutants that cannot express *HO* has identified at least ten *SWI* genes (*SWI1–SWI10*) that encode nuclear factors necessary for *HO* transcription (Stern et al., 1984; Breeden and Nasmyth, 1987). Most encode components of a multisubunit complex that is required for the expression of many genes besides *HO* and is thought to be needed to remodel chromatin structure (Peterson et al., 1994). Others, like *SWI4* and *SWI6*, encode components of a transcription factor (SCB-binding factor [SBF]) that is responsible for confining the activation of *HO* and many other genes to late G1/S phase (Koch and Nasmyth, 1994). None of these factors is mother cell specific.

SWI5, on the other hand, has been implicated in mother/daughter asymmetry. It encodes a zinc finger protein that binds to two sites within the URS1 region of the *HO* promoter (-1000 to -1400), the replacement of which by the *GAL* upstream activating sequence (UAS) causes *HO* to lose its mother/daughter dependence and gain dependence on a carbon source without altering its cell cycle control (Nasmyth, 1987; Stillman et al., 1988; Tebb et al., 1993). Furthermore, ectopic expression either of *SWI5*, which is normally not transcribed during G1, or of alleles that lack a domain in the center of the protein causes *HO* to be expressed in daughter cells (Nasmyth et al., 1987a; Tebb et al., 1993).

Swi5p is not, however, the determinant of the mother cell specificity of *HO*. The protein accumulates in the cytoplasm during G2 and M phases and only enters nuclei upon destruction of cyclin B/Cdc28 kinases during anaphase (Moll et al., 1991), at which point it accumulates to similar levels in mother and daughter nuclei. This accumulation is transient, because the bulk of Swi5p in the nucleus is rapidly degraded during G1 phase (Tebb et al., 1993). The transient nuclear accumulation of Swi5p at the anaphase/G1 boundary is accompanied by the transient activation of several Swi5p-dependent genes (Koch and Nasmyth, 1994), but not of *HO*, whose activation must await activation of SBF in late G1. Mothers and daughters differ in some way with regard to the ability of Swi5p to activate *HO*.

In an accompanying paper (Jansen et al., 1996 [this issue of *Cell*]), we describe the identification of five genes, called *SHE1–SHE5* (for Swi5p-dependent *HO* expression), that encode a very different set of proteins needed for *HO* expression. She proteins are located in the cytoplasm. More surprising still, both She1p/Myo4p

and She3p accumulate preferentially in growing buds and not in mother cells that require their activity. The identification of She1p with the type V minimyosin Myo4p led us to propose that She proteins are involved in the transport from mother cells into their growing buds of a factor that promotes *HO* repression.

Until recently, there seemed no easy way of identifying genes that encode daughter cell–specific repressors. Here we describe the isolation, by two different methods, of mutants defective in repressing *HO* in daughter cells. Most mutants belong to a single complementation group and define a gene, termed *ASH1* (for asymmetric synthesis of *HO*), that encodes a zinc finger protein. Ash1p appears in both mother and daughter nuclei at the end of anaphase, but accumulates to much higher levels in daughter nuclei. This asymmetry is dependent on the activity of *SHE* genes. Ash1p accumulates to equally high levels in mother and daughter nuclei in *she* mutants, and this is responsible for their failure to transcribe *HO*.

Results

Mutants That Express *HO* in Daughter Cells

We used two very different methods to isolate mutants defective in putative daughter cell–specific repressors. The first started with the premise that *she* mutants cannot express *HO* because both mother and daughter cells accumulate a repressor that is normally restricted to daughter cells. If so, mutations that inactivate this repressor should restore *HO* transcription to *she* mutants.

Haploid strains whose sole *ADE2* and *CAN1* genes are fused to *HO* promoters (at the *HO* and *CAN1* loci, respectively) grow in the absence of adenine and are sensitive to canavanine (i.e., they are Ade^+ Can^S). Deletion of *SHE1* causes such a strain to become resistant to canavanine but dependent on exogenous adenine (i.e., it becomes Ade^- Can^R). Following mutagenesis of *she1 HO–ADE2 HO–CAN1* strains with **a** (*mat*Δ) and α mating types, we isolated 80 mutants capable of growth in the absence of adenine that had simultaneously become sensitive to canavanine. Following disruption of the *SWI5* locus, 38 of 40 mutants analyzed again became Ade^- Can^R. Crosses between the mutants with a *SWI5*-dependent phenotype isolated in *mat*Δ and *MAT*α backgrounds showed that 17 of 20 recessive mutations fell into a single complementation group, called *ash1*. In crosses between two mutants in this group (*ash1-2* and *ash1-12*) and a strain with the parental genotype, the Ade^+ Can^S phenotypes cosegregated 2:2. Crosses to *SHE1* strains showed both mutations to be unlinked to *she1*. *ash1-12* was also shown to suppress the Ade^- phenotype of *she3* and *she5* mutants and to cause daughter cells of *she1::URA3 HO* strains to switch mating types (Table 1). The wild-type *ASH1* locus was isolated from a genomic library by virtue of its ability to restore canavanine resistance to *ash1-12* mutant cells.

To identify repressor genes by a method that was not contingent on interpretation of the *she* mutant phenotype, we meanwhile devised a method to isolate mutants capable of expressing *HO* in both mother and daughter cells. *CDC6* encodes an unstable protein necessary for initiation of DNA replication, which is synthesized in two bursts during the cell cycle, at the end of mitosis and in late G1 simultaneously with *HO* (Piatti et al., 1995). We created a strain carrying a deletion of the endogenous *CDC6* locus and fusions of *CDC6* to the *HO* and *GAL1-10* promoters. Upon transfer to glucose medium, in which *GAL–CDC6* is not expressed, Cdc6 synthesis only occurs from the *HO* promoter. Pedigree analysis showed that most mothers, but few if any daughters, were capable of cell division under these circumstances. This strain grows healthily on galactose medium but, owing to the lack of daughter cell proliferation, cannot form colonies on glucose. We isolated 12 spontaneous mutants capable of growth on glucose. This ability depended on *SWI5* and was therefore due to expression of *CDC6* from the *HO* promoter. To test whether the mutations conferred *HO* expression in daughter cells (it was possible that they merely altered Cdc6 protein stability), we crossed several of the mutants to an *HO* strain and analyzed the pedigree of switching in several segregants from each cross. This confirmed that in all cases the mutant strains contained mutations that allowed daughter cells to switch mating types. The ability of 10 of 11 mutants to grow on glucose was abolished by transformation with a centromeric plasmid carrying the *ASH1* gene, and one such mutant (*ash1-61*) was shown to be allelic with *ash1-12*.

Table 1. Mating Type Switching in *ash1* Mutants and *ASH1–myc9* Cells

Strain	Mothers (%)	Daughters (%)
Wild Type (K4709)	70 (33 of 47)	0 (0 of 42)
ash1-12 she1::URA3 (K5861)	76 (22 of 29)	36 (8 of 22)
ash1-61 (K5864)	100 (59 of 59)	85 (50 of 59)
ash1::URA3 (K5862)	100 (33 of 33)	82 (27 of 33)
ASH1–myc9 (K5863)	97 (29 of 30)	0 (0 of 30)

Frequency of mating-type switching in mothers and daughters. Percentages are indicated, together with the actual numbers of switching per division (within parentheses). In all the cases examined, spores never switched mating types.

Table 1 shows that *ash1* mutations not only allow daughter cells to switch mating types, but also cause mother cells to switch with a higher frequency. Mutations in *SIN3/SDI1* and *SDI2* also allow *HO* to be expressed in daughter cells (Nasmyth et al., 1987b; Sternberg et al., 1987). However, these mutations probably do so by allowing *HO* transcription to become independent of Swi5p. We found, in contrast, that expression due to *ash1* mutations of *HO–ADE2* or *HO–CAN1* in *she1/myo4* mutants and *HO–CDC6* in daughter cells was dependent on Swi5p (this was determined by disrupting *SWI5*). *ASH1* is therefore not needed to confer Swi5p dependence on the *HO* promoter, but rather for specifying whether Swi5p can be active at *HO*.

The smallest centromeric plasmids capable of complementing *ash1-12* contained three contiguous open reading frames (ORFs) from yeast chromosome XI (Dujon et al., 1994). When subcloned, the ORF YKL185w

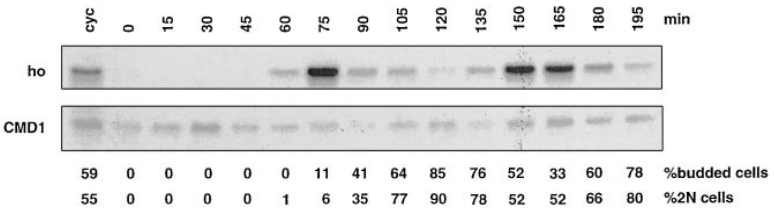

Figure 1. Cell Cycle–Dependent *ho* Expression in *ash1::URA3* Daughter Cells

Cells (K5550) were grown in yeast extract–peptone (YEP)–raffinose. Small unbudded G1 cells were collected by centrifugal elutriation and incubated in YEP–dextrose (YEPD) at 30°C. RNA samples were collected at the timepoints indicated (top). The percentage of budded cells and cells with 2N DNA content (determined by fluorescence-activated cell sorter analysis) is shown underneath the Northern blots. Calcofluor staining showed that 99% of the cells in the starting population were daughters.

alone was sufficient for complementation. To check that YKL185w truly corresponds to *ASH1*, we disrupted the chromosomal locus with *URA3* and showed that it was linked to *ash1-12* (see Experimental Procedures). The *ASH1* disruption suppressed the adenine auxotrophy of *she1 HO–ADE2* mutants and caused mating-type switching in *SHE HO* strains to increase from 0% to 82% in daughter cells and from 70% to 100% in mother cells. Ash1p must therefore be active in mother as well as daughter cells.

To check that daughter cell switching in *ash1* mutants is due to ectopic *HO* expression, we measured *ho* RNA levels during the growth of a culture of G1 daughter cells isolated by centrifugal elutriation (Figure 1). In wild-type cells, *ho* RNA does not appear until cells enter the second cycle (Nasmyth, 1983; Nasmyth et al., 1987a; see also Figure 8B). In *ash1::URA3* cells, *ho* RNA accumulated transiently also during the first cycle, shortly before budding and entry into S phase. *ASH1* is therefore required to prevent *HO* transcription in daughter cells, but it is not needed for restricting *HO* expression to the late G1/S period of the cell cycle.

YKL185w has the potential to encode a 588 amino acid protein, which contains a motif that resembles a GATA factor–like zinc finger (Figure 2). This type of zinc finger motif is found in several regulatory proteins: the vertebrate erythrocyte-specific factor GATA-1 (Evans and Felsenfeld, 1989; Omichinski et al., 1993), Drosophila Pannier (Ramain et al., 1993), and fungal regulatory proteins, such as Aspergillus AreA (Kudla et al., 1990) and yeast Dal80p (Cunningham and Cooper, 1991). The spacing between the two pairs of cysteine residues is, however, increased by three additional amino acid residues in the Ash1p putative zinc finger.

Another interesting feature of the Ash1p amino acid sequence is a basic domain amino-terminal to the putative zinc finger that is very rich in serine and proline. Serine-proline motifs occur at high frequency in other proteins that regulate transcription and are potential substrates for cyclin-dependent kinases (Cdks) and mitogen-activated protein kinases (Shenoy et al., 1989; Langan et al., 1989; Clark-Lewis et al., 1991); indeed, this region of Ash1p contains three potential Cdk1 phosphorylation sites.

Ash1p Accumulates Preferentially in Daughter Cell Nuclei

To detect Ash1p, we replaced the endogenous *ASH1* gene by a version that is tagged at its carboxyl terminus with nine repeats of the Myc epitope (Evan et al., 1985). Western blotting showed that *ASH1–myc9* produces a protein of 90 kDa, which is close to its predicted size of 82 kDa (data not shown). *she1 HO–ADE2 ASH1–myc9* cells are Ade$^-$ Can^R, suggesting that the Ash1p–Myc9 protein is functional. To investigate this further, we analyzed the pedigree of mating-type switching of *ASH1–myc9 HO* cells (Table 1). We found that 29 of 30 mother cells and none of 31 daughter cells switched mating type. Ash1p–Myc9 is therefore sufficiently active to repress *HO* fully in daughter cells. Wild-type Ash1p prevents switching at 30% of mother cell divisions, and Ash1p–Myc9 largely fails to do this. The addition of Myc epitopes might cause this effect either by reducing modestly the activity of Ash1p or by enhancing the asymmetry of its action.

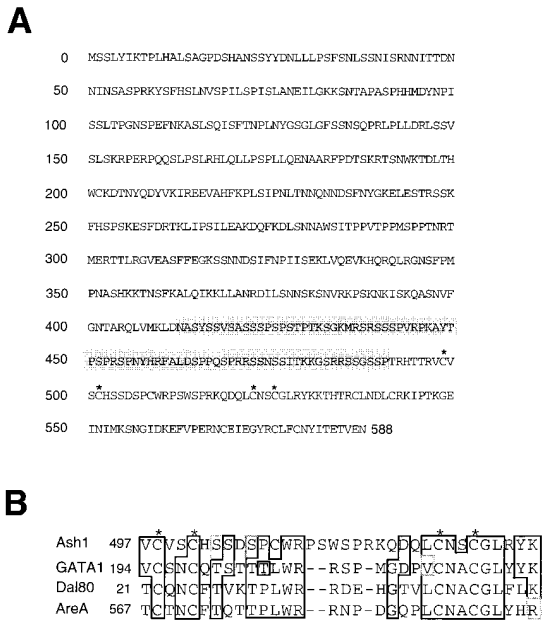

Figure 2. Ash1p Amino Acid Sequence Predicted for ORF YKL185w

(A) A putative zinc finger motif is present at the carboxyl terminus of the protein, and the cysteine residues are marked by asterisks. The serine-proline-rich region is stippled.

(B) Alignment between the potential Ash1p zinc finger and the zinc finger motifs found in GATA-1, Dal80p, and AreA. For GATA-1 transcription factor, only the sequence of the carboxy-terminal finger, which has been demonstrated to bind DNA on its own, is reported. Residues identical to those of Ash1p are boxed with black lines, and conserved residues are boxed with stippled lines. Dashes indicate gaps inserted to optimize the alignment. The cysteine residues forming the zinc finger are indicated with asterisks.

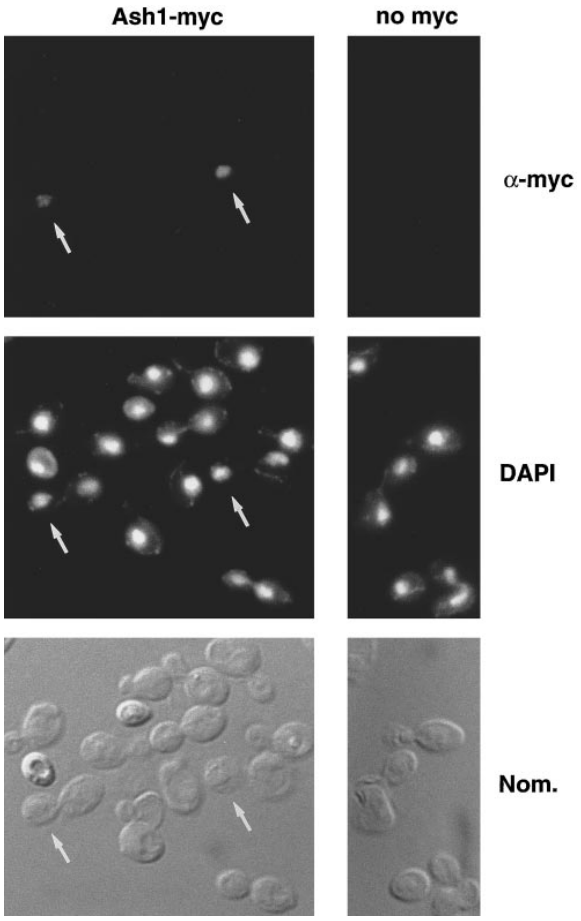

Figure 3. Ash1p Localization

Cells from asynchronous cultures of strain K5552 (*ASH1–myc9*) and K699 (*ASH1*) were fixed for indirect immunofluorescence and stained with the 9E10 anti-Myc antibody. No signal is detected in K699 that does not express the Myc epitope. Ash1p–Myc9-specific staining (indicated by arrows) always colocalized with the nucleus as revealed by diamidophenylindole (DAPI) staining. Nom, Nomarski optics. Note that the pictures portray many cells at different stages of the cell cycle, which can be assessed by the size of buds and by the DAPI staining.

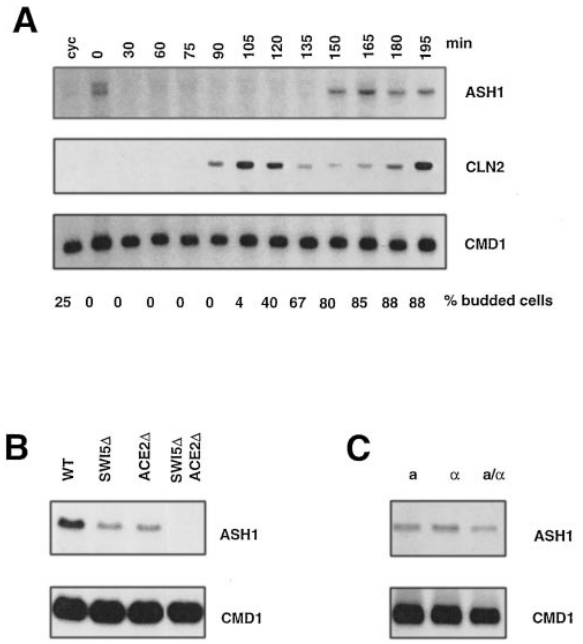

Figure 4. *ASH1* mRNA Regulation

(A) *ASH1* mRNA oscillates during the cell cycle. Small unbudded G1 cells (K1107, wild type) were collected by centrifugal elutriation and incubated in YEPD at 30°C. RNA samples were collected at the timepoints indicated on the top (see Koch et al., 1993). The percentage of budded cells is shown underneath the Northern blots. *ASH1* mRNA accumulation coincides exactly with that of *SIC1* (data not shown). *CMD1* (calmodulin) mRNA was used as an internal loading control.

(B) *ASH1* transcription is dependent on Swi5p and Ace2p transcription factors. *ASH1* and *CMD1* RNAs from exponentially growing wild-type (K699), *swi5* deletion (K1750), *ace2* deletion (K3772), and *swi5 ace2* double deletion (K3773) cells measured by Northern blotting.

(C) *ASH1* mRNA levels are comparable in *MAT***a** (K699), *MAT*α (K700), and *MAT***a**/*MAT*α (K842) diploid cells.

We analyzed the distribution of Ash1p–Myc9 protein by indirect immunofluorescence. Ash1p–Myc9 was detected exclusively in nuclei; no cytoplasmic staining above background could be detected in any cells. Ash1p–Myc9 accumulation was detectable in only certain types of cells in asynchronous cultures (Figure 3). Staining of nuclei was intense in many uninucleate unbudded cells and binucleate budded cells, but was weak if not absent in uninucleate cells with small- to medium-sized buds and was absent in all uninucleate cells with medium- to large-sized buds. This pattern suggests that Ash1p is absent in S, G2, and M phase cells and accumulates in nuclei only as cells undergo nuclear division. Ash1p–Myc9 is absent in many cells whose chromosomes have already segregated, suggesting that the protein does not accumulate until a late stage of anaphase (see cell at lower right of Figure 5C). Most interestingly, we always observed a marked difference in the intensity of Ash1p–Myc9 staining in sister nuclei of postanaphase cells. We always saw higher levels in nuclei situated in the smaller of the two cells, suggesting that Ash1p–Myc9 accumulates to higher levels in buds (i.e., daughters) than in their mothers (Figure 3).

Ash1p Synthesis Is Cell Cycle Regulated

To address whether transcriptional controls contribute to the pattern of Ash1p localization, we measured the abundance of *ASH1* mRNAs during growth of a synchronous culture of unbudded G1 daughter cells isolated by centrifugal elutriation. *ASH1* mRNAs are present in unbudded G1 daughter cells, but decline as these cells enlarge and start budding (Figure 4A). They remain low until cells complete mitosis and reenter G1. The appearance of *ASH1* mRNAs at the end of mitosis is simultaneous with that of *SIC1* (D. Knapp, personal communication; data not shown). *ASH1* mRNAs are absent in pheromone-arrested G1 cells and accumulate following their release only after nuclear division (data not shown).

The accumulation of *ASH1* mRNAs at the end of mitosis is similar to that of *EGT2*, *CTS1*, and *SIC1* mRNAs

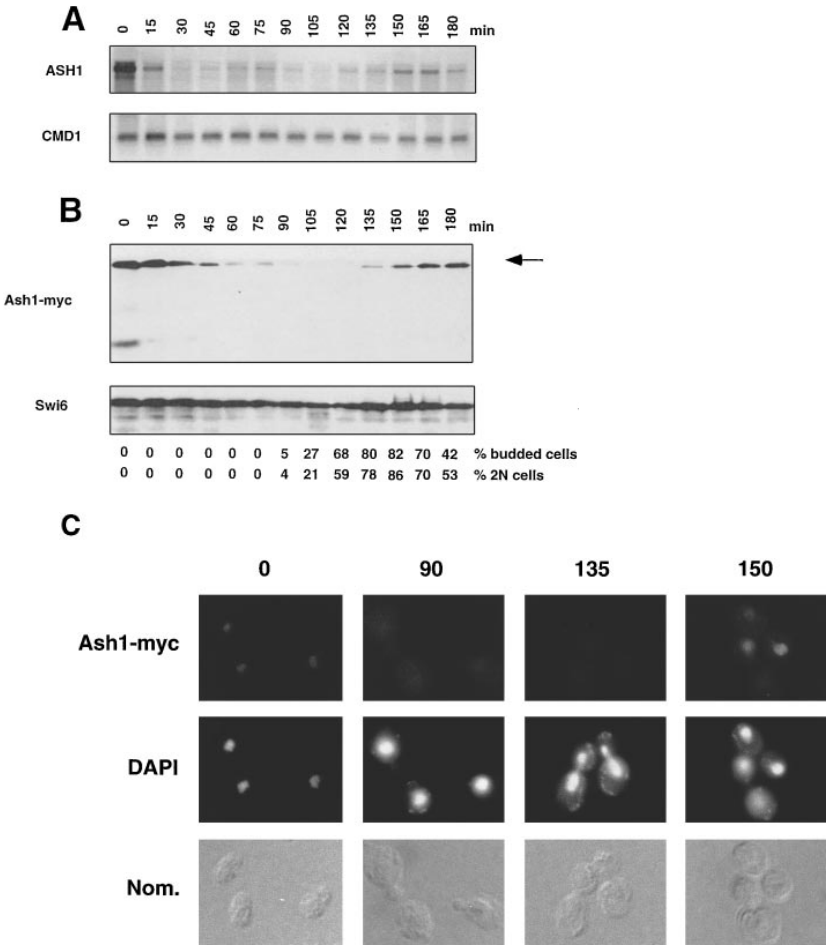

Figure 5. *ASH1–myc9* mRNA, Protein, and Protein Localization in a Synchronous Culture

Small unbudded G1 daughter cells (K5552, *ASH1–myc9*) were isolated by centrifugal elutriation and incubated in YEPD at 30°C.

(A) *ASH1–myc9* and *CMD1* mRNAs at timepoints shown on top. The percentage of budded cells and cells with 2N DNA content at each timepoint is shown at the bottom.

(B) Ash1p–Myc9 and Swi6p levels measured by Western blotting. The arrow indicates the position of intact Ash1p–Myc9 protein.

(C) Localization of Ash1p–Myc9 protein at the indicated timepoints by indirect immunofluorescence. All unbudded early G1 daughter cells isolated by elutriation showed strong Ash1p–Myc9 nuclear staining. This later disappeared and did not reappear until cells underwent anaphase.

(Koch and Nasmyth, 1994), which are known to be regulated by Swi5p and a related factor called Ace2p (Dohrmann et al., 1992). Swi5p and Ace2p accumulate in the cytoplasm upon their synthesis during G2 and M phases and are triggered to enter mother and daughter nuclei, in similar amounts, by inactivation of cyclin B/Cdc28 kinases at the end of anaphase (Moll et al., 1991). Like *EGT2* and *SIC1*, the levels of *ASH1* mRNA are somewhat lower in *ace2* and *swi5* single mutants and drastically reduced in *ace2 swi5* double mutants (Figure 4B). *ASH1* transcription could thus be triggered by entry of Swi5p and Ace2p into the nucleus at the end of anaphase. We do not yet know whether *ASH1* transcription is activated equally in mother and daughter nuclei. We also compared *ASH1* mRNA levels in **a**, α, and **a**/α cell types and found no differences (Figure 4C).

To establish the relationship between *ASH1* mRNA fluctuations and Ash1p accumulation, we analyzed a synchronous culture of *ASH1–myc9* cells (Figure 5). *ASH1–myc9* mRNA and protein levels were high in the starting culture of unbudded G1 daughter cells. Furthermore, in situ immunofluorescence showed that all daughter cells start the cell cycle with high levels of Ash1p in their nuclei (Figure 5C). *ASH1* mRNAs declined rapidly, and protein levels declined somewhat more slowly, as cells enlarged (Figures 5A and 5B). Protein levels and nuclear staining declined more rapidly around the onset of budding, reaching a minimum during G2 and M phases. In this culture, we also noticed a modest transient accumulation of *ASH1* mRNAs just prior to budding. Both Ash1p–Myc9 nuclear staining and protein levels recovered shortly after *ASH1* mRNA reaccumulation in late anaphase. Thus, Ash1p abundance fluctuates during the cell cycle. It is hard to determine from our data the rate at which Ash1p inherited by daughter cells is degraded because some of the reduction seen in the Western blots will be due to dilution mediated by cell growth. The data nevertheless show that most of the protein that appears in daughter cell nuclei at the end of anaphase must be synthesized de novo.

Asymmetry Depends on *SHE* Genes

Both the lack of *HO* expression in *she* mutants and the suppression of this defect by loss of Ash1p activity could be explained if *SHE* genes were involved in preventing accumulation of Ash1p to high levels in mother cells. To test this hypothesis, we analyzed the effect of *she* mutations on the cellular distribution of Ash1p–Myc9. We find that Ash1p–Myc9 accumulates equally in mother and daughter cells in *she1*, *she2*, *she3*, and *she5* mutants (Figure 6; data not shown). The levels in mother and daughter nuclei are above those seen in *SHE*$^+$

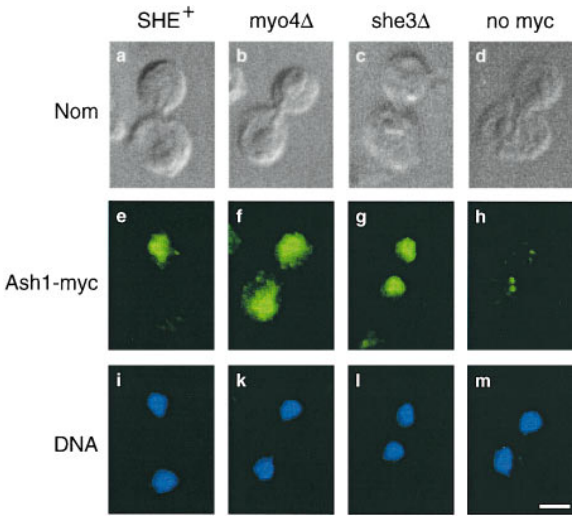

Figure 6. Symmetric Accumulation of Ash1p–Myc9 in Mothers and Daughters in *she* Mutants

Representative examples of Ash1p–Myc9 localization in late anaphase cells of wild-type (K5552), *she1/myo4* (K5679), and *she3* (K5680) strains analyzed by indirect immunofluorescence. A similar loss of asymmetry was also seen in *she2* and *she5* mutants.

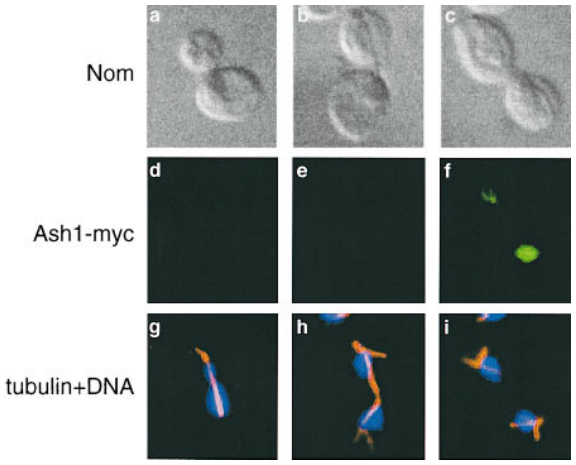

Figure 7. Nuclear Ash1p–Myc9 Protein Accumulation Only Occurs after Mitotic Spindle Breakdown

An asynchronous culture of K5552 (*ASH1–myc9*) cells was prepared for indirect immunofluorescence. Shown are metaphase cells ([a], [d], and [g]; 48 cells counted, 0 positive), anaphase cells with divided nuclei and long spindle ([b], [e], and [h]; 42 cells counted, 0 positive), and telophase cells ([c], [f], and [i]; 59 cells counted, 54 positive). (a)–(c) show Nomarski optics (Nom), (d)–(f) show anti-Myc staining, and (g)–(i) show anti-tubulin and DAPI (DNA) staining.

mother cells and approach those seen in *SHE*$^+$ daughters. *SHE* genes are therefore essential for generating the asymmetry in Ash1p–Myc9 accumulation.

SHE1 encodes a myosin (Myo4p) that accumulates in growing buds along with She3p and She5p/Bni1p. She proteins might be involved in transporting from mothers into their buds substances that promote *HO* repression. A key question is whether Ash1p might be one of the cargos transported by She1p/Myo4p. The levels of She1p/Myo4p and She3p remain higher in buds than in mother cells until middle to late anaphase (Jansen et al., 1996). After this point, the two proteins are found in equal abundance in mother and bud owing to a possible cessation of directional transport. Around this time, mothers direct growth back to the mother cell, which enlarges for a short period before formation of a new bud. To address whether Ash1p accumulates before or after She1p/Myo4p and She3p cease accumulating in buds, we compared the state of mitotic spindles and Ash1p–Myc9 accumulation. In cells with separated sister chromatids and intact mitotic spindles, She3p staining was symmetrical in some cases and asymmetric in others (roughly 50:50; Jansen et al., 1996). In contrast, we found no cells (0 of 42) with intact mitotic spindles that had accumulated Ash1p–Myc9 (Figure 7). We conclude that Ash1p only accumulates in nuclei after She3p has become symmetrical.

Asymmetry and Cytokinesis

Differences in the accumulation of Ash1p in mother and daughter cells appear around the time of cytokinesis. Might formation of the septum be required to maintain differences between the two cells without which Ash1p would accumulate equally? Establishing whether cytokinesis has a role in maintaining asymmetry is also crucial if we are to interpret correctly the loss of Ash1p asymmetry in *she5/bni1* mutants. Is it possible that *she5/bni1* mutants accumulate Ash1p symmetrically merely because of their delay in cytokinesis? If so, might other *she* mutants have subtle defects in cytokinesis that we have so far not detected?

To address this issue, we analyzed Ash1p–Myc9 accumulation in mutants with known cytokinetic defects. We found that Ash1p–Myc9 accumulates asymmetrically in *cla4* mutants, which also have wide bud necks due to a cytokinetic defect (Cvrckova et al., 1995) (Figure 8A). Ash1p–Myc9 even continued to accumulate asymmetrically in mother and daughter nuclei for several hours after *cdc12-1* mutants were shifted to the restrictive temperature (Figure 8A), at which no cytokinesis occurs. After extended incubation at the restrictive temperature, Ash1p accumulation in mother cell nuclei increases, although its distribution remains asymmetric. We also compared the appearance of *ho* mRNAs as wild-type and *cdc12* mutant cells underwent nuclear division at the restrictive temperature and found no major differences (Figure 8B). We conclude that neither Ash1p asymmetry nor *HO* expression in mother nuclei is contingent on mother and daughter nuclei becoming enclosed in separate cells. The loss of asymmetry in *she5* mutants is therefore not trivial. She5p/Bni1p must have a genuine role in generating differences between mother and daughter cytoplasm that affect Ash1p accumulation.

Is Swi5p a Target for Ash1p?

Our data suggest that *she* mutants fail to express *HO* owing to accumulation of Ash1p in both mother and daughter nuclei. A crucial criterion for identifying *she* mutants was their ability to express the hybrid *GAL–HO* promoter despite being completely defective for *HO*

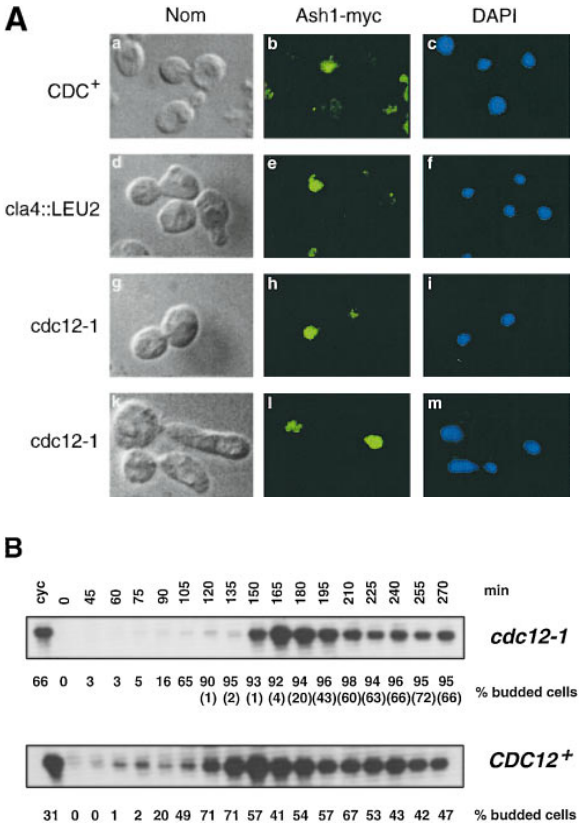

Figure 8. Asymmetric Accumulation of Ash1p–Myc9 and Mother-Specific *ho* Expression Are Independent of Cytokinesis

(A) Asymmetric accumulation of Ash1p–Myc9 and *ho* expression in the absence of cytokinesis. K5552 (*ASH1–myc9*), K5749 (*ASH1–myc9, cla4::LEU2*), and K5750 (*ASH1–myc9, cdc12-1*) cells were grown at 30°C (K5552 and K5749) or 37°C (K5750) for 3–4 hr and prepared for indirect immunofluorescence. (g)–(i) are K5750 cells grown at the restrictive temperature for 3 hr; (k)–(m) are K5750 cells grown at the restrictive temperature for 4 hr. (a), (d), (g), and (k) show Nomarski optics; (b), (e), (h), and (l) show anti-Myc staining; and (c), (f), (i), and (m) show DAPI (DNA) staining.

(B) Unbudded G1 daughter cells were isolated by centrifugal elutriation from asynchronous cultures of wild-type *CDC12*$^+$ and mutant *cdc12-1* growing at 25°C and incubated in fresh YEPD medium at 37°C. *ho* mRNAs measured by Northern blotting (equal amounts of RNA were loaded) and budding index are shown. Numbers in parentheses in the *cdc12-1* strain indicate appearance of a second bud and entry into the second cell cycle. Note that *ho* RNAs only accumulate in the second cycle; the weak signal in the first cycle in *CDC12*$^+$ is due to a small contamination of mothers in this daughter cell population. *ho* RNAs accumulate slightly later but to similar levels in *cdc12-1* mutants.

(Jansen et al., 1996). This suggests that repression of *HO* by Ash1p occurs via the URS1 sequences that are replaced by the *GAL1-10* UAS in *GAL–HO*. Swi5p also mediates its effect via URS1. Moreover, alleles of *SWI5* such as *SWI5Δ59* that lack a central "inhibitory" domain cause *HO* to be expressed in 82% of mother cells and up to 50% of daughter cells (Table 2; Tebb et al., 1993). The similarity of this phenotype to that of *ash1* mutants suggests that Ash1p might repress *HO* by interfering directly or indirectly with Swi5p function via the inhibitory domain of the latter. According to this hypothesis,

Table 2. Mating Type Switching in *swi5Δ59 she* Double Mutant Cells

Strain	Mothers (%)	Daughters (%)
Wild Type (K4709)	70 (33 of 47)	0 (0 of 42)
swi5Δ59 (H990Δ59)	81 (31 of 38)	48 (15 of 31)
swi5Δ59 she1-456 (K4997)	86 (42 of 49)	40 (17 of 43)
swi5Δ59 she2-652 (K4997)	80 (49 of 61)	55 (34 of 61)
swi5Δ59 she3-1440 (K5311)	90 (37 of 41)	51 (20 of 39)

Frequency of mating-type switching in mothers and daughters. Percentages are indicated, together with the actual numbers of switching per division (within parentheses). In all the cases examined, spores never switched mating types. Mother cells carrying these *she1, she2,* and *she3* mutations and a *SWI5*$^+$ allele switch mating types at frequencies of 1.5%, 5.5%, and 0.7%, respectively (Jansen et al., 1996).

SWI5Δ59 allows *HO* to become at least partially refractory to Ash1p. To test this, we analyzed the effect of replacing *SWI5* by *SWI5Δ59* on pedigrees of mating-type switching in *HO she* strains (Table 2). We found, remarkably, that switching induced by *SWI5Δ59* is unaffected by mutations in any of the *SHE* genes. For example, a *she3* mutation reduces mother cell switching to 1% of divisions when driven by *SWI5* (Jansen et al., 1996), but has no effect on the rate of switching in either mothers or daughters when it is driven by *SWI5Δ59*.

Discussion

Only yeast cells that have previously given birth to a bud express the *HO* gene and switch mating types (Nasmyth, 1983; Strathern and Herskowitz, 1979). This mother cell–specific *HO* expression is presumably due to the differential accumulation or modification of factors in the two sister nuclei produced by mitosis. The nuclear "determinants" of *HO* asymmetry could be either activators found in mothers or repressors found in their daughters. Such activators should be essential for *HO* expression. However, none of the ten *SWI* genes or five *SHE* genes required for *HO* transcription encodes mother cell–specific nuclear factors. Swi proteins are nuclear factors present in both mother and daughter cells (Breeden and Nasmyth, 1987; Nasmyth et al., 1990; Peterson et al., 1994), whereas She proteins are all located in the cytoplasm (Jansen et al., 1996). The discovery that *SHE1* encodes a type V myosin, which along with She3p accumulates preferentially in buds, suggests that *HO* expression might depend on the transport from mother cells into their buds of factors that promote *HO* repression. If so, the determinant of *HO* asymmetry might be a repressor, not an activator.

We have used two different mutant screens to identify genes needed to repress *HO* in daughter cells. The first screen started with the premise that the lack of *HO* expression in *she* mutants is due to the accumulation in both mother and daughter cells of a repressor that is

normally daughter cell specific. We duly isolated mutants capable of expressing *HO* in the absence of She function and thereby identified a single gene, *ASH1*, that is essential for preventing *HO* expression in daughter cells.

Our second approach had the advantage of not being model driven. We made a yeast strain in which the *CDC6* gene, which encodes an unstable protein required for DNA replication, was expressed from either the *GAL* or the *HO* promoter. Upon transfer to glucose medium, which represses *GAL*, only mother cells were able to divide, and cells failed to produce viable colonies. We isolated mutants capable of producing colonies on glucose and thereby identified several mutations in a single complementation group, which corresponds to *ASH1*. Sil and Herskowitz (1996 [this issue of *Cell*]) have independently identified an allele of *ASH1* by searching directly for mutants whose daughters switch mating types.

ASH1 encodes a 66 kDa protein that is essential to prevent *HO* expression and switching in daughters, but also has a role in reducing the switching frequency of mothers from 100% to 70%. Analysis of an epitope-tagged version suggests that Ash1p is scarce if not absent in S, G2, and M phase cells and only appears in the nucleus at the end of anaphase. Crucially, Ash1p accumulates to much higher levels in daughter nuclei, and this asymmetry depends on *SHE* genes. The protein lingers in daughter cell nuclei until cells bud, after which it is degraded.

Is Ash1p the Sole Determinant of *HO* Asymmetry?

Is the greater abundance of Ash1p in daughter cell nuclei sufficient to account for the restriction of *HO* expression to mother cells? That is, if Ash1p were to accumulate to the same extent in mother cell nuclei, would it also completely repress *HO* in mother cells? Several data suggest that it would. Our data suggest that some Ash1p does accumulate in mother cell nuclei, and this is apparently sufficient to reduce their frequency of mating-type switching. The protein that accumulates in mothers is therefore not inactive. Is it merely insufficiently abundant to repress *HO* efficiently? The behavior of *she* mutants strongly suggests that is the case. In *she* mutants, Ash1p accumulates to equally high levels in mother and daughter nuclei, and as a consequence *HO* fails to be expressed in mothers as well as daughters. This alone does not prove that the unequal distribution of Ash1p is the sole determinant of *HO* asymmetry, because *SHE* genes might also promote the unequal distribution of additional, as yet unidentified *HO* transcription factors. However, our observation that increased *ASH1* gene dosage reduces *HO–ADE2* and *HO–CAN1* expression in SHE^{+} cells (data not shown) suggests that increasing abundance of Ash1p in mother cells is sufficient to repress *HO*. Thus, differential Ash1p accumulation could well account for most of the differences in *HO* expression in mothers and daughters.

Origin of Ash1p Asymmetry

Ash1p accumulates equally in mother and daughter nuclei in *she1*, *she2*, *she3*, and *she5* mutants (we did not test *she4*). She proteins are therefore essential for generating Ash1p asymmetry. She1p/Myo4p, She3p, and She5p/Bni1p are also required for each others' preferential accumulation in growing buds. *SHE1* encodes Myo4p, a type V myosin related to Myo2p that has been implicated in moving secretory vesicles from mother cells to their buds (Haarer et al., 1994). These facts suggest that She1p/Myo4p, with the help of other She proteins, might generate Ash1p asymmetry by moving substances from mothers into their buds. Do they move Ash1p, and is this the basis for its asymmetric accumulation? The distribution of She3p switches from an asymmetric to a symmetric pattern during anaphase, which indicates that its directional transport ceases shortly before cell division (Jansen et al., 1996). Crucially, this switch occurs shortly before or around the time Ash1p accumulates. It seems, therefore, that the bulk of Ash1p might be synthesized too late in the cell cycle for it to be transported from mother cells into their buds by the She machinery. If She proteins do not move the bulk of Ash1p, they might instead be involved in generating differences in the capacities of mother and daughter cells to synthesize and accumulate Ash1p. These differences could reside in *ASH1* gene transcription, in the translation of its mRNA, or in the stability of Ash1p. It is interesting in this regard that examples of cell-autonomous differentiation in flies and worms rarely concern the segregation of proteins, but rather the capacity to synthesize them (St Johnson, 1995). Another aspect of Ash1p asymmetry reminiscent of flies is the continued generation of differences in the accumulation of Ash1p in sister nuclei in the absence of cytokinesis, which is analogous to the different patterns of gene expression adopted by individual nuclei within the syncytia of early Drosophila embryos.

The accumulation of *ASH1* mRNAs during anaphase depends on Swi5p and its relative Ace2p. This is intriguing given that Ash1p later acts to hinder Swi5p function at the *HO* promoter. Swi5p and Ace2p accumulate in the cytoplasm during G2 and early M phase, but suddenly enter mother and daughter nuclei at the end of anaphase, at which point they activate a battery of genes, including *EGT2*, *CTS1*, *SIC1*, and *CDC6*, involved in cell separation and preparations for DNA replication (T. Schuster, personal communication; Dohrmann et al., 1992; Schwob et al., 1994; Piatti et al., 1995). Both Swi5p and Ace2p accumulate to similar levels in mother and daughter nuclei (Nasmyth et al., 1990; Dohrmann et al., 1992). If *ASH1* is more actively transcribed in daughter cell nuclei, it is not owing to greater levels of Swi5p in these cells.

How Does Ash1p Repress *HO*?

The accumulation of Ash1p predominantly in daughter cell nuclei is essential for repressing *HO* as daughter cells undergo the G1 to S phase transition. In *she* mutants, Ash1p accumulates to equally high levels in mother and daughter nuclei, and this represses *HO* in both progeny. *she* mutants were singled out from a vast number of mutants defective in expressing *HO* because they were not defective in expressing a *GAL–HO* promoter fusion, in which the upstream promoter region

URS1 is replaced by the *GAL* UAS. This means that the Ash1p that accumulates in both mother and daughter nuclei in *she* mutants cannot repress *GAL–HO* and suggests that Ash1p exerts its effect on *HO* via URS1. Swi5p also acts via this region of the promoter; a pair of Swi5p-binding sites within URS1 is essential for *HO* transcription (Stillman et al., 1988; Tebb et al., 1993). Might Ash1p, therefore, specifically inhibit the function of Swi5p? There is some evidence that it might. The lack of mating-type switching due to *she* mutations is completely suppressed by an allele of *SWI5*, *SWI5Δ59*, which encodes a protein lacking a central inhibitory domain. One explanation for this remarkable result is that Ash1p represses *HO* directly or indirectly via the inhibitory domain missing in Swi5Δ59p; that is, the deleted protein is insensitive to inhibition mediated by Ash1p. This hypothesis has the merit of explaining why *SWI5Δ59* causes daughter cells to switch mating types with high efficiency, which we had previously suggested was due to less rapid Swi5p proteolysis (Tebb et al., 1993). This could still be an important factor, but we now suggest that it may not be the sole explanation. Whether Ash1p represses *HO* by binding to sequences in the neighborhood of Swi5p-binding sites is not known. Ash1p contains sequences at its carboxyl terminus that resemble the DNA-binding domain of the GATA-1 transcription factor, suggesting that Ash1p could be a site-specific DNA-binding protein.

The Pedigree of Mating-Type Switching

Our current picture of events that lead to differential *HO* expression in mother and daughter cells is as follows. We propose that the process starts with the accumulation of Cln1p and Cln2p/Cdc28 kinases in late G1 owing to activation of the SBF transcription factor by Cln3p/Cdc28 (Dirick et al., 1995). This initiates polarization of the cytoskeleton, which enables cell growth to be directed into a bud and triggers the transport by She proteins of factors that will be needed later for the accumulation of Ash1p (Jansen et al., 1996). This transport could be effected by the movement of She1p/Myo4p along actin cables that run from the mother cell into the growing bud. The next crucial step is activation of cyclin B/Cdc28 kinases in G2, which activates *SWI5* and *ACE2* transcription (Amon et al., 1994) and leads to the accumulation of their gene products in the cytoplasm (Nasmyth et al., 1990; Dohrmann et al., 1992). Subsequent activation of the anaphase-promoting complex triggers nuclear division and inactivation of cyclin B/Cdc28 kinase (King et al., 1994; Irniger et al., 1995). The loss of Cdc28 kinase causes Swi5p and Ace2p to enter the newly formed mother and daughter nuclei (Moll et al., 1991; Dohrmann et al., 1992), and this activates *ASH1* transcription. We propose that, owing to the prior segregation to the bud of factors involved in Ash1p synthesis or stability, Ash1p accumulates to much higher levels in the daughter than the mother nucleus. Ash1p is sufficiently abundant in daughters (but not in most mothers) to interfere with the function Swi5p at the *HO* promoter. However, this interference is not realized until the cycle makes a full turn with reactivation of SBF by the Cln3p/Cdc28 protein kinase in the subsequent cell cycle.

Swi5p has a pivotal but ambiguous role in these proceedings because it is needed first for the efficient transcription of *ASH1* and subsequently for that of *HO*, which is inhibited by the high levels of Ash1p in daughter nuclei. The efficiency with which the She/Ash system prevents daughter cell switching may depend on *HO* activation needing the successive activation of two factors: first Swi5p and then SBF. The dependence of *HO* on SBF delays its activation relative to other genes activated by Swi5p. This may be important for giving the daughter cell time to accumulate enough Ash1p to inhibit Swi5p (at *HO*), which might explain why some daughter cells switch mating types (between 3% and 20%, depending on the strain) when the process is driven by a version of the *HO* promoter that is SBF independent (Nasmyth, 1985; data not shown).

It has long been strange that not all mother cells switch their mating types (Strathern and Herskowitz, 1979). We now know that this is due to their inheritance of small amounts of Ash1p. The replacement of *ASH1* by a Myc-tagged version increased mother cell switching to nearly 100% without raising the level in daughters. It seems, therefore, that yeast "chooses" to switch mating types with less than 100% efficiency. It might do so either because a lowered rate of switching is itself desirable or because Ash1p must also fulfill some role in mother cells that is unrelated to switching. Ash1p accumulates asymmetrically in diploid cells (data not shown), which suggests that it has functions besides regulating *HO*; that is, it may be involved in establishing other differences between mothers and their daughters that are pertinent to their different developmental fates.

Experimental Procedures

Culture Conditions, Strains, and Media

Unless otherwise stated, all strains were derived from W303 (K699; *MAT***a** *ade2-1 trp1-1 leu2-3 his3-11 ura3 can1-100 ho*) and all methods were as described in Jansen et al. (1996).

Mutant Isolation and Characterization

In screen 1, EMS mutagenesis was performed on *MATα HIS3 leu2* (K5406) and *matΔ:LEU2 his3* (K5407) strains carrying *HO–ADE2*, *HO–CAN1*, and a *URA3*-marked disruption of the *SHE1/MYO4* gene (Jansen et al., 1996). These strains were derived from K5212 (*MAT***a** *she1/myo4::URA3 HO–ADE2 HO–CAN1*). After mutagenesis to 70% survival and growth for two generations in yeast extract–peptone–dextrose (YEPD), 2×10^6 cells were plated at 25°C on medium lacking adenine. Approximately 1500 mutants formed white colonies after 3 days, among which 80 of 80 were shown to be canavanine sensitive. Mutants whose *HO–ADE2* and *HO–CAN1* expression was *SWI5* dependent (38 of 40) were crossed to a parental strain of the opposite mating type to test for dominance; 18 were dominant or semidominant, and the rest were recessive.

In screen 2, to isolate mutants capable of activating the *HO* promoter in daughter cells, we constructed a strain whose endogenous *CDC6* locus had been deleted and which expressed *CDC6* from both the *HO* and *GAL1-10* promoters (K6001; *MAT***a** *cdc6::hisG HO–CDC6 GAL–UbiRCDC6::URA3*) (Piatti et al., 1995). To construct the *HO–CDC6* fusion, NheI sites were generated by PCR-mediated mutagenesis 5′ of the *HO* ATG and before the ATG of the *CDC6* gene. This strain grows on galactose but not glucose medium. We isolated 12 independent spontaneous mutants after streaking on glucose plates. Their ability to grow on glucose medium was lost following disruption of the *SWI5* locus. To generate a strain carrying *ash1-61* and *HO–ADE2*, strain K6061 (*MAT***a** *cdc6::hisG HO–CDC6 GAL–UbiRCDC6::URA3 ash1-61*) was crossed to strain K5211 (*MATα HO–ADE2 HO–CAN1 she1::URA3*). After sporulation and tetrad dissection, strain K6062 (*MAT***a** *CDC6 HO–ADE2 HO–CAN1 she1::URA3 ash1-61*) was isolated. Analysis of the cross between K6062 and

K5694 (*MATα HO–ADE2 HO–CAN1 she1::URA3 ash1-12*) showed *ash1-61* and *ash1-12* mutations to be allelic.

Mating-Type-Switching Strains

K5861 (*ash1-12 she1::URA3 HO*) was isolated from a cross between strain K5694 (*MATα HO–ADE2 HO–CAN1 she1::URA3 ash1-12*) and the homothallic strain RPY53 (*HO she1::URA3*). K6061 (*MAT***a** *cdc6::hisG HO–CDC6 GAL–UbiRCDC6::URA3 ash1-61*) was crossed to the homothallic strain K4709 (*TRP1 HO*) to isolate K5864 (*ash1-61 CDC6 HO*). K5862 (*ash1::URA3 HO*) was isolated by crossing K5550 (*MAT***a** *ash1::URA3 ho*) to the homothallic strain K4709, while K5863 (*ASH1–myc9 HO*) was isolated from a cross between K5552 (*MATα ASH1–myc9 ho*) and the homothallic strain K4709.

To isolate strain K4996 (*she1-456 SWI5Δ59 HO*), a version of strain K4872 (*she1-456 HO*) carrying a *URA3*-marked disruption at the *SWI5* locus was created (K4938). K4938 was sporulated and crossed to H990 (*SWI5Δ59 HO* YEp13-*LEU2*). Diploids were selected for uracil and leucine prototrophy. After sporulation and tetrad dissection, progeny that carried *SWI5Δ59*, *she1-456*, and *HO* were selected.

Following a similar procedure, K4997 (*she2-652 SWI5Δ59 HO*) and K5311(*she3-1440 SWI5Δ59 HO*) were obtained from strain K4873 (*she2-652 HO*) and K4874 (*she3-1440 HO*), respectively.

Cloning and Disruption of the *ASH1* Gene

Plasmids containing the *ASH1* gene were isolated from a YCplac111 genomic library after transformation of K5694 (*MATα HO–ADE2 HO–CAN1 she1::URA3 ash1-12*) and K5695 (*MATα HO–ADE2 HO–CAN1 she1::URA3 ash1-2*) and selection for canavanine resistance and growth in the absence of leucine (YCplac111 contains *LEU2*). Two different plasmids restored canavanine resistance and adenine auxotrophy to *ash1-2* and *ash1-12 HO–CAN1 HO–ADE2 she1::URA3* mutant strains. These same two plasmids also caused *ash1-61 cdc6::hisG HO–CDC6 GAL–UbiRCDC6::URA3* to lose the ability to grow on glucose. The smallest insert contained sequences from chromosome XI and carried 3 complete ORFs. The *ash1*-complementing activity resided in a SalI–PvuII fragment containing only the complete ORF YKL185w.

ASH1 was disrupted by inserting the *URA3* gene into the single NcoI site within the *ASH1* ORF. The disruption DNA was released from the YCplac111 vector by digestion with EcoRI, gel purified, and used to transform a diploid wild-type strain K842 (*MAT***a**/*MATα SHE ho*) to uracil prototrophy. Heterozygosity for the correct *ash1* gene disruption was shown by Southern blot analysis, and haploid *ash1::URA3* progeny were obtained after tetrad analysis. To demonstrate that the disrupted gene corresponds to *ASH1*, we crossed *HO–ADE2 HO–CAN1 she1::URA3* strains carrying *ash1::URA3* with syngenic strains carrying *ash1-12* and *ash1-61*. All spores (16 tetrads were analyzed) resulting from these crosses were able to express *HO–ADE2*, which indicates tight linkage between the cloned locus and *ash1-12* and *ash1-61*.

Epitope Tagging Ash1p

To introduce an epitope tag at the carboxyl terminus of Ash1p, a BamHI site was introduced by PCR-mediated mutagenesis in front of the *ASH1* stop codon. BamHI fragments containing three Myc epitopes were cloned into this site. We selected clones that contained two or three copies of the Myc cassette in the correct orientation. *ASH1–myc6* and *ASH1–myc9* constructs were linearized and integrated at the genomic locus by replacing the *ash1::URA3* disruption. Yeast strains with the correct replacements were selected on 5-fluoroorotic acid–containing medium and checked by Western blot analysis for expression of the epitope-tagged protein and by Southern blotting for integration. Strains K5552 (*MATα ho ASH1–myc9*) and K5555 (*MATα ho ASH1–myc6*) were chosen and used for indirect immunofluorescence microscopy as described by Jansen et al. (1996). Similar staining patterns were observed using *ASH1–myc6* and *ASH1–myc9* strains; protein made from the latter was much more easily detectable in Western blots.

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